Changes in iso- and n-alkane distribution during biodegradation of crude oil under nitrate and sulphate reducing conditions

Marion Hasinger a,1, Kerstin E. Scherr a,1, Tserenlyam Lundaa b, Leopold Bräuer c, Clemens Zach c, Andreas Paul Loibner a

a University of Natural Resources and Life Sciences Vienna, Department for Agrobiotechnology, Institute for Environmental Biotechnology, Konrad Lorenz Strasse 20, 3430 Tulln, Austria
b School of Agrobiology, Mongolian State University of Agriculture, Zaisan 17026, Ulaanbaatar, Mongolia
c OMV Exploration and Production GmbH, Technology Development and Application, Trabrennstraße 6-8, 1020 Vienna, Austria

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A B S T R A C T

Crude oil consists of a large number of hydrocarbons with different susceptibility to microbial degradation. The influence of hydrocarbon structure and molecular weight on hydrocarbon biodegradation under anaerobic conditions is not fully explored. In this study oxygen, nitrate and sulphate served as terminal electron acceptors (TEAs) for the microbial degradation of a paraffin-rich crude oil in a freshly contaminated soil. During 185 days of incubation, alkanes from n-C11 to n-C39, three n- to iso-alkane ratios commonly used as weathering indicators and the unresolved complex mixture (UCM) were quantified and statistically analyzed. The use of different TEAs for hydrocarbon degradation resulted in dissimilar degradative patterns for n- and iso-alkanes. While n-alkane biodegradation followed well-established patterns under aerobic conditions, lower molecular weight alkanes were found to be more recalcitrant than mid- to high-molecular weight alkanes under nitrate-reducing conditions. Biodegradation with sulphate as the TEA was most pronounced for long-chain (n-C32 to n-C39) alkanes. The observation of increasing ratios of n-C17 to pristane and of n-C18 to phytane provides first evidence of the preferential degradation of branched over normal alkanes under sulphate reducing conditions. The formation of distinctly different n- and iso-alkane biodegradation fingerprints under different electron accepting conditions may be used to assess the occurrence of specific degradation processes at a contaminated site. The use of n- to iso-alkane ratios for this purpose may require adjustment if applied for anaerobic sites.

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1. Introduction

Crude oil represents a highly heterogeneous mixture of environmental contaminants that are released worldwide into soil, air and water. Bioremediation, the engineered promotion of naturally occurring microbial pollutant breakdown processes, is a powerful approach for the decontamination of environmental matrices (Johnston et al., 1998; Okoh and Trejo-Hernandez, 2006; Scherr et al., 2009). Oxidative biodegradation of crude oil, or petroleum hydrocarbons, can take place with a variety of terminal electron acceptors (TEAs), where the highest degradation rates are reported for oxygen, yielding the largest amount of energy per unit hydrocarbon degraded (Thauer et al., 1977; Widdel and Rabus, 2001). The mechanisms governing the aerobic degradation of petroleum hydrocarbons have been elucidated in numerous studies (Atlas, 1981; Leahy and Colwell, 1990; Prince et al., 2003; Watson et al., 2002). Beside environmental factors (Leahy and Colwell, 1990; Okoh, 2006) and pollutant bioaccessibility (Scherr et al., 2007; Ehlers et al., 2010), intrinsic structural properties of an individual compound determine the degree and rate of degradation. Most prominent petroleum hydrocarbon structures include linear (normal or n-), branched (iso- or i-) and cyclic alkanes and alkenes, mono- and polyaromatic compounds, resins and asphaltens (Peters et al., 2005). For aerobic degradation, an inverse relationship between hydrocarbon molecular weight and degradability applies (Peters et al., 2005; Setti et al., 1993; Scherr et al., 2007). Similarly, higher complexity reduces degradability due to steric hindrance of the enzymatic attack (Setti et al., 1993). Most studies agree on a distinct succession in the microbial depletion of structural groups under aerobic conditions, with,
in order of decreasing biodegradation: \( n \)-alkanes > \( i \)-alkanes > low-molecular weight aromatics > high-molecular weight aromatics and cyclic alkanes (Leahy and Colwell, 1990; Wang and Fangas, 2003). This succession reflects progressive compositional changes in petroleum chemistry in the field, i.e. the development of distinct hydrocarbon `fingerprints’, as a function of exposure time to a variety of weathering processes (Wang and Fangas, 2003; Roques et al., 1994). This concept is used for the age dating of oil spills and to assess the occurrence of biodegradation in situ during bioremediation or natural attenuation (Christensen and Larsen, 1993; Wang et al., 1998; Fisher et al., 1996). Different mathematical models were developed for these purposes (e.g. Howard et al., 2005; Haeuser et al., 2010). Also in this context, ratios of selected \( n \)- to iso-alkanes, such as \( n \)-heptadecane to pristane (\( n \)-C17 to i-C19) and \( n \)-octadecane to phytane (\( n \)-C18 to i-C20) are employed to indicate biodegradation, since specifically branched alkanes are degraded at lower rates than linear alkanes with a roughly similar carbon number (Wang et al., 1998).

In contrast, the occurrence of crude oil biodegradation under anaerobic conditions was considered quantitatively negligible until the early 1980s (Atlas, 1981). At present, the degradation of petroleum hydrocarbons connected to the reduction of different non-oxygen electron acceptors like nitrate (Callaghan et al., 2009), sulphate (Fuku et al., 1999; Caldwell et al., 1998), ferric iron and under methanogenic conditions (Jones et al., 2008; So and Young, 2001) have been demonstrated. The relation of a compound’s structure or weight to its degradability as known for aerobic conditions, however, does not necessarily apply for anaerobic degradation. These relations have not been fully explored. The understanding of the underlying degradative processes appears to be limited to compounds with less than 20 carbon atoms (Grossi et al., 2008). In the absence of molecular oxygen, hydrocarbon activation follows different mechanisms and at least two different, bacteria-specific pathways exist (Wilkes et al., 2002; So et al., 2003). Moreover, electron acceptors other than oxygen yield considerably less energy. These factors could contribute to the development of distinctly different degradation patterns for crude oil under anaerobic conditions. The interpretation of such fingerprints can yield valuable information concerning the age, the rate and extent of natural attenuation or the efficiency of bioremediation measures, provided an understanding of the governing processes. Therefore, the present study sets out to characterize the biodegradation behavior of selected crude oil constituents with oxygen, nitrate and sulphate as TEA, respectively. Biodegradation of a paraffin-rich crude oil spiked freshly to a soil was monitored in lab-scale experiments over 185 days. Using GC–FID, changes in (i) the abundance of 29 \( n \)-alkanes, (ii) three commonly used linear-to-branched-alkane ratios and (iii) the bulk of complex hydrocarbons represented by the unresolved complex mixture (UCM) were monitored and statistically analyzed.

2. Materials and methods

2.1. Soil

The soil used in the experiments was an uncontaminated surface soil sampled in lower Austria, and was classified as a sandy loam according to US soil taxonomy. Prior to use in the experiments, the soil was sieved to \(< 2 \text{ mm} \) and homogenized. This soil was spiked with crude oil to exclude ageing effects on petroleum hydrocarbon bioavailability, as observed earlier by Scherr et al. (2007).

2.2. Crude oil

A paraffin-rich crude oil from the Vienna Basin was used as the soil contaminant. For this crude oil, gas chromatographic analysis and other aspects of aerobic and anaerobic biodegradation are described elsewhere (Scherr et al., 2007; Scherr et al., this issue). A chromatogram is provided in the supplementary information (Fig. S1) and Scherr et al. (this issue). The oil was a donation by OMV Exploration & Production Company Ltd. It contains \( n \)-alkanes from C6 to C44, with a maximum abundance over the range of C10–C20. Compositional data was supplied along with the oil. Table 1 displays mass fractions of saturated and aromatic compounds and of resins and asphaltenes (`SARA’-analysis), which were determined via thin layer chromatography on a IATROSCAN MK-6s (SES Analysensysteme, Bechenheim, Germany).

The crude oil was added to the homogenized, air-dry soil using a Pasteur pipette to achieve a concentration of 10,000 mg total petroleum hydrocarbons (TPH) per kilogram soil dry weight (dw) and mixed thoroughly by stirring with a spoon before use in the experiments.

2.3. Soil slurry microcosms

Biodegradation experiments using crude oil-contaminated soil under different electron accepting conditions (oxygen, nitrate and sulphate) were carried out in batch soil slurries as described before (Scherr et al., 2009) with modifications for the nitrate- and sulphate-amended (`anaerobic’) microcosms. Three microcosms per experiment were sacrificed on each sampling day. Slurry analysis included TPH, nitrate, nitrite, sulphate, sulphide, ammonia, phosphates, pH and oxidation–reduction potential (ORP). All analyses were carried out in triplicate.

Microcosms were set up in 100 mL Pyrex® bottles containing 10 g of contaminated soil dry weight supplied with mineral medium at a soil:medium ratio of 1:2 (w/v) for aerobic and of 1:4 (w/v) for anaerobic microcosms. In addition, anaerobic microcosms were supplemented with microorganisms from an anaerobic contaminated site as described below. Initial pH values were 7.1 for the aerobic experiment and 6.6 with nitrate and 7.3 with sulphate as TEA. Microcosms were incubated at 20 ± 2 °C in the dark on an orbital shaker (GFL 3020, GFL, Burgwedel, Germany). Anaerobic bottles were shaken at 150 rpm and aerobic microcosms at 200 rpm. The anaerobic test bottles were shut tight with caps coated with polytetrafluorethylene on the inside. To maximize oxygen diffusion to the aerobic microcosms, the bottles’ openings were covered with a needle-punctured aluminum foil. Abiotic losses were quantified, in order to calculate net aerobic biodegradation, from a killed control containing mercury(ll)-chloride (Sigma Aldrich, St. Louis, MO, USA) at a concentration of 1 g/L (Trevors, 1996). Two additional bottles for each anaerobic degradation experiment were set up to monitor the oxidation–reduction potential (ORP). Further specifications are provided below.

2.4. Mineral media

Macronutrients were supplied to the aerobic microcosms with \( \text{NH}_4\text{NO}_3, \text{Na}_2\text{HPO}_4 \) and \( \text{KH}_2\text{PO}_4 \) to yield a stoichiometric C:N:P ratio of 100:10:1 based on contaminant carbon (assuming 85% (w/w) carbon in the crude oil, Peters et al., 2005) and with the phosphate compounds doubling as buffer (equimolar ratio; Scherr et al., 2009). For the anaerobic microcosms, a C:N:P ratio of 300:10:1, assuming

<table>
<thead>
<tr>
<th>Abundance in % (w/w)</th>
<th>Saturated compounds</th>
<th>Aromatic compounds</th>
<th>Resins</th>
<th>Asphaltenes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>52.5</td>
<td>29.5</td>
<td>16.7</td>
<td>1.3</td>
</tr>
</tbody>
</table>

Table 1 Mass fractions of saturated and aromatic compounds, resins and asphaltenes in the paraffin-rich crude oil used in the experiments as quantified with thin layer chromatography (`SARA’-analysis).
lower biomass production (Thauer et al., 1977), was applied, and NH₄Cl was used as a nitrogen source. All chemicals were obtained from Fluka, Buchs, Switzerland and dissolved in bi-distilled water.

For all experiments, mineral media were supplemented with 10 mL/L of a sulphate free trace element solution according to Widdel and Pfennig (1981) and 1 mL/L of a vitamin solution containing (concentrations in mg/L): p-aminobenzoic acid (5), biotin (2), folic acid (2), lipoic acid (5), nicotinic acid (5), pantothenic acid (5), pyruvexin/HCI (10), riboflavin (5), thiamin (5) and vitamin B12 (0.1); all from Sigma Aldrich, St. Louis, MO, USA. The anaerobic media were purified in a glove-box (field pyramid®, Erlab D.F.S S.A.S, Köln, Germany) flushed with argon (Messer Austria GmbH, Gumpoldskirchen, Austria) at a flow of 200 mL/min for 30 min before use to remove dissolved oxygen.

2.5. Inoculum preparation and community composition

To ensure the presence of anaerobic hydrocarbon degrading bacteria, the anaerobic microcosms were supplemented with microorganisms from an anaerobic site that was historically contaminated with the paraffin-rich crude oil used in this study. The GC-FID chromatogram of a site soil sample is displayed in Fig. 1.

About 200 g of soil sample from the saturated zone was immersed in 400 mL of the anaerobic mineral medium, described above, in a 1000 mL Pyrex® bottle, and placed on a shaker over night. Soil manipulation was conducted in the argon-flushed glove-box to maintain anaerobic conditions. One milliliter of the supernatant was added to each microcosm bottle of the anaerobic experiments at the start of the experiments.

The 16S rDNA community analysis of this soil revealed the identified dominant phylum to be Bacteroidetes (33%), followed by Proteobacteria (23%) and Actinobacteria (8%). The majority (85%) of Bacteroidetes was unclassified, while the phylum of Proteobacteria was constituted by γ-class (53%), δ-class (24%) and β-class (16%). Many strains capable of anaerobic degradation of hydrocarbons belong to the phylum of Proteobacteria (Widdel and Rabus, 2001; Pedersen et al., 2010; Mbadinga et al., 2011). Thus, a certain capacity for hydrocarbon degradation was assumed. This is further supported by the fact that the oil composition at the site exhibits distinct signs of degradation with a depletion in n-alkanes in the entire analyzed carbon range (Fig. 1). The microbial analysis is described in more detail in the supporting information.

2.6. Terminal electron acceptors (TEAs)

The experimental setup (open bottles, shaking) was designed so as to supply oxygen by diffusion from air to the aerobic microcosms.

Nitrate and sulphate were supplemented initially at concentrations of 25 mM as potassium nitrate or sodium sulphate (Fluka, Switzerland) according to Crawford et al. (1998) and Motamedi and Pedersen (1998), respectively. Nitrate and sulphate consumption were monitored over the incubation time in order to re-supply acceptors if indicated (<40% of initial concentration), however this was not the case.

2.7. Analysis of total petroleum hydrocarbon (TPH) and individual alkanes

The determination of the total petroleum hydrocarbon (TPH) content of the soil slurry was performed according to DIN ISO 16703 and as published by Scherr et al. (2007). Briefly, following ultrason-aided hexane extraction, gas chromatographic separation and detection via flame ionization detection (GC-FID), chromatograms were integrated between the retention time window (RTW) markers (n-C10 and n-C40, Fig. 1) to calculate TPH concentrations. The signal in this RTW was also the subject of compositional TPH analysis in the present study. The twenty-nine n-alkanes between RTW markers, three chromatographically distinct branched alkanes and the unresolved complex matrix (UCM) were included in the compositional analysis. Individual alkanes were identified in the chromatogram using an external standard mixture (Chiron, Norway). Concentrations of n- and i-alkanes were calculated based on their peak area with the unresolved complex matrix (UCM) as base line, using the sample’s TPH response factor, sample weight, dry weight and extract volume. The UCM was calculated by subtracting the area sum of individual compounds from total TPH area.

Three methylated alkanes were used for the calculation of mass ratios with chromatographically ‘neighboring’ n-alkanes (approximately similar retention time), such as are used for quantifying the degree of hydrocarbon weathering (Wang et al., 1998). Compounds farnesane (FAR; 2,6,10-trimethyldecane), pristane (PRI; 2,6,10,14-tetramethylpentadecane) and phytane (PHY; 2,6,10,14-tetramethylhexadecane) were compared with their chromatographic neighbors as follows: n-C14:FAR, n-C17:PRI and n-C18:PHY.

2.8. Measurement of oxidation–reduction potential (ORP) and pH

ORP and pH were measured in the continuously argon-flushed glove box. The measurements were conducted with a portable pH meter type HQD 30 (Hach Lange GmbH, Düsseldorf, Germany) equipped with the Intellicl® ORP-REDOX MTC 301 ORP and pH-C 101 probes (both Hach Lange GmbH).

2.9. Analysis of phosphate, TEAs and reduction products in anaerobic microcosms

Ahead of TPH analysis of the anaerobic microcosms, the microcosm bottles were centrifuged at 2000 rpm (Beckman 5810; Beckman Coulter Inc., Brea, CA, USA). Twenty milliliter of the aqueous supernatant were removed with a Pasteur pipette and analyzed for phosphate, ammonia, TEAs and degradation products (nitrite and sulphide, respectively). Nitrite and sulphide were analyzed immediately after the removal of the supernatant to minimize oxidation.

The supernatants were filtered using 0.45 μm syringe filters and analyzed (ÖNORM EN ISO 10304-1:1995) for nitrate, sulphate and phosphate concentrations. Ion chromatography was performed on a Dionex ICS 5000 system (Dionex Corporation, Sunnyvale, CA, USA) equipped with a micro membrane suppressor AMMS 300 (4 mm), guard column AG 14A, separation column AS 14A and a conductivity detector (DS 5).

Analysis of nitrite, ammonia and sulphide was carried out with photometrical test kits LCK 302, 305, 341, 342 and LCK 653 from Hach Lange (Hach Lange GmbH, Düsseldorf, Germany) on a DR2800 photometer (Hach Lange GmbH, Düsseldorf, Germany).

2.10. Statistical analysis

The observed degradative patterns were object of statistical analysis using methods described by Scherr et al. (2009).

Crude oil degradation (Figs. 2 and 3 and Table 3) is expressed as remaining relative mass of any compound or UCM in percent of that at day zero. Calculations in Table 2 are based on these values. For all experiments, results for the 29 alkanes were first grouped by three, four and five successively eluting compounds and subjected to two-way analysis of variance (ANOVA), followed by the Bonferroni post test (Graphpad Prism Software 5.0; \( P < 0.05; n = 3 \)).

This test was chosen to detect the influence of two factors (n-alkane group and type of treatment) on the results. All groupings resulted in a similar trend. However, groups of three showed the most pronounced effects and will therefore be discussed. Furthermore, one-way ANOVA followed by Tukey’s multiple comparison test (Graphpad Prism 5.0; \( P < 0.05; <0.01; <0.001; n = 3 \)) was applied to analyze the degradative performance of n-alkane groups with different chain length (Table 3) and n-alkane/l-alkane ratios (Table 2).
Table 2: Ratios of selected n-alkanes to chromatographically neighboring i-alkanes by area during biodegradation using oxygen, nitrate and sulphate as terminal electron acceptors (TEAs).

<table>
<thead>
<tr>
<th>TEA/incubation time</th>
<th>Area ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n-C14 to FAR</td>
</tr>
<tr>
<td>Start</td>
<td>1.77 (0.04)</td>
</tr>
<tr>
<td>Oxygen</td>
<td></td>
</tr>
<tr>
<td>5 days</td>
<td>1.24 (0.11)</td>
</tr>
<tr>
<td>9 days</td>
<td>0.23 (0.06)</td>
</tr>
<tr>
<td>Nitrate</td>
<td></td>
</tr>
<tr>
<td>116 days</td>
<td>1.59 (0.08)</td>
</tr>
<tr>
<td>185 days</td>
<td>1.22 (0.02)</td>
</tr>
<tr>
<td>Sulphate</td>
<td></td>
</tr>
<tr>
<td>116 days</td>
<td>2.19 (0.02)</td>
</tr>
<tr>
<td>185 days</td>
<td>1.88 (0.00)</td>
</tr>
</tbody>
</table>

The value given in parenthesis is one standard deviation, n = 3; FAR, Farnesane; PRI, pristane; PHY, phytane.

* Significant difference (ANOVA/Tukey’s, α = 0.05) to start values.

3. Results

3.1. Crude oil biodegradation with oxygen as TEA

TPH biodegradation proceeded rapidly in the aerobic microcosms. After 16 days of incubation, TPH degradation amounted to 78%, corresponding to a rate of 470 mg TPH/kg soil dry weight/d. In the chromatogram, only the UCM and no individual alkane peaks were identifiable after 16 days (data not shown). For fingerprint analysis, the sample taken after five days of incubation (17% TPH degradation) was chosen. Concentrations of n-alkanes groups relative to day 0 and after correction for abiotic losses are displayed in Fig. 2A. Degradation of individual alkanes after five days ranged between 35% for n-C11, where abiotic losses accounted for 16.5%, and 20% for n-C39. Table 2 provides area ratios for selected linear to branched alkanes that are co-eluting within approximately one minute. Decreasing ratios over incubation time indicate a preferential degradation of normal over branched alkanes, which was the case for all three investigated n- to i-pairs in the aerobic microcosms (Table 2 and Fig. 3A).

3.2. Crude oil biodegradation under nitrate reducing conditions

The oxidation–reduction potential (ORP) determined in the nitrate-amended microcosms indicated the occurrence of reducing conditions, with an ORP of around −40 mV (day 116) and −75 mV (day 185). Nitrate consumption amounted to 46% and to 59% by days 116 and 185, respectively, with an average decrease of 0.1 mmol NO₃⁻/L/d. On day 185, 24% of the added nitrate was stoichiometrically recovered as nitrite. Ammonia was present in the mineral medium, and no increase was detected.

The average TPH degradation rate amounted to 28 mg TPH/kg/d, equal to 59% degradation over 185 days. For the calculation of the stoichiometric acceptor demand for TPH degradation, n-C25 was used as a model mid-weight hydrocarbon. Accordingly, 4.3 mmol of nitrate were reduced per mmol n-C25 consumed (day 185).

Recoverable n-alkane concentrations amounted to 52% and 30% of day 0 for n-C11 after 116 and 185 days of incubation, respectively, and to 79% and 64% for n-C39, respectively. C11–C13 alkanes were the most recalcitrant alkane group (Fig. 2B), while mid-weight hydrocarbons were degraded to less than half of their initial concentration after 185 days.

All ratios of n- to i-alkanes decreased (Table 2) over the incubation time, however with generally lower residual concentrations after 185 days (Fig. 3B) than under aerobic and sulphate-reducing conditions (Fig. 3A and C). This reflected the pronounced degradation of mid-weight alkanes under nitrate-reduction mentioned above (Fig. 2B).

3.3. Crude oil biodegradation under sulphate reducing conditions

The ORP measured in sulphate-amended microcosms, between −215 mV and −387 mV for days 116 and 185, respectively, indicated strongly reducing conditions. The calculated TEA consumption rates until days 116 and 185 both amounted to 0.012 mmol SO₄²⁻/L/d, with roughly 90% of the initial sulphate concentration recovered on day 185. Approximately 0.07% of added sulphate was recovered as dissolved sulphide after 185 days. The color change from brown to grey in the microcosms likely indicated the formation of ferrous sulphide precipitates.

The average TPH degradation rate of 18 mg TPH/kg/d was the lowest of all experiments. Following the approach described above, the hypothetical acceptor demand over 185 days amounted to 1.2 mmol sulphate per mmol n-C25.

Under sulphate reducing conditions, extent of n-alkane degradation and chain length were directly related (Fig. 2C). On day 185, most high molecular weight alkanes (carbon numbers 32–34 and 38–39) were significantly more depleted than compounds in the

Table 3: Comparison of degradation performance of n-alkanes, selected i-alkanes and the unresolved complex mixture (UCM) in the three treatments. Numbers are relative concentrations (% of day 0). At time of analysis (day 116 for nitrate and sulphate as TEA and day 5 for aerobic microcosms), approximately 83% of initial TPH concentration is remaining.

<table>
<thead>
<tr>
<th>Terminal electron acceptor/Compound group</th>
<th>Oxygen</th>
<th>Nitrate</th>
<th>Sulphate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% of day 0 (17% degraded)</td>
<td>% of day 0 (17% degraded)</td>
<td>Difference to aerobic test? If yes, α</td>
</tr>
<tr>
<td>n-Alkanes, carbon number range</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11–13</td>
<td>67 (8.0)</td>
<td>79 (3.5)</td>
<td>Not significant</td>
</tr>
<tr>
<td>14–16</td>
<td>68 (6.5)</td>
<td>56 (2.7)</td>
<td>Not significant</td>
</tr>
<tr>
<td>17–19</td>
<td>69 (6.7)</td>
<td>54 (6.5)</td>
<td>0.05</td>
</tr>
<tr>
<td>20–22</td>
<td>70 (7.2)</td>
<td>55 (5.6)</td>
<td>0.05</td>
</tr>
<tr>
<td>23–25</td>
<td>71 (7.3)</td>
<td>53 (6.0)</td>
<td>0.01</td>
</tr>
<tr>
<td>26–28</td>
<td>72 (7.2)</td>
<td>52 (5.6)</td>
<td>0.001</td>
</tr>
<tr>
<td>29–31</td>
<td>71 (4.5)</td>
<td>58 (4.4)</td>
<td>Not significant</td>
</tr>
<tr>
<td>32–34</td>
<td>75 (3.3)</td>
<td>62 (5.5)</td>
<td>Not significant</td>
</tr>
<tr>
<td>35–37</td>
<td>73 (2.3)</td>
<td>63 (7.0)</td>
<td>Not significant</td>
</tr>
<tr>
<td>38–39</td>
<td>74 (2.9)</td>
<td>58 (7.6)</td>
<td>0.05</td>
</tr>
<tr>
<td>UCM</td>
<td>85 (3.5)</td>
<td>88 (4.3)</td>
<td>Not significant</td>
</tr>
<tr>
<td>∑(FAR, PRI, PHY)</td>
<td>95 (3.0)</td>
<td>73 (7.0)</td>
<td>0.001</td>
</tr>
</tbody>
</table>

The value given in parenthesis is one standard deviation (n = 3). Statistics were ANOVA/Bonferroni for group identification and Tukey’s test for significance, α. Significance level of statistical analysis.

sulphate-reducing conditions the branched alkanes pristane and phytane were significantly better degraded that their unbranched chromatographic neighbors.

3.4. Comparison of hydrocarbon composition at similar concentrations with different acceptors

Samples with a similar residual hydrocarbon concentration, roughly 8.400 mg TPH/kg soil dry weight (approximately 17% degradation), were chosen for comparative compositional analysis from the three experimental lines. Due to different degradation rates, these were taken from different incubation times.

Results of the statistical analysis of n-alkane, i-alkane and UCM degradation under the three TEA-conditions are summarized in Table 3.

The largest fraction of TPH contamination is made up by the UCM. Its degradation was found to be not significantly influenced by the type of electron acceptor present (on average 13%, Table 3). Under aerobic conditions, the degradation of n-alkanes decreased with increasing carbon number. In contrast, sulphate reduction was connected to less degradation of n-alkanes up to n-C19 and more pronounced degradation for compounds of n-C35 and higher molecular weight. Alkanes with a chain length of up to 31 carbon atoms were significantly better degraded under nitrate reducing than under sulphate reducing conditions. With nitrate addition, mid-weight compounds (n-C17–n-C28) were more readily degraded than under aerobic conditions. The selected iso-alkanes (sum of FAR, PRI and PHY) were degraded significantly better under both anaerobic than under aerobic conditions, with 73% and 79% remaining versus 95% with nitrate, sulphate and oxygen as terminal electron acceptors, respectively.

4. Discussion

4.1. Electron acceptor ratios in anaerobic biodegradation of crude oil hydrocarbons

TPH degradation rates under different electron accepting conditions were decreasing from oxygen (470 mg TPH/kg/d, over 16 days), and nitrate (28 mg TPH/kg/d, over 185 days) to sulphate (18 mg TPH/kg/d, over 185 days). This result is a decreasing reactivity of the electron acceptors and consequently, of a strongly decreasing energy gain from the corresponding oxidation/reduction reactions (Thauer et al., 1977). Gibbs free energy (ΔG°) per mol hydrocarbon mineralized with nitrate and sulphate reduction, however, differs by a factor of around 15 (Widdel and Rabus, 2001; Heider et al., 1999).

In this study, the fraction of the TPH mixture that was degraded (in mol C, assuming 15% (w/w) hydrogen; Peters et al., 2005) was converted to the degradation of a hypothetical model compound, n-C25, to calculate the stoichiometric TEA amount required for this degradation. Using n-C25 as a model compound appears realistic since an n-alkane with 25 carbon atoms elutes in the middle of the presently investigated retention time window, which is delimited by n-C10 and n-C40 (Fig. 1). Using the molecular weight of a saturated rather than an aromatic compound, which may differ significantly with differing hydrogen contribution for the same carbon number, appears justified. Saturates compose the majority of well degradable compounds in the oil used (Table 1). This assumes resins and asphaltenes to be rather recalcitrant (Peters et al., 2005).

The theoretical stoichiometric electron acceptor demand for n-C25 mineralization, assuming no biomass production, would amount to 25:3:1 for nitrate and to 15:2:1 for sulphate as TEA, expressed in mM TEA (reduced to HS- or N2) per mM n-C25 mineralized (e.g. Haeseler et al., 2010). Considerably lower ratios were
noted in this study, with 4.3:1 for nitrate and 1.2:1 (over 185 days) for sulphate. The regeneration of reduced electron acceptors on the expense of other oxidized species, such as sulphide re-oxidation via geogenic Fe(III)-compounds, can induce apparently stoichiometric ratios. On the other hand, since the crude oil fractions eluting before n-C10 and after n-C40, which may also be degraded, are not accounted for in these calculations, actual ratios are probably even lower. In general, these stoichiometric ratios indicate the occurrence of incomplete hydrocarbon mineralization under both anaerobic conditions. The accumulation of metabolites in anaerobic aquifers is often observed (Gieg and Sulfila, 2002).

Several authors have postulated that petroleum hydrocarbon degradation in the absence of molecular oxygen, which is an essential reactant in hydrocarbon activation as well as a TEA, would need to follow profoundly different activation mechanisms (e.g. Grossi et al., 2008; Widdel and Rabus, 2001; Spormann and Widdel, 2000) than those present under aerobic conditions. Two proven (plus one hypothetical) mechanisms for the anaerobic oxidation of n-alkanes are known. One is the activation of the subterminal (C-2) carbon atom followed by addition to fumarate (Wilkes et al., 2002). The other proven mechanism is carboxylation with inorganic carbon at C-3 (So et al., 2003). The theoretical possibility for intracellular oxygen production via chlorate respiration (Mehboob et al., 2009) or nitrite (Ettwig et al., 2010) has not been demonstrated yet.

Intermediate products such as fatty acids (Callaghan et al., 2009) and other partially oxidized hydrocarbons exhibit a higher polarity than their parent compounds (Cravo-Lauere et al., 2005; Beller et al., 1995; Gieg and Sulfila, 2002) and are therefore not resolved with unpolar chromatographic systems, as used presently. One practical approach for the simultaneous measurement of polar and unpolar contaminants is via two-dimensional gas chromatography (Vasilieva et al., this issue), which may contribute to the elucidation of the reasons for the incomplete degradations of hydrocarbons under anaerobic conditions (Gieg and Sulfila, 2002).

4.2. Alkane fingerprints under different TEA-conditions

4.2.1. Aerobic biodegradation

Distinct n-alkane degradation patterns evolved under different reducing conditions (Fig. 2). By and large, degradation under aerobic conditions followed previously published results (Leahy and Colwell, 1990; Wang and Fangas, 2003), with (i) preferential degradation of lower over higher molecular weight (Table 3) and (ii) of linear over branched alkanes (Table 2 and Fig. 3). Furthermore, the UCM, which is composed of compounds with higher complexity, showed limited degradation (Table 3).

4.2.2. Sulphate reducing conditions: high molecular weight linear alkanes

The application of nitrate and sulphate as terminal electron acceptors induced a profoundly different petroleum hydrocarbon degradation pattern than under aerobic conditions. Under aerobic conditions, short chain alkanes are degraded faster than long chain alkanes. While our results confirm this degradation pattern, we observed an inverted behavior under sulphate reducing conditions (Fig. 2C). We found lesser removal for lower molecular weight compounds and an increased degradation of higher molecular weight compounds.

The preferential aerobic degradation of short chain alkanes is attributed to biological (enzymatic activity, steric hindrance) and chemical factors (water solubility, surface tension; Setti et al., 1993). For anaerobic degradation, most of the research performed focused on alkanes in the liquid range (C6–C16). Metabolic reactions involving alkanes >n-C20 have not been characterized thoroughly (Grossi et al., 2008 and Grossi et al., 2011). One possible explanation, however, for the observed preferential degradation of higher molecular weight n-alkanes is offered in terms of biochemical energy yield. In the initial activation reaction, fumarate addition to alkanes is exergonic (ΔG° < 0; Rabus et al., 2001), while alkane carboxylation is energetically feasible only under distinct physiological conditions (Thauer and Shima, 2008). On the other hand, the calculatory overall biochemical energy yield from alkane mineralization is directly related to alkane chain length. According to Spormann and Widdel (2000), the Gibbs free energy per mol of TEA that is reduced on the expense of hydrocarbon oxidation is increasing with increasing hydrocarbon chain length. For denitrification, ΔG° is equal to −492.8 kJ/mol NO3 for n-C6 and −493.7 kJ/mol NO3 for n-C16. For sulphate reduction, one mol of sulphate yields −442.1 kJ on the expense of n-C6 oxidation and −456.6 kJ with n-C16 (Spormann and Widdel, 2000). Accordingly, the additional energy gain from n-C16 degradation compared to n-C6 degradation is 0.9 kJ/mol NO3 and 1.4 kJ/mol SO42−. Thus, the ΔG° obtained from the reduction of one mol TEA is a function of the oxidized hydrocarbon’s chain length. Hydrocarbon oxidation under sulphate reducing conditions is connected to a particularly low ΔG° compared to denitrification (e.g. −45.6 kJ/mol SO42− versus −493.7 kJ/mol NO3 for n-C16 oxidation; Spormann and Widdel, 2000). Thus, the relative increase in ΔG° from n-hexane to n-hexadecane is 0.2% for nitrate and 3.2% for sulphate. In these calculations, however, the energy investment for hydrocarbon activation is not explicitly accounted for. It can be hypothesized that a small increment in energy yield led to the preferential degradation of long- over short-chain alkanes under sulphate-reducing conditions that was observed (Fig. 2C).

4.2.3. Nitrate and sulphate reducing conditions: low molecular weight n-alkanes

Under nitrate reducing conditions, three low molecular weight compounds (C11–C13) were significantly more recalcitrant to biological attack than the majority of other n-alkane groups (Fig. 2B). Thermodynamic constraints likely do not account for the higher degradation rates of alkanes >C13.

Considering the physical properties of these compounds, it appears more likely that hydrocarbon toxicity contributed to inhibiting their degradation. At room temperature liquid hydrocarbons, up to n-C17, have solvent characteristics and exert physiological toxic effects by solubilizing cell membranes (Heipieper and Martinez, 2010). Under aerobic conditions, n-C11 and n-C12 concentration reductions were found to be initially controlled by evaporation. In contrast, such an evaporation, which is limited to compounds with less than 15 carbon atoms (Payne et al., 1991), was impossible in the tightly shut anaerobic microcosms. It can be therefore assumed that toxic effects of lower molecular weight compounds were attenuated by their quick volatilization under aerobic conditions. That is, the degradation of these compounds was possibly inhibited by their toxicity under anaerobic conditions.

4.3. Sulphate reducing conditions: branched alkanes

Under sulphate-reducing conditions, the increase of the three determined n- to i-alkane ratios (Table 2) indicated the preferential degradation of branched over corresponding linear alkanes. Relative concentrations of pristane and phytane after 185 days of incubations were in fact significantly lower than those of n-C17 and n-C18 (Fig. 3). While iso-alkanes were long regarded as relatively inert biomarkers (Bailey et al., 1973; Hostettler and Kvenvolden, 2002), pristane biodegradation under nitrate-reducing and methanogenic conditions has been observed (Bregnard et al., 1997). The herein presented results are, to our knowledge, the first observation of a preferential degradation.
of n- over chromatographically neighboring iso-alkanes. Interestingly, the chromatogram of the soil site that was used for inoculation showed distinct iso-alkane peaks but was depleted of n-alkanes (Fig. 1). However, the predominant electron accepting process at the site was not determined. A possibly reduced bioavailability of branched alkanes via sorption to soil organic matter (Zhu and Pignatello, 2005) may also contribute to these effects. Due to the small differences of the paired alkanes’ carbon numbers, e.g. n-C18 and i-C20 (phytane), it is unlikely that a similar energetic explanation as for the increased degradation of long chain alkanes under sulphate-reducing conditions would apply here.

While there is little known about the mechanisms governing the anaerobic degradation of iso-alkanes, the presently obtained results may have a profound effect on the applicability of n- to i-alkane ratios for the assessment of sites contaminated with crude oil and its products (Christensen and Larsen, 1993; Wang et al., 1998). For sulphate reducing conditions, the present results contradict the widely accepted assumption of higher degradation rates for linear than branched alkanes (Leahy and Colwell, 1990; Wang and Fingas, 2003). According to the present data, this concept could lead to misleading results when aerobic degradation patterns of linear and branched alkanes are applied to anaerobic conditions. Further research is warranted on this issue.

4.4. Anaerobic bioremediation of crude oil contaminated sites

Anaerobic bioremediation of petroleum hydrocarbon contaminated sites may be applied where the introduction of oxygen into the subsurface is costly or inefficient. Many TPH-contaminated sites are depleted of oxygen due to the natural attenuation, requiring the addition of massive amounts of oxygen for the re-establishment of aerobic conditions (Boopathy, 2003). The presently used TEAs have an aqueous solubility exceeding that of gaseous oxygen (8.6 mg/L at 101.1 kPa and 25°C in fresh water) by several orders of magnitude (Crawford et al., 1998; Motamedi and Pedersen, 1998). In addition to the potential benefits of long-distance TEA transport with the groundwater flow (Bouwer and McCarty, 1984) this renders the application of anaerobic TEAs an attractive alternative. Aspects requiring further attention include lower degradation rates than under aerobic conditions and the behavior of TEA reduction products such as nitrite and sulphide precipitates in the subsurface.

5. Summary and conclusions

In the present study, the development of distinct alkane fingerprints was observed when different TEAs were applied to support the biodegradation of crude oil. Remarkably, under sulphate-reducing conditions, higher degradation rates were observed for higher molecular weight compounds. This was, to a minor extent, also observed under nitrate reducing conditions. A small increment in Gibbs free energy (ΔG°) per mol sulphate reduced at the expense of increasingly longer n-alkanes or energy investments for alkane activation provide an explanation for this behavior. This appears more plausible for degradation reactions with a particularly low energy yield, such as sulphate reduction, than for aerobic degradation.

First evidence of the preferential degradation of iso- over chromatographically neighboring n-alkanes under sulphate reducing conditions is provided. While the identification of the underlying mechanisms requires further in-depth research, this may profoundly influence the way alkane ratios are used presently for the assessment of natural attenuation processes or of the efficiency of remediative measures for petroleum hydrocarbon contaminations in the field. The distinct, TEA-specific hydrocarbon fingerprints identified in the present study provide the possibility to obtain a more comprehensive picture of ongoing subsurface processes.

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Appendix A. Supplementary data


References


