Sequential Hydrocarbon Biodegradation of a soil from arid coastal Australia oil-treated under laboratory controlled conditions.

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Keywords: Biodegradation, Petroleum, Soil, Hydrocarbon, Methylnapthalenes, Methylphenanthenes.

Abstract
A general consistency in the sequential order of petroleum hydrocarbon reduction of previous biodegradation studies has led to the proposal of several molecular based biodegradation scales. Few studies have investigated the biodegradation susceptibility of petroleum hydrocarbon products in soil media, however, and metabolic preferences can change with habitat type. A laboratory based study comprising the GCMS analyses of extracts of oil-treated soils incubated for up to 161 days was conducted to investigate the biodegradation of crude oil exposed to sandy soils of Barrow Island, home to both a class A nature reserve and Australia’s largest on-shore oil field. Biodegradation trends of the Barrow soils were largely consistent with previous reports but some unusual behavior was recognised both between and within hydrocarbon classes. For example, the n-alkanes persisted at trace levels from day 86 - 161 following the removal of typically more stable dimethyl naphthalenes and methyl phenanthenes. The relative susceptibility to biodegradation of different di- tri- and tetramethylnaphthalene isomers also showed several features distinct from previous reports. The unique biodegradation behavior of Barrow Is. soil likely reflects difference in microbial functioning with physiochemical variances of the environment. Correlation of molecular parameters, reduction rates of selected alkynaphthalene isomers and CO₂ respiration values to a delayed (61 d) oil-treated soil identified a slowing of biodegradation with microcosm incubation, and a reduced function or population of incubated soil flora might also influence the biodegradation patterns observed.
1. Introduction

Numerous studies have assessed the effects of environmental biodegradation of petroleum hydrocarbons (e.g., Volkman et al., 1984; Rowland et al., 1986, Loehr, 1991; Sugiura K et al., 1997; Wenger et al., 2001; Fisher, 2002; George et al., 2002; Huang et al., 2004; Barman Skaare et al., 2007). Biodegradation is a sequential process, \( n \)-alkanes typically amongst the first hydrocarbons to be removed, with lighter homologues (C\(_6\)-C\(_{12}\)) being most susceptible. This is followed by the removal of other hydrocarbon classes depending on the capabilities of the microorganisms, but the general order is iso-alkanes, cycloalkanes, 1-3 ring aromatics, polyaromatics, asphaltenes and resins.

Biodegradation of aromatic products typically decreases with increasing molecular weight (MW), number of methyl substituents and number of rings (e.g. Loehr, 1991; Skiba et al., 1991; Budzinski et al., 1998; Sauer et al., 1998; Bispo et al., 1999; Leblond et al., 2001). Naphthalenes degrade prior to phenanthrenes and dibenzothiophenes and chrysenes are much more resistant (Sauer et al., 1998). Differing susceptibility to biodegradation of isomers of alkyl naphthalenes, alkyl phenanthrenes, diphenylmethanes and alkyl biphenyls have also been reported (Solanas et al., 1984; Volkman et al., 1984; Rowland et al., 1986; Williams et al., 1986; Budzinski et al., 1995; 1998; Cassani and Eglinton, 1991; Fisher et al., 1996, 1998; Ahmed et al, 1999; Trolio et al., 1999; Fisher, 2002), and are largely controlled by the size and ring position of the alkyl substituents (Huang et al., 2004; Le Blond et al., 2001).

The general consistency of hydrocarbon biodegradation trends has led to the development of several biodegradation scales based on the relative abundances of different groups and biomarker ratios of sequentially more susceptible structures (Volkman et al., 1984; Peters and Moldowan, 1993; Wenger et al., 2001; Wenger and Isaksen, 2002; Fisher, 2002; Peters et al., 2005). However, few studies have investigated the biodegradation of petroleum hydrocarbons in soils and different physiochemical conditions may lead to variances in the metabolic function of microbial communities. In-reservoir biodegradation was historically attributed to aerobic biodegradation, but anaerobic
biodegradation is now thought to be more important (Head et al., 2003). Simultaneous aerobic and anaerobic conditions can exist in soil. The few studies to have investigated the aerobic biodegradation of hydrocarbon constituents of petroleum impacted soils (Solanas et al., 1984, Rowland et al., 1986, Loehr, 1991; Chang et al., 2002) have largely been consistent with the biodegradation trends in reservoired oils, although subtle differences in behavior have been noted.

Here we report the relative vulnerability of aliphatic and aromatic hydrocarbon constituents of Barrow crude oil to biodegradation following its exposure to the arid coastal soil of Barrow Island (WA). Oil treated soils were maintained in a regulated microcosm environment for 161 days and the composition of solvent extractable hydrocarbons was analysed on several occasions using GCMS to follow the biodegradative progress.
2. Experimental

2.1. Field Site

The soil samples were from Barrow Island, off the northwest coast of Western Australia. As a result of its pristine environment and populations of Australian and endemic species, some of which are listed under the Wildlife Conservation Act as threatened species, Barrow Island has been classified a Class A Nature Reserve. Despite its intrinsic environmental value, it is home to Australia’s largest on-shore oil field. Production commenced in 1967 and is expected to continue for at least another two decades (http://www.gorgon.com.au).

2.2. Samples

Non oil contaminated soil of 0-10 cm depth was collected from Barrow Island at a location 2 km north of the airstrip (UTM Grid 50K 331442 E, 7696050 N). The soil was collected using clean non-hydrocarbon containing tools, placed in Hessian bags to avoid hydrocarbon contamination and stored at 4ºC. Prior to incubation, the soil was sieved to 2 mm using a metal sieve.

Typical of the sandy soils of Western Australia, Barrow soils are characterised by low carbon (1.33 g total C/kg soil) and macronutrient (0.07 g total N/kg soil; trace $\text{PO}_4^{3-}$) values. Their mineral content is predominantly clay (5-10 %), hematite (5 %) and calcite (5 %) with traces of illite and feldspar. Their porous texture and an arid, low rainfall climate conspire to maintain very low moisture levels at < 2 % (Barron, 2004; Davie, 2004).

Crude oil (5 L) was extracted from the Barrow Island production flow line station and stored in a metal container at 4ºC to minimise volatilisation and microbial activity. The production oil represents a composite oil from several producing wells. The two main Barrow sub-Basin oils are a Late Jurassic or Early Cretaceous highly paraffinic oil and a Cretaceous naphthenic-aromatic oil almost devoid of $n$-alkanes (Volkman et al., 1983). Barrow oils have also shown different degrees of biodegradation (Volkman et al., 1983) and aromatic content with Volkman et al. (1984) reporting a range of 12-34 % aromatics.
for a selection of four Barrow oils. Column chromatography (on a larger scale to that described in section 2.5) showed the present production oil comprises 49.9 % aliphatics and 9.4 % aromatics.

2.3 Incubation Study

Soil samples (~1 kg), housed in glass jars (385 mL) were either exposed or not to Barrow crude oil (50 mL/kg). A concentration of 50 mL oil/kg soil had previously been found to produce the highest microbial respiration response over the range 0.5 - 100 mL kg\(^{-1}\) (Barron, 2004; Davie, 2004). The oil-treated soils were additionally amended with 100 mg/kg of both phosphorus and nitrogen and deionised water to 60 % water holding capacity (WHC) to promote microbial activity (Barron, 2004; Davie, 2004). The oil, water, macronutrients and soil were combined by gentle rolling in Al foil. Millipore 45 µm membrane filters were placed over jars to prevent contamination from outside microorganisms, while allowing diffusion of gases. The jars were incubated at 25ºC in the dark, typical laboratory incubation conditions for mesotrophic microorganisms (Linn and Doran, 1984; Carter and Tibbett, 2006), for 161 d. This temperature also is close to the annual mean average temperature of Barrow Island of 25.8ºC (http://www.bom.gov.au/products/IDW60801/IDW60801.95304.shtml). The soils were further watered on a weekly basis from day 61 to offset moisture losses (determined on a gravimetric basis) and maintain WHC at 60 %.

Concurrent incubations of a non oil-treated soil, a delayed 61 d oil-treated soil and a sterilised oil-treated soil were also studied. The non-oil exposed soil helped distinguish extractable soil organic matter (SOM) and petroleum hydrocarbons of the oil-treated soils. The delayed treatment allowed a temporal assessment of the effect of microcosm incubation on biodegradation and the sterilised treatment helped distinguish losses from abiotic processes. Duplicate experiments were performed for all soil treatments.

2.4. CO\(_2\) respiration

Microbial respiration of CO\(_2\) was measured from day 61 of treatments using an Infrared Gas Analyser (IRGA, Series 225 Gas Analyser, Hoddesdon, UK) as described by Clegg
et al. (1978). Between 0.25 and 1 mL of head space gas was extracted from each microcosm and analysed against a CO\textsubscript{2} standard (4.66, 4.86 or 4.95 % v/v). Measurement of CO\textsubscript{2} respiration is a popular method for the assessment of biodegradation studies because of its simple, nondestructive, and representative nature (Sharabi and Bartha, 1993, Huesemann and Moore, 1993).

2.5. Extraction and GC-MS

Sub samples of microcosm soil (10 g) were collected for GCMS analysis on days 61, 86, 111 and 161 of incubation. The soil samples were extracted with a 9:1 mixture of dichloromethane (DCM) and methanol using a Dionex accelerated solvent extractor. Polarity based fractions of the extracts and crude oil were obtained using column chromatography. The extract/oil (2 mL) was applied to the top of a small column (5.5 x 0.5 cm i.d.) of activated silica gel with a cotton wool filter at the base of the column. A combined saturate and aromatic (SAA) fraction was obtained by elution with 2 mL DCM in hexane solution (0.3:0.7; v/v).

Analysis of the SAA fraction was performed with either an Agilent 6890/5975b GCMS or a 6890/5973 GCMS, depending on availability. Both instruments were equipped with a 60 m x 0.25 mm x 0.25 μm ZB-5 (Phenomenex) capillary column and used a constant helium carrier gas flow of 1.1 mL/min. The chromatograph was used in pulsed splitless mode and the oven temperature programme was: 40°C (held 2 min) at 4°C/min to 300°C (held 20 min). Full scan (m/z 50 – 550) and selected ion mass spectral data were either simultaneously (5975b) or separately (5973) acquired. Relatively standard MS conditions, including an electron energy of 70 eV; source temperature of 230°C and transfer line temperature of 300°C, were used.

Component identification was based on comparison to library mass spectra and GC retention profiles. The identity of alkylnaphthalene isomers was confirmed by correlation to oil fractions with well defined alkyl naphthalene profiles from previous analyses and correlation in our laboratories, including with several Barrow sub-Basin oils (e.g., Volkman et al., 1984, Fisher et al., 1998, Fisher, 2002). The areas of the diagnostic
parent ion peaks of the polymethyl naphthalenes and m/z 191 peak of 17α(H), 21β(H)-hopane (C$_{30}$αβH) from selected ion recorded (SIR) data were integrated to report product abundances and biomarker ratios. With its extremely low solubility, biodegradability and volatility, C$_{30}$αβH is preserved until very severe levels of biodegradation and is commonly used as an internal conservative standard for quantifying the extent of biodegradation (Bragg et al., 1994; Prince et al., 1994; Pollard et al., 1999; Peters et al., 2005; Gallego et al., 2006).

3. Results and discussion

3.1. Compositional changes

The total and selected m/z 85 ion chromatograms of the SAA fractions of Barrow crude oil and the oil-treated soil at day 61 are shown in Figure 1. Consistent with a mixture of the paraffinic and naphthenic oils of the region (Volkman et al., 1983), the composition of the production oil includes a homologous series of n-alkanes from C$_{10}$ to beyond C$_{28}$, (Fig. 1a, b) and distinctive distributions of alkyl naphthalenes. Combined integration of peaks associated with specific product classes relative to the entire total ion chromatogram showed a production oil composition of 10.5 % n-alkanes and 10.2 % C$_0$-C$_4$ naphthalenes. A pronounced baseline attributed to hydrocarbons of an unresolved complex mixture (UCM) or hump typical of a biodegraded oil represented a significant proportion (77.3 %) of the overall GCMS signal.

The SAA fraction of the 61 d oil-treated soil showed several features indicative of further biodegradation, including the increasing prominence of the UCM (Fig. 1c) and significant depletion in n-alkanes, particularly < C$_{20}$ (Fig. 1d). The parent ion profiles of C$_2$-C$_4$ alkyl naphthalenes and C$_1$-C$_2$ alklyphenanthrenes of the crude oil and extracts of the contaminated soil at different stages of the 161 day treatments are shown in Figs 2-5.
The ratios of selected aliphatic and aromatic hydrocarbons, reflecting low to moderate levels of biodegradation (Fisher, 2002; Peters et al., 2005), were measured at days 61, 86, 111 and 161 (Table 1). The tabulated values are means of duplicate analyses for each treatment. Alkynaphthalene concentration data of each analytical replicate are shown in Figure 6. At day 61, the aliphatic parameters \( n-C_{17}/Pr \) (Pr = pristane) and \( n-C_{18}/Ph \) (Ph = phytane) were 70 % and 75 % lower, respectively, than their fresh oil values (Table 1). These \( n \)-alkane and isoprenoid hydrocarbons were not detected by analysis of corresponding 61 d non-oil treated soils indicating that the soil organic matter (SOM) does not directly contribute to the value of these aliphatic parameters.

The day 61 analysis also showed a significant reduction in \( C_2 \) naphthalenes (Fig. 2) and methyl phenanthrenes (Fig. 5), with more susceptible isomers removed completely. The day 61 values for the biodegradation-sensitive dimethylnaphthalene (1,6-/1,5-) and methylphenanthrene (2-/9-) isomer ratios were 94 % and 86 %, less than their fresh oil values, respectively (Table 1). A decrease in abundance of the \( C_3 \) naphthalenes and \( C_2 \) phenanthrenes was also evident at day 61, with slight to moderate alteration of their isomeric profiles. The \( C_3 \) trimethylnaphthalene ratios (Table 1) had decreased from fresh oil values by 68 % (1,3,6-/1,2,4-) and 86 % (2,3,6-/1,2,4-), respectively. The decrease in abundance of tetramethyl naphthalenes and dimethyl phenanthrenes was not as significant, reflecting the increasing resistance to biodegradation of alkylaromatics with increasing MW. General depletion of the \( n \)-alkanes and alteration of the \( C_2-C_3 \) naphthalenes and the methyl phenanthrenes is consistent with a biodegradation level of 2-3 on the scales proposed by Fisher (2002) and Peters et al. (2005).

Progressive biodegradation was evident from analysis of the SAA fractions of days 86, 111 and 161 oil-treated soils. All \( C_2 \) naphthalenes and methyl phenanthrenes (apart from trace levels of more resilient isomers) were removed by day 86. The aliphatic and \( C_3-C_4 \)-methyl naphthalene biodegradation parameters also continued to decrease. These two classes were largely reduced to trace levels at days 86 and 161, respectively. A gradual decrease in the abundance of \( C_2 \) phenanthrenes was observed throughout the experiment (Fig 5), but the overall distribution was largely unaltered with isomers reflecting few
differences in relative susceptibility.

3.2. Temporal influence of microcosm incubation on biodegradation

The 161 d values of the molecular parameters listed in Table 1 indicate that a moderate level of biodegradation was reached by the completion of the treatments. There was only partial reduction of residual \( n \)-alkanes, \( C_3 \)-\( C_4 \) naphthalenes and \( C_2 \) phenanthernes, with at least trace levels of these hydrocarbons detected at day 161. The 70 % and 75 % decrease in the values of \( n-C_{17} \)/Pr and \( n-C_{18} \)/Ph, respectively, mentioned above to day 61 was followed by much more modest decreases of 7 % and 15 % over the final 100 days.

A study investigating the biodegradation of incubated inoculums of oil-treated sea floor sediments in a separate laboratory set-up (i.e., 0.15 mL oil/500 mL sediments constrained within a flask) showed a much more advanced level of biodegradation after just 14 days (Rowland et al., 1986) with all of the \( C_2 \)-naphtalenede and \( C_3 \)-naphtalenenes (apart from \( \alpha \beta \)-ethylmethylnaphthalene) removed. The relatively slower biodegradation evident in the sandy Barrow Is. soils is probably due to historically severe moisture limitation of hydrocarbon degrading activity, associated with the low rainfall and high rate of evaporation on Barrow Island. The lower oil concentration of oil pollutants used by Rowland et al (1986) may also aid rapid hydrocarbon biodegradation. The metabolic quotient of microbial biomass relative to respired CO\(_2\) following exposure of Barrow soil to crude oil in a similar manner to the present laboratory treatments indicated more efficient degradation of lower concentrations of oil (Davie, 2004; Barron, 2004).

Significantly, the day 61 values of the aliphatic parameters, 1,6-/1,5-dimethylnaphthalenes and 2-/9- methylphenanthernes and the day 86 trimethyl naphthalene and 1237/1267 tetramethyl naphthalene biodegradation parameters were all distinctly lower than the corresponding 161 d values of the delayed 61 d oil treatment (Table 1). This indicates that biodegradation of these hydrocarbons was less over the 100 day period following the delayed (61 d) oil treatment, compared to the shorter 61 or 86 day period following the initial (0 d) oil treatment and therefore that their biodegradation was faster at the beginning of the microcosm treatments than the end.
The concentration data of selected alkyl naphthalene isomers (relative to C₃₀αβH) from both 0 d and 61 d oil treatments fitted to a first order kinetic model to derive a biodegradative decay constant are shown in Figure 6. In each of these cases, the delayed oil-treatment showed a lower rate constant than the initial oil-treatment providing a quantitative measure of the slower rates of biodegradation occurring over the final 100 d period of the treatments. Data from each of the analyses of 61, 86, 111 and 161 d treatments are also separately shown to provide an indication of the reproducibility of the duplicate analysis.

Microbial CO₂ respiration provides an indication of microbial activity (Sharabi and Bartha, 1993, Huesemann and Moore, 1993) and has been previously been correlated to the extent of hydrocarbon degradation (Baptista et al., 2005). Accumulated CO₂ evolution over the corresponding 70-100 d post oil addition period for both the initial and delayed oil-treatments was 32 µg C-CO₂/g soil and 24 µg C-CO₂/g soil, respectively. The lower CO₂ respiration of the delayed oil treatment provides further evidence that it was biodegraded more slowly than the oil of the initial (0 d) treatment.

The temporal slowing of biodegradation is most likely due to poor microcosm maintenance of the soil microbial community. The relatively small scale and confined nature of 1 kg of soil within glass jars may not be particularly representative of open soil environments. Heterotrophic bacterial counts of a laboratory controlled microenvironment used to study the bioremediation of diesel oil contaminated soils showed consistently higher numbers at day 55 of treatment than at the beginning of the treatment (Mariano et al., 2007). This suggests the temporal slowing of biodegradation evident in the present data may be due to a change in microbial function, rather than a reduction in bacterial populations.

3.3. Relative susceptibility to biodegradation of different hydrocarbon classes

A general consistency in the orderly loss of hydrocarbons has been reflected by most
studies of petroleum related biodegradation. The removal of 1-3 ring aromatics generally follows the removal of \( n \)-alkanes, which are particularly susceptible to biodegradation. The Barrow soil data however, shows that the \( n \)-alkanes persist to day 161, following the removal of typically more recalcitrant dimethyl naphthalenes and methyl phenanthrenes by day 86. Such unusual biodegradation behavior may be due to the hydrocarbon metabolic preferences of the soil microbial community. The physiological and ecological characteristics of micro-organisms are likely to be extremely diverse in different habitats (Atlas and Bartha, 1993). Whilst a general persistence of higher molecular weight polyaromatic hydrocarbons (PAHs) in soils has been attributed to a lack of specialised bacteria (Straube et al., 1999), the Barrow soil may contain bacteria which are relatively efficient at degrading lower MW PAHs. Notwithstanding this, the stressed nature of the soil microbes in the microcosm environment of the experiments, inferred from the apparent slowing of biodegradation with microcosm incubation time, might also lead to a divergence from natural biodegradation behavior.

Whilst most petroleum hydrocarbon biodegradation studies accord with the biodegradation scale outlined in Peters et al (2005), there have been several reports of notable divergence from these trends, including the more favored degradation of aromatic hydrocarbons (Fedorak and Westlake, 1981; Jones et al., 1983; Rowland et al., 1986; Leblond et al., 2001). The laboratory-based data of Rowland et al. (1986) showed that the \( n \)-alkanes of a crude oil were substantially utilized only after dimethyl naphthalenes had been severely depleted and the trimethyl napthalenes also affected. The early sequential degradation of these aromatics was suggested to be due to either promotion of a more efficient mechanism of aromatic degradation or because of an altered microbial composition - possibly influenced by nutrient amendment (Rowland et al., 1986). Varied N and P nutrient amendment has been shown to cause differences in the patterns of metabolic utilization of aromatic and saturated hydrocarbons of crude oils (Fedorak and Westlake, 1981). Li et al. (2008) also recently showed that inocula of certain bacterial and fungal isolates can selectively promote the biodegradation of particular aromatic hydrocarbons. The varied adsorption of different hydrocarbon classes to soil particles or aggregates, effectively rendering them unavailable to metabolic processing, can also
contribute to preferential degradation of certain PAHs (Leblond et al., 2001).

Hydrocarbon susceptibility to biodegradation may also be influenced by the proportions of aliphatic and aromatic degraders in the Barrow soil and the new carbon substrate available to them following addition of the oil. Whereas \(n\)-alkanes are major constituents of crude oils including the Barrow oil used in this study (Fig 1a, b), they are typically present in much lower concentrations in pristine soil. No \(n\)-alkanes were detected in the corresponding analysis of the non oil-treated soils of the present study. Aromatic rich higher plant precursors of soil organic matter (Wilson, 1988) may favor aromatic degrading micro-organisms. These may subsequently be able to cope better with the aromatic hydrocarbons of the oil, than the historically subdued concentrations of aliphatic degraders may be able to cope with the high alkane concentrations of the oil.

3.4. Relative susceptibility to biodegradation of individual \(C_2-C_4\) naphthalenes

The significant loss of the \(C_2\)-naphthalenes evident at day 61 and the slow reduction of the \(C_3\) and \(C_4\) naphthalenes from days 86 – 161 allowed a detailed investigation of relative susceptibility to biodegradation of individual polymethyl naphthalene isomers. The abundance of individual \(C_2-C_4\) polymethyl naphthalenes relative to \(C_{30}\alpha\beta\)H (recalcitrant to biodegradation) for all analysis periods is shown in Table 2.

The overall polymethyl naphthalene biodegradation trends of the present Barrow soil microcosm study are generally in accord with previous studies concerned with the specific biodegradation behavior of individual alkynaphthalene isomers (Solanas, et al., 1984; Volkman et al., 1984; Williams et al., 1986; Rowland et al., 1986; Ahmed et al., 1999; Fisher et al., 1996; 1998; Fisher, 2002). There are several examples (DMN: 1,6-; TMN: 1,6,7-; TeMN: 1,2,3,6-, 1,3,6,7-) of the relatively high vulnerability of components with substituents at C-1 and C-6  (Fisher et al., 2002) and the relatively slower biodegradation for isomers with adjacent methyl substituents (e.g., DMN: 1,2-,2,3- {&1,4}, TMN: 1,2,6-, 1,2,4-;TeMN: 1,2,6,7) attributed to steric effects (Volkman et al., 1984). However, the Barrow soil data also showed several variances from the biodegradation behavior of previous reports.
3.4.1. Dimethylnaphthalenes (DMNs)

The order of susceptibility of biodegradation of the dimethyl naphthalenes in the oil-spiked Barrow soils (Fig. 2; Table 2) was:

13 and 17 > 16 > 26 and 27 > 12 > 14 and 23 > 15

A similar susceptibility of 1,2- 2,6- and 2,7- dimethylnaphthalenes relative to 1,4- or 2,3-dimethylnaphthalene was identified in a controlled microenvironment investigation of biodegradation by Solanas et al. (1984) and was also evident in a series of biodegraded Barrow oils studied by Rowland et al. (1986). The oil data of the latter study showed 2,7-dimethylnaphthalene was more susceptible to biodegradation than 2,6-dimethylnaphthalene and the ratio of these two isomers was used as an indicator of the extent of biodegradation (Rowland et al. 1986). No distinction in relative susceptibility of dimethyl naphthalene biodegradation, however, was evident in a laboratory simulation of biodegradation of the same study (Rowland et al., 1986).

The Barrow soils also showed several contrasts to previous reports of C₂-naphthalene biodegradation behavior. 1,6-dimethylnaphthalene has been consistently reported to be the most susceptible isomer to biodegradation (Volkman et al., 1984, Fisher et al. 1996; 1998; Fisher, 2002), but here (Fig. 2) was less susceptible than the combined 1,3- and 1,7- dimethyl naphthalene pair. As a result of their co-elution, the separate effect of biodegradation on the 1,3- and 1,7- dimethylnaphthalene isomers cannot be distinguished on the basis of GCMS data alone. GC-Fourier transform infrared (FTIR) analysis has been used to determine the relative contribution of co-eluting alkyl naphthalenes and in turn their discrete biodegradation behavior (Budzinski et al. 1993, Fisher, 2002).

The most recalcitrant of the dimethylnaphthalene peaks was 1,5-dimethylnaphthalene, whilst co-eluting 2,3- and 1,4-dimethylnaphthalene showed the next least reduction. The 2,3-dimethylnaphthalene isomer is usually the most recalcitrant of the dimethylnaphthalenes (Volkman et al., 1984; Rowland et al., 1986; Fisher, 2002), but here its biodegradation behavior could not be discretely defined due to co-elution with
1,4-dimethylnaphthalene.

The concentration data of the most (1,3-\&1,7-) and least (1,5-) susceptible dimethyl naphthalene isomers of the Barrow soil data are both consistent with a first order reaction model as shown in Figure 6(a) and (b), respectively, albeit with the 1,3-\&1,7-dimethylnaphthalene data set following initial oil treatment limited to the original oil and SAA fraction of the 61 d incubation.

3.4.2. Trimethylnaphthalenes (TMNs)

Apart from 1,2,4-trimethylnaphthalene, which proved most recalcitrant of the trimethyl naphthalenes and was detected throughout the 161 day treatment, the other trimethylnaphthalene isomers were reduced by between 30-85 % by day 61 and by between 90-99 % by day 86, but remained at trace levels to day 161. The order of susceptibility of the assigned C$_3$-naphthalenes (Fig. 3; Table 2) was:

236 > 167 > 136 > 125 > 126 > 146 and 135 > 124

The recalcitrance observed for 1,2,4-trimethylnaphthalene is consistent with previous reports of its stability to biodegradation (Fisher et al., 1998; Fisher, 2002). However, the 2,3,6 isomer was the most susceptible, where previous reports have generally indicated 1,3,6-trimethylnaphthalene as the most susceptible of the trimethylnaphthalenes to biodegradation (Fisher et al., 1998; Fisher, 2002). Here, the 1,3,6 isomer showed comparable reduction to each of 1,6,7-, 1,2,5- and 1,3,7-trimethylnaphthalene. The 1,3,7-isomer was one of the more recalcitrant of the trimethylnaphthalenes to biodegradation in sea floor sediments adjacent to drilling operations (Fisher et al., 1998), although Rowland et al. (1986) observed greater reduction of the 1,3,7 (and 2,3,6) isomer compared to 1,3,6-trimethylnaphthalene.

The concentration data of the most (2,3,6-) and least (1,2,4-) susceptible trimethyl naphthalene isomers of the Barrow soil data have been fitted with a first order reaction model as shown in Figure 6(c) and (d), respectively. The reduction of both isomers shows a reasonable approximation to first order principles.
3.4.3 Tetramethylnaphthalenes (TeMNs)

The TeMNs were reduced to 28-81% of fresh oil abundances by day 86 and to 83-94% by day 161. The following distinctions in the order of susceptibility of tetramethylnaphthalene isomers were evident:

\[ 1237 > 1236 \sim 1367 > 2367 \sim 1357 > 1235&1256 > 1257 > 1267 > 1246&1247&1467 \]

The 1,2,3,7 isomer was the most susceptible of the tetramethyl naphthalenes to biodegradation, just ahead of the 1,2,3,6- and 1,3,6,7- isomers. The generally high susceptibility of these isomers is consistent with several previous studies (Fisher et al.; 1998; Fisher, 2002; Ahmed et al., 1999), although there have been different accounts of their relative order of susceptibility. For instance, 1,2,3,6-tetramethylnaphthalene was identified to be the most susceptible of the tetramethyl naphthalenes in sea floor sediments in the proximity of oil drilling operations (Fisher et al. 1998) and was also reported to degrade faster than the 1,2,3,7-isomer in the free hydrocarbon fraction of biodegraded coals (Ahmed et al., 1999).

The high biodegradation susceptibility of 2,3,6,7- and 1,3,5,7-tetramethylnaphthalenes in the Barrow soils, represents a further contrast to the sea floor sediments studied by Fisher et al. (1998; see also Fisher, 2002) in which they showed relatively greater recalcitrance. Here, the incubated oil-treated soils showed the co-eluting 1,2,4,6-, 1,2,4,7- and 1,4,6,7-tetramethylnaphthalenes to be the most stable. As a result of their co-elution, their relative concentrations or susceptibilities to biodegradation could not be distinguished. GC-FTIR was used to show that the relative proportions of these isomers in a crude oil isolate (Lambert-1) was 38% 1,2,4,6-, 34% 1,2,4,7- and 28% 1,4,6,7-tetramethylnaphthalene (Fisher, 2002).

The concentration data of the most (1,2,3,7-) and least (1,2,4,6- & 1,2,4,7- & 1,4,6,7-) susceptible tetramethylnaphthalene isomers of the Barrow soil data have been fitted with a first order reaction model as shown in Figure 6(c) and (d), respectively. The reduction of both isomers shows a reasonable approximation to first order principles.
4. Conclusions

The oil treated Barrow Island soils, showed several notable variances from the usual order of sequential hydrocarbon loss. \(n\)-Alkanes, whilst significantly removed by day 61 remained at trace levels from day 86-161. Dimethyl naphthalenes and methyl phenanthrenes, usually more stable to biodegradation than the \(n\)-alkanes, were absent from day 86. This behavior is probably due to the metabolic hydrocarbon preference of the soil microbial community, although the artificial conditions used in this study may also be influential. \(n\)-Alkane substrate saturation following oil exposure or reduced aliphatic degrading function of the soil microbial community with increased incubation may contribute to the relatively high recalcitrance of the \(n\)-alkanes. Lower CO\(_2\) respiration levels, hydrocarbon reduction rates and a decline in biodegradation sensitive hydrocarbon parameters from a delayed oil treatment indicated that biodegradation slowed with incubation time. Poor maintenance of the soil flora may contribute to unnatural functioning or representation of the indigenous soil microbial community and in turn the unusual biodegradation behavior observed. Improvements in the design of our microcosm incubation are needed for further laboratory based biodegradation studies and the outcomes of this research also need to be extended to open field environments.

Convincing evidence of the unique metabolic traits of the Barrow soil microbial community was evident from the unusually high susceptibilities to biodegradation shown by several di- (1,3- or 1,7-), tri- (2,3,6- and 1,3,7-) and tetra- (1,2,3,7) methyl naphthalenes. Environmental variations in biodegradation susceptibility due to the enzymatic adaption of different microbes to different substitution patterns may have implications for the use of previously ascribed alkyl naphthalene biodegradation parameters. Different isomeric pairings may be necessary to best represent biodegradation processes of distinct settings, highlighting the need for local site assessment of oil spill incidents. For example, the 2,3,6-/1,2,4- trimethyl naphthalene and 1,2,3,7-/1,2,6,7- tetramethyl naphthalene ratios are more responsive to the biodegradation
of Barrow soils than the traditionally used 1,3,6-/1,2,4- trimethylnaphthalene and 1,3,6,7-/1,3,5,7- tetramethylnaphthalene biodegradation parameters.

Acknowledgements:
Soil and oil samples and additional financial and logistical support was provided by Chevron Australia Pty Ltd. Sarah Wibrow’s European Joint Masters studies were supported by an Erasmus Mundus Scholarship. Reviews of an earlier manuscript by Kathy Burns, Mark Yunker and an anonymous reviewer greatly improved the quality of this manuscript and were much appreciated.

References


**Table Captions**

**Table 1.** Biodegradation parameters from hydrocarbon fraction extracted from Barrow soil exposed to Barrow crude oil in incubated microcosms. Pr=Pristane; Ph=Phytane.

**Table 2.** Parent ion abundance of C$_2$-C$_4$ naphthalenes vs. m/z 191 for C$_{30}$ αβ hopane for SAA fraction of Barrow oil and treated soil extracts (% decrease from original oil in parenthesis). All data represent the mean of duplicate analysis.

**Figure Captions:**

**Figure 1** (a) Total ion chromatogram and (b) m/z 85 chromatogram from SAA fraction of Barrow crude oil; (c) total ion chromatogram; (d) m/z 85 chromatogram from SAA fraction of soil treatments at 61 days incubation. Each chromatogram is normalised to the most abundant peak in the displayed region, the corresponding mass spectral signal of which is indicated in italics. C$_{17}$ = C$_{17}$ n-alkane; C$_2$N = C$_2$-naphthalenes.

**Figure 2** Partial m/z 156 chromatogram highlighting C$_2$-naphthalenes in SAA fraction of (a) Barrow Island oil; (b) oil-treated soil at 61 days incubation (numbers correspond to methyl locations). Each chromatogram is normalised to the most abundant peak in the displayed region, the corresponding mass spectral signal of which is indicated in italics. C$_2$-naphthalene profiles of subsequent days all show little more than baseline noise.

**Figure 3** Partial m/z 170 chromatogram highlighting C$_3$-naphthalenes in SAA fraction of (a) Barrow Island oil; (b) oil-treated soil at 61 days incubation; and (c) oil-treated soil at 86 days incubation (numbers correspond to methyl locations). Each chromatogram is normalised to the most abundant peak in the displayed region, the corresponding mass spectral signal of which is indicated in italics. The 86 d, 111 d and 161 d C$_3$-naphthalene profiles were similar, apart from showing further temporal decline in overall concentrations.

**Figure 4** Partial m/z 184 chromatogram highlighting C$_4$-naphthalenes in SAA fraction of (a) Barrow Island oil; (b) oil-treated soil at 86 days incubation; and (c) oil-treated soil at 161 days incubation (numbers correspond to methyl locations). Each chromatogram is normalised to the most abundant peak in the displayed region, the corresponding mass spectral signal of which is indicated in italics. Very similar C$_4$-naphthalene profiles were obtained for the fresh oil and 61 d data; and the 111 d and 161 d data.

**Figure 5** Partial m/z 192+206 chromatogram highlighting C$_1$-C$_2$ phenanthrenes in SAA fraction of (a) Barrow Island oil; (b) oil-treated soil at 61 days incubation; and
(c) oil-treated soil at 111 days incubation (numbers correspond to methyl locations). Each chromatogram is normalised to the most abundant peak in the displayed region, the corresponding mass spectral signal of which is indicated in italics. Very similar C_1-C_2 phenanthrene profiles were obtained for the 61 d and 86 d data; and the 111 d and 161 d data.

**Figure 6.** A first order decay model of log concentration of (a) 1,3- & 1,7-dimethylnaphthalene; (b) 1,5-dimethylnaphthalene; (c) 2,3,6-trimethylnaphthalene; (d) 1,2,4-trimethylnaphthalene; (e) 1,2,3,7-tetramethylnaphthalene; and (f) 1,2,4,6- & 1,2,4,7- & 1,4,6,7-tetramethylnaphthalene relative to C_{30}αβH with incubation time following 0 d (◦; full line) and delayed 61 d (■, broken line) oil treatment. These isomers representing the most (left) and least (right) susceptible of each of the C_{2-4} alkyl naphthalene isomers classes. Rate constants (k) are corresponding (r^2) values are indicated.
Figure 1

(a) TIC: 7.1e5

(b) m/z 85: 7.2e4

(c) TIC: 3.9e6

(d) m/z 85: 9.6e4
Figure 2

(a) Fresh oil: $2.2 \times 10^5$

(b) 61 d: $1.7 \times 10^3$
Figure 3

(a) Fresh oil: $1.5 \times 10^5$

(b) 61 d: $3.0 \times 10^4$

(c) 86 d: $5.5 \times 10^3$
Figure 4

(a) Fresh oil: $4.1 \times 10^4$

(c) 86 d: $6.5 \times 10^3$

(e) 161 d: $4.2 \times 10^3$
Figure 5

(a) Fresh oil: 4.2e4

(b) 61 d: 1.2e4

(d) 111 d: 5.9e3

Ret time (mins)
Figure 6

(a) 13&17 DMN

\( k = -3.9 \times 10^{-2} \) (1.00)
\( k = -2.4 \times 10^{-2} \) (0.92)

(b) 15 DMN

\( k = -2.3 \times 10^{-2} \) (0.90)
\( k = -1.8 \times 10^{-2} \) (0.99)

(c) 236 TMN

\( k = -2.6 \times 10^{-2} \) (0.78)
\( k = -1.3 \times 10^{-2} \) (0.95)

(d) 13&17 DMN

\( k = -7.4 \times 10^{-3} \) (0.70)
\( k = -1.2 \times 10^{-3} \) (0.40)

(e) 1237 TeMN

\( k = -7.3 \times 10^{-3} \) (0.82)
\( k = -5.4 \times 10^{-3} \) (0.94)

(f) 1246+ TeMN

\( k = -5.9 \times 10^{-3} \) (0.75)
\( k = -2.8 \times 10^{-3} \) (0.76)

Log \[ \text{[aN]/[C}_{30}\alpha\beta\text{H}] \] vs. Time (day)
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