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Potential for aerobic and anaerobic biodegradation of petroleum hydrocarbons in boreal subsurface

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Abstract

We studied the role of aerobic and anaerobic petroleum hydrocarbon degradation at a boreal, light-weight fuel and lubrication oil contaminated site undergoing natural attenuation. At the site, anoxic conditions prevailed with high concentrations of CH_4 (up to 25% v/v) and CO_2 (up to 18% v/v) in the soil gas throughout the year. Subsurface samples were obtained mainly from the anoxic parts of the site and they represented both the unsaturated and saturated zone. The samples were incubated in microcosms at near *in situ* conditions (i.e. *in situ* temperature 8 °C, aerobic and anaerobic conditions, no nutrient amendments) resulting in the removal of mineral oil (as determined by gas chromatography) aerobically as well as anaerobically. In the aerobic microcosms on average 31% and 27% of the initial mineral oil was removed during a 3- and 4-month incubation, respectively. In the anaerobic microcosms, on average 44% and 15% of the initial mineral oil was removed during a 12- and 10-month anaerobic incubation, respectively, and e.g. *n*-alkanes from C_{11} to C_{15} were removed. A methane production rate of up to 2.5 μg CH_4 h^{-1} g^{-1} dwt was recorded in these microcosms. In the aerobic as well as anaerobic microcosms, typically 90% of the mineral oil degraded belonged to the mineral oil fraction that eluted from the gas chromatograph after C_{10} and before C_{15}, while 10% belonged to the fraction that eluted after C_{15} and before C_{40}. Our results suggest that anaerobic petroleum hydrocarbon degradation, including *n*-alkane degradation, under methanogenic conditions plays a significant role in the natural attenuation in boreal conditions.

Introduction

During recent years, natural attenuation has gained both interest and acceptance as an optional method to remediate contaminated soil and groundwater (ASTM 1998; Azadpour-Keeley et al. 2001; USEPA 1999). In addition, natural attenuation by biodegradation plays a significant role in active remediation (Chapelle 1999). Low temperatures may result in low microbial activity and poor availability of the contaminants to the microorganisms (Borden et al. 1995; Richmond et al. 2001). However, recent studies have shown that there is a significant potential for the use of active bioremediation in contaminated soil and ground water remediation in the boreal or arctic environments (Bradley & Chapelle 1995; Eriksson et al. 2001; Margesin 2000; Whyte et al. 1998).

It is known that BTEX compounds, that is benzene, toluene, ethylbenzene and xylenes, are biodegradable under anaerobic conditions (Edwards & Grbic-Galic 1992; Hutchins et al. 1991; Kazumi et al. 1995). Very recently it has become apparent that also aliphatic hydrocarbon degradation takes place under anaerobic conditions. Aliphatic hydrocarbon degradation linked to denitrification (Bregnard et al. 1997; Chayabutra & Ju 2000; Wilkes et al. 2002), sulfate reduction (Coates et al. 1997; Kropp et al. 2000; Rueter et al. 1994; So & Young 1999) and methanogesis (Anderson & Lovley 2000; Zengler et al. 1999) has been demonstrated to take place. This is of particular relevance because BTEX compounds constitute only a minor portion of crude oil and many refined petroleum products. There are though few reports on natural attenuation of pet-

roleum hydrocarbons other than BTEX compounds in the subsurface in boreal environments.

The metabolic pathways behind anaerobic alkane biodegradation are not well understood. For the methanogenic degradation of alkanes, BTEXs or other highly reduced compounds, syntrophic association between fermentative bacteria and methanogenic archaea is required (Conrad & Klose 1999; Heider et al. 1999). Subsequently, aceticlastic methanogenesis has been proposed for the terminal step in anaerobic alkane degradation pathway in several studies using various tools (Anderson & Lovley 2000; Bolliger et al. 1999; Dojka et al. 1998; Revesz et al. 1995). Zengler et al. (1999) found that both aceticlastic methanogenesis and methanogenesis by CO_2 reduction occurred during anaerobic alkane degradation, but aceticlastic methanogenesis was considered to be the predominant pathway. Bolliger et al. (1999) and Dojka et al. (1998) used molecular phylogeny tools and reported that the methanogenic zones in petroleum hydrocarbon contaminated aquifers studied were dominated by acetogenic and aceticlastic methanogenic micro-organisms. Methane production via the acetate fermentation pathway was supported also by isotope fractionation (Bolliger et al. 1999; Revesz et al. 1995) and soil microcosm studies (Anderson & Lovley 2000). Most of the reports related to the anaerobic mineralization of aliphatic hydrocarbons are studies with pure cultures or enrichment cultures in laboratory scale (Chayabutra & Ju 2000; So & Young 1999; Zengler et al. 1999). Hence, the significance of these results in the environment e.g. contaminated soils and sediments, is not yet known and the evidence for the anaerobic degradation of alkanes in environmental samples has been reported only in few cases (Anderson & Lovley 2000; Caldwell 1998; Coates et al. 1997).

In this study, laboratory microcosms for soil samples and field methods (soil gas and ground water analyses) were employed to estimate the efficiency of the aerobic and anaerobic biodegradation of petroleum hydrocarbons in the subsurface of a closed industrial dump-site contaminated with diesel fuel and lubrication oil.

Materials and methods

Case description

We studied an abandoned industrial dumpsite (Trollberget) located in Southern Finland (N 59°, 53′; E 23°, 3′). At the site, soil and groundwater have been contaminated with lightweight fuel, lubrication oil and partly with heavy metals (Figures 1 and 2). The total contaminated area is approx. 2,300 m^2. The contaminating activities such as waste dumping and burning were continued until 1984. The origin of the petroleum hydrocarbon contamination at the site is a pit that was used for dumping of oily wastes. After closing down the operation, the pit was filled with a mixture of sand and gravel-containing soil and garbage. The shore zone (the area north-west of points G12, G1 and G7) is former wetland onto which soil and slag-containing heavy metals have been spread. Located on the continuation of a glacifluvial ridge, Salpausselkä I, the surface geology of the site is characterized by strata of fine and coarse sand and gravel. At the site, silt and clay typically comprise less than 5% and 0.1% of the soil, respectively. However, in the deeper subsurface below the well-permeable layers, the fraction of silt and clay occasionally rises to 14% and 3%, respectively. In combination with fine sand and silt, a layer of poor permeability has developed at the hot spot (the area at the oil pit) contributing to formation of perched groundwater at this point. The studied aquifer was shallow with groundwater or perched groundwater level ranging from 0.8 m to 2.5 m below the ground level.

Groundwater sampling and analysis

At the site, eight groundwater monitoring wells were installed between 1999 and 2001 (Figure 1). From the wells, samples were taken two times a year and analyzed for NO^-_{2+3}, NH^+_4, SO^{2-}_4, N-tot, P-tot and PO^{3-}_4. An inertial pump consisting of a length of Teflon tubing and a foot valve was used to obtain the samples. Temperature, oxygen concentration, pH and redox potential were determined in the field with a WTW P3 Multi-line meter (WTW, Weilheim, Germany). Total nitrogen and the sum of NO^-_2, NO^-_3 (SFS 1990), NH^+_4 (SFS 1976b), PO^{3-}_4 (SFS 1976a), SO^{2-}_4 (SFS 1992) were determined according to Finnish standard protocols (SFS, Finnish Standards Association). Groundwater samples for methane analysis were collected into 120-mL serum bottles. Methane

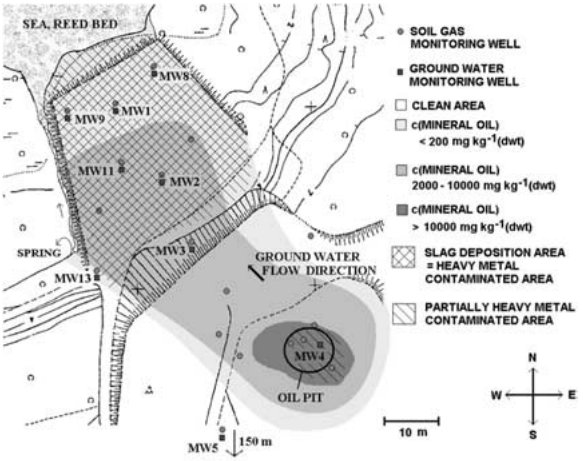

Figure 1. Estimate of the spatial distribution of the petroleum hydrocarbon and heavy metal contamination, groundwater flow direction and the positions of the groundwater monitoring wells at Trollberget, Hanko.

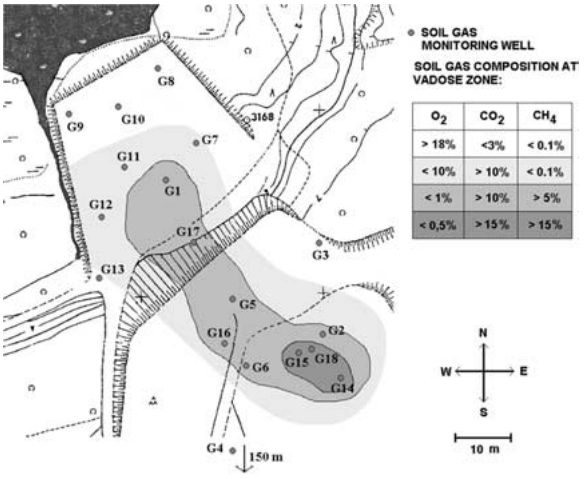

Figure 2. Estimate of the spatial soil gas composition and the positions of the soil gas monitoring wells at Trollberget, Hanko.

concentration was determined from the headspace of the bottles with an HP5890 series II gas chromatograph equipped with a FID and a Hayesep Q column. Nitrogen was used as a carrier gas.

Soil gas monitoring

In 2000 and 2001, 39 soil gas-monitoring wells were installed into different depths in the soil at 17 points at the site (Figure 2). Tubes were introduced both into contaminated and non-contaminated soil. Soil gas composition was measured on average every second month. O_2, CO_2 and H_2S concentrations were determined in the field with a Dräger Multiline II multigas monitor (Drägerwerk, Lübeck, Germany). Subsamples from the soil gas were collected into 10-mL vacuum tubes and analysed for CH_4 as described above.

Soil sampling

A large scale sampling was carried out in May 2000. Soil samples (n = 41) were taken from points G1–G16 from one to five different depths (Figure 2). From all of the samples, respiration rates, mineral oil and heavy metal concentrations were determined. From the mineral oil contaminated samples, long-term aerobic degradation experiments were performed in microcosms. Soil samples for depth profiles were taken In July 2000 from points G1 and G15 and in January 2001 from points G17 and G18. Samples were taken at 0.5 m intervals down to 5 m at the most. From these samples, mineral oil concentration, aerobic respiration rate, methane production rate and bacterial cell count were determined. Furthermore, experiments to study the long-term aerobic and anaerobic degradation of the mineral oil were performed. Soil samples for BTEX- and naphthalene analyses were taken from two points (G17 and G18) in May 2001.

Soil samples were obtained with an engine driven auger, sieved through an eight mm sieve and homogenized in the field. For respiration and methane production experiments, soil was immediately distributed into 120-mL serum bottles and the bottles were tightly closed with a butyl rubber septum. The bottles prepared for the methane production experiments were immediately flushed with oxygen-free N_2 gas for 4 min in the field. For BTEX and naphthalene analysis, 10 g of soil was immediately distributed into a 50-mL glass bottle into which 20 mL of methanol was added. The bottle was then tightly closed with a Teflon-coated cap. Prior to analysis, samples were stored in +4 °C in the dark. For mineral oil analysis, samples were distributed into tightly closed glass bottles and the samples were frozen at −18 °C until the analysis. Samples for the enumeration of the microbial cells and mineralization measurements were stored in +4 °C and prepared within 2 days. For heavy metal analysis, a subsample of at least 200 g of soil was taken into a plastic box and frozen at −18 °C until the analysis. Dry weight (dwt) of the soil was determined after drying the soil sample for 15–20 h at 105 °C and the organic matter content (% of dwt) was determined thereafter by burning the sample at 550 °C for 2 h. All microbiological assays and chemical analyses

were performed on duplicate subsamples of one soil sample if not otherwise stated.

Aerobic respiration

Soil samples of 10 g were incubated at 8 °C in the dark. The accumulation of CO_2 in the headspace was measured every other day for two weeks by IR-spectrometry with a carbon analyzer (Easy Quant, Lammi, Finland) (Laine & Jörgensen 1997). The first CO_2 measurement was performed within 12 to 24 hours from the sampling. Thus, a lag phase shorter that max. 24 hours could not have been observed. The bottles were flushed with atmospheric air (2–3 times the headspace volume) during the incubation period to ensure the supply of oxygen.

Methane production rate

The anoxicity of the headspace of the serum bottles containing 30 g of soil was verified in the laboratory by measuring the O_2 concentration in the headspace with an HP5890 series II gas chromatograph equipped with an ECD and Porapak Q column. Argon-methane was used as the carrier gas. The samples were incubated at +8 °C. CH_4 accumulation in the headspace was monitored as described earlier. The first CH_4 measurement was performed within 12 to 36 hours from the sampling. Thus, a lag phase shorter that max. 36 hours could not have been observed.

Long-term aerobic and anaerobic incubation

The microcosms used for respiration and methane production rate measurements were further incubated to study the long-term aerobic and anaerobic biodegradation of the mineral oil, respectively. The samples were incubated in the dark at 8 ± 1 °C in total for 3 to 4 and 10 to 12 months under aerobic and anaerobic conditions, respectively. From the aerobic microcosms, CO_2 accumulation was monitored every other week as described above. From the anaerobic microcosms, CH_4 accumulation was measured once a month. At the end of the experiment, the cumulative amount of carbon dioxide accumulated in the headspace was determined. After the incubation period, the mineral oil concentration was determined from all of the samples as described below.

A separate long-term control experiment under both aerobic and anaerobic conditions was carried out to study the possibility for abiotic loss of the mineral oil in the microcosms. Two contaminated soil samples from Trollberget were sterilized by autoclaving (3 ± 20 min, 121 °C). The soil samples were then aseptically amended with diesel-fuel to reach mineral oil concentrations of 4,900 and 6,700 mg kg^{-1} dwt^{-1}, respectively. From these soils, aerobic and anaerobic long-term incubations in microcosms were initiated and performed as described above. The controls were incubated for three months.

Enumeration of micro-organisms

Micro-organisms were extracted from 10 g of soil in a solution containing 90 mL of 0.9% NaCl, 1 mL of 10.4% $Na_5P_3O_{10}$ and 0.1 mL of 2% Tween 80. The soil slurry was homogenized for 2 minutes in a homogenizer (Waring Laboratory Blender 8010). A 10-mL subsample was taken from the slurry and fixed with 0.2 mL of 37% formaldehyde. For microscopy (Leitz Diaplan), 0.5–1.5 mL of fixed sample was filtered through a 5-μm pore size filter which was rinsed with the same volume of 1 M KCl. Micro-organisms from the filtered sample were harvested onto black nuclepore membranes (pore size 0.2 μm), stained with DAPI (4′,6′-diamidino-2-phenylindole) as described in Porter & Feig (1980).

Mineral oil analysis

Mineral oil was extracted by sonication from 10 g of fresh soil with acetone-heptane containing internal standards (*n*-decane and *n*-tetracontane). The solution was washed with H_2O. Then, the organic phase was separated and H_2O and the polar compounds were removed with sodium sulfate and florisil, respectively. An aliquot of the purified extract was analyzed by GC-FID (Agilent 6890) equipped with an SGE BPX5 capillary column (SGE). The total peak area between the internal standards *n*-decane and *n*-tetracontane was measured and the content of mineral oil in the sample was quantified. With this method, all hydrocarbons with boiling point ranging from 175 °C to 525 °C were determined. The compounds included in the extraction/analysis were *n*-alkanes from C_{11} to C_{39} isoalkanes, cycloalkanes, alkyl benzenes and alkyl naphthalenes (ISO 2001).

BTEX and naphthalene analyses

A 100-μl aliquot of the methanol phase of the fixed sample was transferred into a headspace bottle containing 10 mL of H_2O and internal standards

(α,α,α-trifluorotoluene and toluene-d_8). The headspace bottles were equilibrated at 80°C for an hour. An aliquot of the gaseous phase was injected into a gas chromatograph (HP5890) equipped with a DB624 capillary column (J&W Scientific) and a mass selective detector (HP5970).

Volatile hydrocarbons in the headspace

The concentration of volatile hydrocarbons including BTEX compounds, methyl tert-buthyl ether (MTBE) and tertiary-amyl methyl ether (TAME) in the headspace of the serum bottles for the long term aerobic and anaerobic degradation experiments was determined with a gas chromatograph (HP5890) equipped with a DB624 capillary column (J&W Scientific) and a mass selective detector (HP5970). The analyses were performed to study the possibility for volatilization of mineral oil or the degradation products from the soil under the incubation. Volatilization would lead to false positive results if mineral oil removed via volatilization instead of biodegradation. Prior to sampling, the serum bottle containing the soil sample was allowed to warm up to room temperature. Then, a 1-mL gas sample from the headspace was injected into the chromatograph. The analysis was not performed for all samples but a number of well representing samples.

Metal analysis

The samples frozen for the metal analysis were freeze-dried. Then, a 250-mg subsample was digested in 5.0 mL of concentrated HNO_3 in a microwave oven (Milestone MLS-1200 mega; 250 W $\times$ 5 min + 400 W $\times$ 5 min +500 W $\times$ 10 min). The digested sample was quantitatively transferred into a 30-mL polypropylene tube and diluted with H_2O to a volume of 25 mL. Prior to the analysis by ICP-MS (PE-Sciex Elan 6000), the samples were further diluted 100 to 1,000 times. With this semi-quantitative analysis (Total Quant), the concentrations of 60 elements were scanned.

Results

Type and distribution of the contamination

Soil and groundwater were contaminated with petroleum hydrocarbons of at least two kinds. The contamination caused by light-weight fuel contained mostly mineral oil constituents that eluted from the gas chromatograph after C_{10} and before C_{15}. Another source of contamination was lubrication oil that contained mineral oil constituents that eluted from the chromatograph after C_{15} and before C_{35}. Light-weight fuel constituted typically over 70% (w/w) and lubrication oil less than 30% (w/w) of the mineral oil contamination (Figure 3A). In the soil samples, BTEX compounds, naphthalene, MTBE or TAME were detected in amounts of <6.2 mg kg^{-1} dwt of each individual compound. The spatial differences in the type of the contamination were minor at the site whereas the vertical differences showed weathering of the contaminants in the top-soil at some points (data not shown).

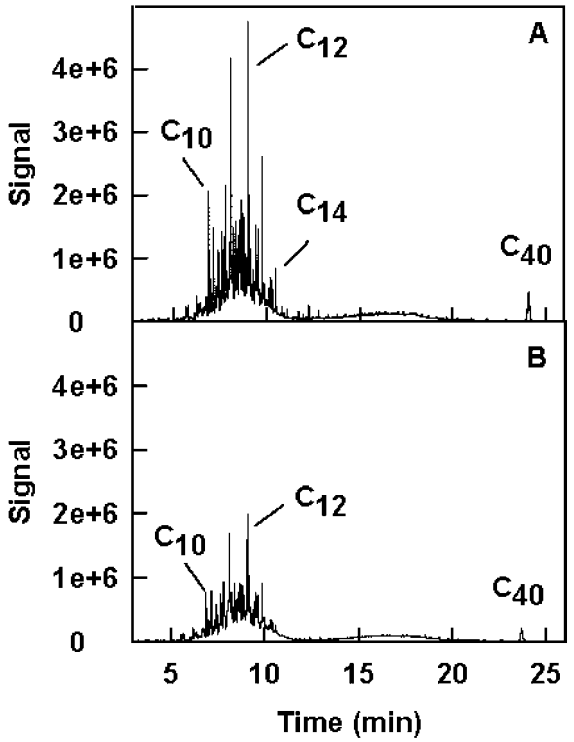

Figure 3. Mineral oil chromatogram from an individual microcosm soil sample (from point G15, depth 2.75 m) before (A) and after (B) one year of anaerobic incubation.

The highest mineral oil concentrations were found at the former oil pit (Figure 1), where the concentrations were up to 68,600 mg kg^{-1} dwt. They were found at 3 m depth, just above the perched groundwater table. At the slag deposition area, the highest mineral oil concentrations (9,200 mg kg^{-1} dwt) were found at approx. 1 m depth, just above the groundwater table. The organic matter content varied from 0.3 to 5.7% with the exception of samples at points G11, G14 and G18, where separate layers contained organic

matter up to 44%. The contribution of the mineral oil to the total organic matter in the soil samples is listed in Tables 1 and 2.

The heavy metal contamination was found mostly at the slag deposition area (Figure 1). However, high concentrations of heavy metals were occasionally found in the surroundings of the oil pit as well. Of the heavy metals, the major polluting agents were lead (0.8–3,400 mg kg^{-1} dwt), zinc (0–7,200 mg kg^{-1} dwt) and copper (1.9–46,000 mg kg^{-1} dwt). Barium (6–1,700 mg kg^{-1} dwt), boron (0–90 mg kg^{-1} dwt), cadmium (0–62 mg kg^{-1} dwt), arsenic (0–61 mg kg^{-1} dwt) and nickel (0–1,400 mg kg^{-1} dwt) exceeded the regulatory threshold values as well.

Groundwater chemistry

Groundwater parameters were measured from eight groundwater monitoring wells. The groundwater at the site was neutral (pH 6.6–7.8), anoxic ($O_2 < 0.2$ mg L^{-1}) with redox-potential ranging from -190 to $+70$, and its temperature varied between 8 °C and 12 °C. The annual changes in the groundwater level were minor. The ranges of the concentrations of the analyzed groundwater constituents were 12–139 μg L^{-1} of NO^{-}_{2+3}–N, 1.9–62 mg L^{-1} of SO^{2-}_{4}, 0.2–5.4 mg L^{-1} of CH_4, 1,870–11,480 μg L^{-1} of NH^{+}_{4}–N, 2,440–26,100 μg L^{-1} of N-tot and 0.9-330 μg L^{-1} of PO^{3-}_{4}–P.

Soil gas composition

The soil gas composition at the site changed drastically from clean to contaminated areas (Figure 2). At the clean area, the unsaturated subsurface soil was oxic with CO_2 concentrations typically less than 3% (v/v) and oxygen concentrations higher than 18% (v/v) even in deeper layers (∼5m). At the most contaminated area, concentrations up to 18 and 27% (v/v) of carbon dioxide and methane, respectively, were measured. At these points, depressed oxygen concentrations were detected in the subsurface above the contamination (0–0.7 m) leading to the development of complete anaerobic conditions ($O_2 < 0.1\%$ (v/v)) in the deeper layers (Figure 4). In the upper layers (0–0.7 m), trace concentrations of methane were measured. Dihydrogen sulfide was not detected in concentrations higher than 3 ppm in any part of the site.

Soil gas composition was monitored on average once every second month for 16 months. During that period, temporal changes in the soil gas composition were minor both at contaminated and non-contaminated areas (data not shown).

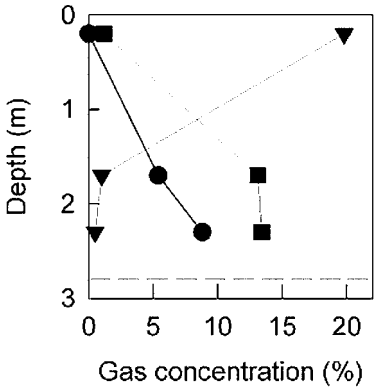

Figure 4. Depth profile of the soil gas composition in the subsurface at point G14. (- -, ground water level, ■, CO_2; ●, CH_4; ▼, O_2).

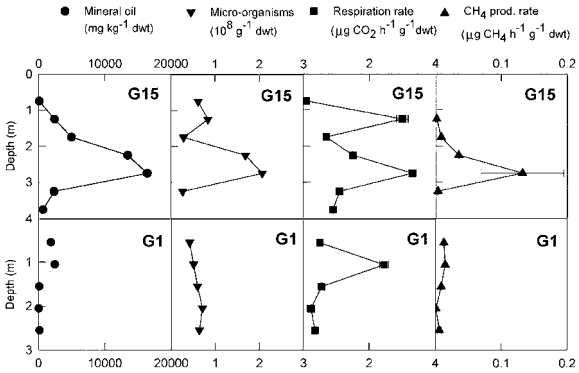

Figure 5. Depth profile of mineral oil concentration, number of microorganisms, respiration and methane production rates in subsurface samples at points G15 and G1. Ground water level is indicated by the dashed line. Error bars indicate standard deviation.

Total microbial activity

The highest aerobic and methanogenic activities (ranges: 0.1–3.3 μg CO_2 h^{-1} g^{-1} and ($< 2.0 \times 10^{-6} - 2.5$ μg CH_4 h^{-1} g^{-1} dwt, respectively) as well as numbers of micro-organisms coincided with the highest petroleum hydrocarbon contamination (Figure 5). In most of the experiments, aerobic respiration showed no lag phase. The methane production rate accelerated in most of the samples during the first months of incubation. Most of the samples did not show a lag phase.

Table 1. Removal of mineral oil in subsurface samples during anaerobic incubation in microcosms

Sampling		Organic matter content	Incubation	Mineral oil			CH_4-C[a]/ oil removed[d]	CO_2-C[b]/ oil removed[d]
Point	Average		Duration	Initial concentration	Removal[c]	Contribution to organic matter[d,e]		
	(m)	(% of dwt)	(month)	(mg kg^{-1} dwt)	(%)	(%)	(%)	(%)
G15	1.25	1.9	12	2400 ± 60	42 ± 11	17	10	25
	1.75	1.1	12	5000 ± 40	64 ± 3	59	4	8
	2.25	3.2	12	13500 ± 120	47 ± 1	55	6	3
	2.75	3.8	12	16400 ± 540	34 ± 8	56	70	20
	3.25	1.1	12	2300 ± 490	61 ± 4	27	13	8
G17	0.55	1.8	10	4200 ± 40	0	31	–	–
	1.05	1.7	10	7800 ± 270	6 ± 3	60	0.2	17
	1.55	1.0	10	7600 ± 90	38 ± 3	99	0.1	2
	2.05	1.1	10	2900 ± 100	17 ± 0.3	34	0.2	33
	2.55	0.5	10	750 ± 20	21 ± 4	20	0.3	14
G18	2.45	17	10	14500 ± 40	0	11	–	–
	3	44	10	68600 ± 2200	0	20	–	–
	3.5	0.6	10	260 ± 40	35 ± 8	6	71	56
	4.5	0.6	10	660 ± 20	18 ± 0	14	>100	>100

[a]The amount of CH_4–C accumulated during the incubation.
[b]The amount of CO_2–C accumulated during the incubation.
[c]The amount of mineral oil removed during duplicate incubations as a percentage of the initial mineral oil concentration. The average and standard deviation of the removal percentage have been calculated using the average of the initial mineral oil concentration.
[d]The amount of mineral oil has been converted to C assuming that 85% of mineral oil is carbon.
[e]Ignition loss has been converted to C assuming that 65% of overall organic matter is carbon.

Anaerobic degradation of the mineral oil in microcosms

Anaerobic biodegradation of the petroleum hydrocarbons was studied using long-term incubation in microcosms accompanied with mineral oil analyses. During 12- and 10-month incubation in microcosms, on average 44% and 15%, respectively, of the mineral oil present in the original sample was removed (Table 1). The comparison between the amount of oil removed and the amount of carbon dioxide and methane produced showed that the amount of CO_2 and CH_4 could account for 2% to 90% of the amount of mineral oil degraded when the initial mineral oil concentration exceeded 1,300 mg kg^{-1} (Table 1). This theoretical calculation is based on an assumption that all gas production originated from mineral oil even though the contribution of mineral oil to total organic matter is low in some of the samples (Tables 1 and 2). A chromatogram from a mineral oil analysis of an individual soil sample (G15, depth 2.75 m) before and after the anaerobic incubation demonstrates the disappearance of mineral oil (Figure 3). In the anaerobic long-term microcosms, for example at point G15, 90% (range: 82–100%) of the mineral oil belonged to the mineral oil fraction that eluted from the gas chromatograph after C_{10} and before C_{15}, while 10% (range: 0–18%) belonged to the fraction that eluted after C_{15} and before C_{40}. However, exceptions were also found: for instance, the heavily contaminated samples from point G18 showed high anaerobic activity (CH_4 production rate ranged from 0.07 to 2.5 μg CH_4 h^{-1} g^{-1} dwt) but no mineral oil degradation occurred in these samples during the incubation (Table 1). In the control experiment excluding biological activity, neither production of carbon dioxide or methane nor loss of the mineral oil was observed.

Aerobic degradation of the mineral oil in microcosms

Aerobic biodegradation of the petroleum hydrocarbons was studied using long-term incubation in microcosms accompanied with mineral oil analyses. During a three- and four-month aerobic incubation in microcosms, on average 31% and 27%, respectively, of the mineral oil present in the original samples was removed (Table 1). On average 90% (range: 86–100%) of the mineral oil degraded belonged to the mineral oil

Table 2. Removal of mineral oil in subsurface samples during aerobic incubation in microcosms

Sampling		Organic matter content	Incubation	Mineral oil			CO_2-C[a]/ oil removed[d]
Point	Average		Duration	Initial concentration	Removal[b]	Contribution to organic matter[c,d]	
	(m)	(% of dwt)	(month)	(mg kg^{-1} dwt)	(%)	(%)	(%)
G7	0.3	3.7	3	5800 ± 130	20 ± 2	20	48
	0.6	5.7	3	9300 ± 260	16 ± nm	21	56
	1.15	4.4	3	1300 ± 300	58 ± nm	4	70
	2.25	1.2	3	520 ± 20	17 ± 5	6	>100
G11	0.5	8.8	3	500 ± 60	44 ± 1	1	>100
	1.5	10	3	5100 ± 210	22 ± 9	7	74
	2.55	5.7	3	3000 ± 200	29 ± 0.1	7	47
G14	0.9	1.9	3	380 ± 4	19 ± 16	3	>100
	2.15	16	3	10600 ± 70	53 ± 4	9	12
	3.45	1.3	3	8300 ± 360	75 ± 3	83	3
G15	0.45	2.1	3	740 ± 20	2 ± 7	5	>100
	1.8	3.1	3	9300 ± 120	44 ± 0.9	39	28
	3.4	2.0	3	14900 ± 270	31 ± 4	97	16
G16	1	1.4	3	55 ± 1	16 ± 8	1	>100
	2.75	1.5	3	3500 ± 100	65 ± 2	31	24
	3.25	0.3	3	4700 ± 10	76 ± nm	>100	2
G17	0.55	1.8	4	4200 ± 40	15 ± 3	31	49
	1.05	1.7	4	7800 ± 270	17 ± 4	60	33
	1.55	1.0	4	7600 ± 90	44 ± 7	99	10
	2.05	1.1	4	2900 ± 100	21 ± 0.4	34	43
	2.55	0.5	4	750 ± 20	32 ± 1	20	60
G18	2.45	17	4	14500 ± 40	0	11	–
	3	44	4	68600 ± 2200	0	20	–
	3.5	0.6	4	260 ± 40	52 ± 0.3	6	96
	4.5	0.6	4	660 ± 20	60 ± 2	14	>100

[a]The amount of CO_2 accumulated during the incubation.
[b]The amount of mineral oil removed during duplicate incubations as a percentage of the initial mineral oil concentration. The average and standard deviation of the removal percentage have been calculated using the average of the initial mineral oil concentration.
[c]Ignition loss has been converted to C assuming that 65% of the overall organic matter is carbon.
[d]The amount of mineral oil has been converted to C assuming that 85% of mineral oil is carbon.

fraction that eluted from the gas chromatograph after C_{10} and before C_{15}, while 10% (range: 0–14%) belonged to the fraction that eluted after C_{15} and before C_{40}.

In most of the samples (n = 82) CO_2 accumulation was constant during the incubation. The comparison between the amount of oil removed and the amount of CO_2 produced showed that the amount of CO_2 could account for 2% to 81% of the oil degraded when the initial mineral oil concentration exceeded 1,300 mg kg^{-1}. Some of the samples showed no loss of mineral oil despite CO_2 production. Especially, in the heavily contaminated samples from point G18, the mineral oil remained unaffected during the incubation although high rates of aerobic CO_2 production (1.4–2.9 μg CO_2 h^{-1} g^{-1} dwt) were measured. During the aerobic incubation, no methane accumulated in the headspace nor other volatile hydrocarbons were detected in significant amounts as determined by GC-MS. Thus, the disappearance of the petroleum hydrocarbons through volatilization was excluded. In the control experiment excluding biological activity, neither production of carbon dioxide or methane nor loss of the mineral oil was observed.

Discussion

Degradation of the mineral oil under anaerobic conditions was revealed by the laboratory experiments in this study. The anaerobic biodegradation of various crude oil components has been reported but anaerobic alkane biodegradation is regarded to have little or no importance on the total degradation of these compounds in the environment (Madigan et al. 2000; Parkes 1999). Recently, however, Anderson and Lovley (2000) identified a rapid methanogenic degradation of ^{14}C-labelled hexadecane in aquifer sediment samples contaminated with crude oil. These findings support our results that showed significant removal of mineral oil (on average 44% y^{-1}) under anaerobic conditions. The mineral oil removal in our experiment was obtained at 8 °C and without any nutrient amendments.

Aerobic biodegradation of mineral oil by the endogenous micro-organisms seemed to be effective as demonstrated by the long-term aerobic incubation experiments: on average, 27% of the initial mineral oil was degraded in the aerobic microcosms during a three month period at *in situ* temperature (Table 1). Margesin (2000) obtained a mineral oil degradation rate of 0.3% d^{-1} for Alpine soil with an aged oil contamination at 10 °C when no amendments were made.

The mineral oil analyses showed that the mineral oil fraction that eluted from the gas chromatograph after C_{10} and before C_{15} was more readily biodegradable than the fraction that eluted after C_{15} and before C_{40} both under aerobic and anaerobic conditions. Also, removal of C_{11}- to C_{15}-*n*-alkanes was observed both in aerobic and anaerobic microcosms (Figure 3). This degradation pattern is well known for aerobic petroleum hydrocarbon degradation but this is one of the first reports on the diesel fuel and lubrication oil degradation pattern under methanogenic conditions in subsurface samples incubated at near *in situ* conditions. Anaerobic diesel fuel or crude oil degradation pattern under sulfate reducing conditions have been reported by e.g. Coates et al. (1997) and Rueter et al. (1994).

CO_2 or CH_4 production does not, however, necessarily indicate a concomitant removal of mineral oil as was seen for example with the heavily contaminated samples obtained from point G18: the samples showed high microbial activity both under aerobic and anaerobic conditions but no removal of mineral oil under either conditions. The reason behind this is likely the presence of organic material other than oil in these particular samples (Table 1). At point G18, a layer of waste material with very high organic content apparently has been deposited and this does not represent a typical situation at this site. On the other hand, the presence of organic matter other than oil may also enhance the biodegradation of the petroleum hydrocarbons at the site. Altogether, in the anaerobic as well as aerobic microcosms, the correspondence between the removed mineral oil and the CO_2 and/or CH_4 produced varied greatly among the samples (Tables 1 and 2). In many strongly contaminated samples the amount of CO_2 and/or CH_4 produced could theoretically account for less than 5% of the mineral oil removed in the microcosms indicating that the mineral oil consumed by the micro-organisms in these samples was assimilated into biomass or not completely mineralized (that is, for example, production of polar metabolites). Thus, the actual petroleum hydrocarbon mineralization cannot be estimated solely from these microcosms but total mass balance would require the use of e.g. specific isotopes of the determination of possible intermediates in the soils samples. CO_2 and CH_4 production in the soil gas is often used as measure of petroleum hydrocarbon degradation in practical bioremediation applications (Azadpour-Keeley et al. 2001) Our results, however, show that this practice is not always valid due to the fact that the degradation of other organic matter may significantly interfere with the gas production.

The heavy metals present in the samples had impact on both respiration and methane production activities. For example, in the soil samples obtained from the point G1, methanogenesis prevailed *in situ*, as indicated by the high (up to 25% v/v) CH_4 concentrations in the soil gas. Also, in the anaerobic microcosms, initial CH_4 formation was observed. The methanogenic activity, however, declined at the very beginning of the experiment (data not shown) resulting in CH_4 production rates significantly slower at point G1 than at point G15 (Figure 5). This phenomenon was never observed with the samples that contained only mineral oil but no heavy metal contamination. Furthermore, in the long-term aerobic experiment, the longest lag phases were observed in the samples with the highest heavy metal concentrations.

The methane production rates were low ($< 2.0 \times 10^{-6}$ μg g^{-1} dwt h^{-1}) in the clean soil with low organic matter content (0.75% of dwt) and orders of magnitude lower than in any samples from the contaminated area. At the oil pit, even the samples with low

mineral oil and organic matter content (100 mg kg^{-1} dwt and 0.88% of dwt, respectively) showed orders of magnitude higher methane production rate (5.4×10^{-3} μg g^{-1} dwt h^{-1}) than the soil samples from the clean area. The CH_4 production rates obtained from the contaminated area (up to 2.5 μg g^{-1} dwt h^{-1}) were in the same order of magnitude as for instance the rates obtained for samples from pristine environment rich in organic carbon (boreal bog) at 20 °C (Kettunen et al. 1999) and at 10 °C (Galand et al. 2002). As a comparison, the CH_4 production rates obtained for soil samples at 10 °C from an aquifer contaminated with landfill leachate were significantly lower (Ludvigsen et al. 1998).

The estimation of the predominating microbial processes at the different parts of the site is not straightforward. Clearly, in the surroundings of the oil pit (Figure 1) the terminal process in the anaerobic decomposition of organic substances is methanogenesis (Figures 2 and 4): strong methane production was observed in the anaerobic microcosms (Figure 5) and in the field. Interestingly, high aerobic and anaerobic activities were detected in the same individual samples.

Downgradient, at point G17, methanogenesis seemed to have less pronounced role: The highest measured methane concentration in the soil gas was 8% (v/v). In addition, in the anaerobic microcosms, CO_2 rather than CH_4 was produced with concomitant disappearance of the mineral oil (Table 1) suggesting a dominance of other anaerobic processes than methanogenesis (i.e. iron or sulfate reduction). Also, the aerobic respiration rates were considerably low at this point. Low microbial activity may have resulted from the limited availability of substrates and electron acceptors in fine sand with low permeability. Further downgradient, points with high methanogenic activity were again found (Figure 2). At the Trollberget site, nitrate and sulfate supplies were too low to support microbial denitrification or sulfate reduction. Hence, methanogenesis may easily become the predominating process in this environment. Also, the aquifer material at the Bemidji site (Anderson & Lovley 2000) lacked nitrate, HCl-extractable ferric iron and sulfate leaving methanogenesis the dominant electron accepting process. In contrast, in marine sediment contaminated with petroleum hydrocarbons, sulfate is abundant enabling mineral oil degradation by the sulfate reducing organisms (Coates et al. 1997; Rueter et al. 1994; So & Young 1999).

Although considerable potential for the aerobic biodegradation of the mineral oil was measured in the samples, the relevance of the aerobic biodegradation might not be high in the plume where anaerobic conditions prevail. Aerobic biodegradation may, however, restrict the spreading of the contaminants in the periphery of the plume. Based on the removal of the mineral oil in the microcosms, clean-up time up to 30 years for the mineral oil is to be expected for this site if relied on natural attenuation by biodegradation. Due to the heterogeneity of the subsurface and the uneven distribution of the contaminants, the actual rate of the mineral oil disappearance *in situ* was impossible to evaluate during this 2-year study.

In conclusion, we found that mineral oil, including *n*-alkanes, was biodegraded under methanogenic conditions. Our data suggest that the endogenous microorganisms at this boreal study site are fully capable of degrading the mineral oil both under aerobic and anaerobic conditions.

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