Biodegradation (2009) 20:27–38
DOI 10.1007/s10532-008-9196-0

ORIGINAL PAPER

Effect of salt on aerobic biodegradation of petroleum hydrocarbons in contaminated groundwater

Ania C. Ulrich · Selma E. Guigard · Julia M. Foght · Kathleen M. Semple · Kathryn Pooley · James E. Armstrong · Kevin W. Biggar

Received: 19 October 2007 / Accepted: 17 April 2008 / Published online: 25 April 2008

Abstract Hydrocarbon-contaminated soil and groundwater at oil and gas production sites may be additionally impacted by salts due to release of produced waters. However, little is known about the effect of salt on the in-situ biodegradation of hydrocarbons by terrestrial microbes, especially at low temperatures. To study this effect, we prepared a groundwater-soil slurry from two sites in Canada: a former flare pit site contaminated with flare pit residue (Site A), and a natural gas processing facility contaminated with natural gas condensate (Site B). The slurry with its indigenous microbes was amended with radiolabeled hydrocarbons dissolved in free product plus nutrients and/or NaCl, and incubated in aerobic biometer flasks with gyrotory shaking at either 25 or 10°C for up to 5 weeks. Cumulative production of $^{14}CO_2$ was measured and the lag time, rate and extent of mineralization were calculated. For Site A, concentrations of NaCl $\geq$1% (w/v) delayed the onset of mineralization of both ^{14}C-hexadecane and ^{14}C-phenanthrene under nutrient-amended conditions, but once biodegradation began the degradation rates were similar over the range of salt concentrations tested (0–5% NaCl). For Site B, increasing concentrations of NaCl $\geq$1% (w/v) increased the lag time and decreased the rate and extent of mineralization of aliphatic and aromatic substrates. Of particular interest is the observation that low concentrations of salt ($\leq$1% NaCl) slightly stimulated mineralization in some cases.

Keywords Aerobic biodegradation · Groundwater · Petroleum hydrocarbons · Pollutants · Salt

A. C. Ulrich (✉) · S. E. Guigard · K. Pooley · K. W. Biggar
Department of Civil and Environmental Engineering, University of Alberta, Edmonton, AB, Canada T6G 2W2
e-mail: aulrich@ualberta.ca

J. M. Foght · K. M. Semple
Department of Biological Sciences, University of Alberta, Edmonton, AB, Canada

J. E. Armstrong
WorleyParsons Komex International Inc., Calgary, AB, Canada

Present Address:
K. W. Biggar
BGC Engineering Inc., Edmonton, AB, Canada

Introduction

Contamination of sub-surface sediments and groundwater can occur as a result of oil production (Amatya et al. 2002). Biodegradation can be a significant mechanism of hydrocarbon remediation in the subsurface, but it can be limited by several factors in-situ including low concentrations of dissolved oxygen and other terminal electron acceptors, low nutrient concentrations (particularly nitrogen and phosphorus), low temperatures, and potentially low numbers of

indigenous hydrocarbon-degrading microbes. In addition, salt is a common co-contaminant that can adversely affect the bioremediation potential at sites such as flare pits and drilling sites (i.e., "upstream" sites) contaminated with saline produced formation water, or at oil and gas processing facilities contaminated by refinery wastes containing KCl and NaCl salts (Pollard et al. 1994). The effect of salinity on microbial cells varies from disrupted tertiary protein structures and denatured enzymes to cell dehydration (Pollard et al. 1994), with different species having different sensitivities to salt.

A range of organic pollutants, including hydrocarbons, has been shown to be mineralized by marine or salt-adapted terrestrial microorganisms that are able to grow in the presence of salt (Margesin and Schinner 2001; Oren et al. 1992; Nicholson and Fathepure 2004). In naturally saline soils, Riis et al. (2003) determined that bioremediation of diesel fuel is possible at salinities up to 17.5% w/v. Similar results were found by Kleinsteuber et al. (2006). However, Ward and Brock (1978) observed an inverse relationship between salinity and the biodegradation of petroleum hydrocarbons by halophilic enrichment cultures from the Great Salt Lake, which were unable to metabolize petroleum hydrocarbons at salt concentrations above 20% (w/v) in this hypersaline environment (Ward and Brock 1978). An inhibitory effect of salinity at concentrations above 2.4% (w/v) NaCl was found to be greater for the biodegradation of aromatic and polar fractions than for the saturated fraction of petroleum hydrocarbons in crude oil incubated with marine sediment (Mille et al. 1991). The above-mentioned studies describe ex-situ petroleum hydrocarbon degradation by salt-adapted terrestrial microorganisms. In contrast the research described in this paper focuses on in-situ petroleum hydrocarbon degradation by non-salt adapted microorganisms in groundwater systems. The intent of our research is to understand natural attenuation mechanisms where non-salt adapted microorganisms are suddenly impacted with salt and petroleum hydrocarbons as co-contaminants, which commonly occurs at upstream oil and gas sites.

In contrast to the large body of literature documenting hydrocarbon biodegradation by soil and freshwater microbes in the absence of salt and substantial literature on naturally saline environments, there are few reports describing the effects of salt as a co-contaminant on hydrocarbon degradation in naturally non-saline systems. For example, deCarvalho and daFonseca (2005) examined the degradation of C_5 to C_{16} hydrocarbons at 28°C in the presence of 1.0, 2.0 or 2.5% (w/v) NaCl by the isolate *Rhodococcus erythropolis* DCL14. Results showed that the lag phase of the cultures increased and growth rates decreased with increasing NaCl concentrations. Similar results were reported in a study completed by Rhykerd et al. (1995). Soils were fertilized with inorganic N and P, and amended with NaCl at 0.4, 1.2, or 2% (w/w). After 80 days at 25°C, the highest salt concentration had inhibited motor oil mineralization. However, there is almost no literature examining combinations of factors limiting biodegradation of petroleum hydrocarbon contamination at upstream oil and gas production facilities. A laboratory solid-phase bioremediation study observed that high salinity levels reduced the degradation rate of flare pit hydrocarbons (Amatya et al. 2002), and recently Børresen and Rike (2007) found that addition of NaCl to a petroleum-contaminated Arctic soil decreased hexadecane mineralization rates in the initial stages of bioremediation and increased lag times, but that the final extent of mineralization was comparable over a narrow range of salinity from 0 to 0.4% w/w. Because of increasing emphasis and interest in the viability of intrinsic bioremediation as a remedial alternative, the impact of salt on these processes is of interest. A first step was to study the impact on aerobic systems.

This paper reports for the first time the combined effect of four stressors that could potentially influence in-situ sub-surface biodegradation of petroleum hydrocarbons: presence of free product; incubation temperature (10°C vs. 25°C); addition of nutrients (no addition versus nitrogen and phosphorus amendments); and salt concentrations (ranging from 0 to 5% (w/v) NaCl). We tested these effects on indigenous microbes associated with cold sub-surface soil and groundwater collected from a former oil well flare pit site in northern Alberta (Site A) that had previously been contaminated with flare pit residue (middle to high molecular weight hydrocarbons) and a site contaminated with natural gas condensate (low molecular weight hydrocarbons) from southern Alberta (Site B). It is important to note that the free product from both sites was obtained from monitoring wells, where transformation of the

original contaminant (by dissolution, volatilization, etc.) had already occurred. This makes the study more relevant because we tested the biodegradability of the residual hydrocarbon rather than the original contaminant (which would contain the readily degradable hydrocarbons). To our knowledge, only some areas of Site A had been previously contaminated with salt during oil production. Concentrations up to 6,000 mg/l chloride were detected near the source area, but salinity was not found where the soil and groundwater were obtained for this study.

To quantify the effects of these stressors, we used selected radiolabeled substrates to represent major classes of contaminants at the two sites, and dissolved them in free product from the sites. Sub-surface soil and groundwater were amended with nutrients and/or salt and incubated aerobically for up to 850 h (5 weeks). Cumulative production of $^{14}CO_2$ resulting from mineralization of the radiolabeled substrate was measured.

Materials and methods

Subsurface material

Subsurface soils and groundwater were collected from a former oil well flare pit site (Site A) in northern Alberta, and from a natural gas condensate production facility (Site B) in southern Alberta. Groundwater was collected from monitoring wells at the sites and stored at 4°C in a sealed 200-l drum with an argon headspace; soil was collected from auger flights during monitoring well installation and stored at 4°C in 20 l plastic containers filled to the top with groundwater. The soil samples were obtained from the hydrocarbon-impacted zone. After amendment with salt and/or nutrients (see below), 5 g (wet weight) of uniformly mixed soil were weighed aseptically into modified sterile biometer flasks (Bartha and Pramer 1965) and 50 ml of groundwater were added aseptically to each flask, providing replicate indigenous microbial communities presumably adapted to biodegradation of the hydrocarbon contaminant. This set-up produced a small-scale aerated, slurry-based bioremediation test with the hydrocarbon-adapted natural microbial community as the inoculum.

Addition of salt and/or nutrients

As required, solid NaCl was added to flasks which were then sealed and sterilized by autoclave. Soil and groundwater were subsequently added to the cooled sterile flasks and swirled immediately to dissolve any solid phase salt. NaCl amendments of 0.5, 1.0, 2.5, and 5.0% (w/v) were tested. Solid NaCl was used rather than a stock solution of concentrated NaCl to avoid any dilution effects. NaCl was the only salt tested in this work, as recommended by field personnel.

Nutrients were added to certain flasks as heat-sterilized aqueous solutions of ammonium nitrate and potassium phosphate. The normal amendment (1× N,P) was selected based on previous work by Fedorak and Westlake (1981) and Foght et al. (1999) and provided a final concentration of 12.5 mM nitrate, 12.5 mM ammonium and 6 mM potassium phosphate (pH 7). No other supplements were provided.

Addition of radiolabeled substrates

The following radiolabeled hydrocarbons were purchased: n-[1-^{14}C]decane, 99% radiochemical purity (American Radiochemical Co., St. Louis, MO); n-[1-^{14}C]hexadecane, 95% purity (Sigma, St. Louis, MO); ring-[UL-^{14}C]toluene, 98% purity (Sigma); [9-^{14}C]phenanthrene, 97% purity (Amersham, Arlington Heights, IL). Free product (i.e., the liquid contaminant recovered from monitoring wells at each site) was filter sterilized through a 0.22-μm pore size Millex-FG filter (Millipore Corp; Billerica, MA). The radiolabeled substrates were separately dissolved in the appropriate free product to provide approximately 3 μCi/ml (hexadecane or phenanthrene in Site A free product; decane or toluene in Site B free product). Fifty microlitres (0.1 vol%) of radiolabeled free product (ca. 300,000 dpm) was added to each flask. To measure general metabolic activity, one set of flasks received D-[U-^{14}C]glucose plus 0.01% (w/v) yeast extract to approximately 400,000 dpm, in the presence or absence of 50 μl unlabelled filter-sterilized free product. Flasks were prepared in triplicate except for controls and ^{14}C-glucose tests which were typically single flasks.

Controls

Positive and negative controls were included in each test series. Positive controls consisted of flasks

prepared in parallel to test flasks, additionally inoculated with a laboratory-maintained mixed microbial culture known to degrade hydrocarbons at 10 and 25°C. Activity in the positive control flasks verified that mineralization was possible under the incubation conditions provided. Negative control flasks ("heat-killed controls") contained soil and groundwater that were autoclaved for 20 min on each of two successive days. These controls were used to account for any abiotic processes liberating $^{14}CO_2$. Positive and negative controls received the same radiolabeled substrate as the test flasks and were incubated under the same conditions.

Summary of experimental design

The first set of experiments was used to define appropriate test conditions and to determine the general effect of free product, nutrient amendments, temperature and NaCl on the activity of the indigenous microbial communities (Table 1). The effect of free product on ^{14}C-glucose degradation was tested in volumes of 0 or 50 µl for Site A and 0, 10, 50, or 500 µl for Site B with all amendments containing 1× N,P, and no salt.

The second set of experiments was performed with triplicate flasks to determine the effects of multiple stressors such as nutrient amendments, temperature and NaCl on the activity of the indigenous microbial communities using model substrates (Table 2). The effect of nutrient amendments (1× N,P vs. 0× N,P) on ^{14}C-phenanthrene, ^{14}C-toluene and ^{14}C-decane degradation was tested in the presence of free product and a range of NaCl concentrations. The effect of temperature (10°C vs. 25°C) on ^{14}C-hexadecane degradation was tested in the presence of free product, nutrients and a range of NaCl concentrations and the effect of NaCl on all four of the above mentioned substrates was also tested in the presence of nutrients at 10°C.

Incubation conditions

Flask necks were sealed with neoprene stoppers during experiments with the low volatility substrates hexadecane and phenanthrene, whereas screw-cap biometer flasks with teflon liners were used for experiments with the high volatility substrates toluene and decane. The side arm was sealed with a neoprene stopper pierced by an 18-gauge needle fitted with fine gauge teflon tubing extending to the bottom of the side arm. The needle collar was sealed with a 1-ml plastic syringe. Side arms contained 10 ml of 1 N KOH to trap $^{14}CO_2$ generated during biodegradation. Flasks were incubated in the dark at 10 or 25°C with 150 rpm gyratory shaking. Although a large excess of molecular oxygen was present in the ~300 ml of aerobic headspace to enable complete oxidation of 50 µl free product, in addition, at weekly intervals the flasks were unsealed briefly (<1 min) to introduce fresh oxygen into the headspace.

Measurement of mineralization

At intervals, 0.5 ml of KOH was withdrawn from the sidearm via the syringe and placed in a scintillation vial containing 10 ml ACS fluor (Amersham Biosciences UK Ltd., Little Chalfont, England) plus 1.5 ml water and 0.1 ml glacial acetic acid to neutralize the KOH. Vials were incubated in the dark for 30 min to reduce chemiluminescence, and then counted on a Beckman model LS3801 scintillation counter with automatic quench correction. Cumulative production of $^{14}CO_2$ was reported as a percentage of the original radiolabel added, correcting after each sampling for the volume of KOH in the sidearm and for background radiation (typically 25 dpm).

In addition to presenting graphs of the cumulative release of $^{14}CO_2$ (mineralization), the data have also been analyzed to present three measurements describing the effect of incubation conditions on mineralization:

- "rate of mineralization" is measured as the maximum slope of $^{14}CO_2$ release, usually determined immediately after the lag time, and reported as % of radiolabel mineralized per hour (%/h).
- "lag time" is the time elapsed before onset of $^{14}CO_2$ release, determined as the X-axis intercept of the line used to determine mineralization rate, and reported in hours (h).
- "maximum extent of mineralization" is the maximum cumulative $^{14}CO_2$ release after the period of maximum rate of mineralization. In some cases a plateau was not achieved during the incubation time, as the cultures continued to evolve $^{14}CO_2$ slowly and linearly over the course of incubation. In this case, the extent of mineralization at the end of the experiment is reported.

Table 1 Experimental conditions for initial experiments with Site A soil and groundwater

Substrate	Incubation conditions	Variable	Average maximum rate of $^{14}CO_2$ production (% h^{-1})	Conversion of labeled substrate to $^{14}CO_2$ (Max. extent, %)	Average lag (h)
Effect of nutrient amendment (nitrogen and phosphorus) on hydrocarbon mineralization					
^{14}C-hexadecane	25°C, FP[a], no NaCl	0× N,P	0.082[b]	17[b]	240[b]
		0.1× N,P	0.54 ± 0.18	45 ± 7	105 ± 9
		0.5× N,P	0.48 ± 13	48 ± 2	119 ± 5
		1× N,P	0.58 ± 0.20	54 ± 0	120 ± 9
^{14}C-phenanthrene	10°C, FP, no NaCl	0× N,P	0.03 ± 0.01	14 ± 7	0 ± 0[c]
		1× N,P	0.59 ± 0.17	54 ± 4	46 ± 6
Effect of temperature on hydrocarbon mineralization					
^{14}C-hexadecane	1× N,P, FP, no NaCl	10°C	0.25 ± 0.07	43 ± 3	138 ± 4
		25°C	0.58 ± 0.20	56 ± 0	120 ± 9
Effect of salt concentration on general heterotrophic activity					
^{14}C-glucose	1× N,P, FP, 10°C	0% w/v NaCl	0.36	51	0
		0.5% w/v NaCl	0.31	45	0
		1% w/v NaCl	0.33	50	0
		2.5% w/v NaCl	0.24	39	18
		5% w/v NaCl	0.20	35	101

Results are presented as the mean of 3 data points ±1 standard deviation except where noted

[a] FP = 50µl free product

[b] A single flask of three replicates showed activity; the other two exhibited no mineralization

[c] Slow, linear increase in $^{14}CO_2$ production without a measurable lag

Table 2 Summary of NaCl effects on mineralization of model substrates at 10°C with 1× N,P amendment

Salt conc. (% w/v)	0	0.5	1.0	2.5	5.0[a]
Average maximum rate of $^{14}CO_2$ production (% h^{-1})					
Hexadecane	0.25 ± 0.07	0.13 ± 0.05	0.28 ± 0.10	0.36 ± 0.11	0.20 ± 0.01
Phenanthrene	0.59 ± 0.17	0.73 ± 0.06	0.68 ± 0.10	0.46 ± 0.04	0.21, 0.17[1]
Decane	0.022 ± 0.005	0.018 ± 0.004	0.014 ± 0.002	0.005 ± 0.003	0.023[b]
Toluene	0.13 ± 0.05	0.15 ± 0.05	0.06 ± 0.03	0.01 ± 0.00	0.03[b]
% Conversion of labeled substrate to $^{14}CO_2$ (Max. extent, %)					
Hexadecane	43 ± 3	31 ± 7	34 ± 3	25 ± 4	25 ± 15
Phenanthrene	54 ± 4	55 ± 5	48 ± 2	51 ± 1	41, 51[a]
Decane	5 ± 0.6	4 ± 0.6	3 ± 0	1 ± 0	6[b]
Toluene	61 ± 14	62 ± 8	34 ± 18	4 ± 2	27[b]
Average lag (determined from x-intercept in hours)					
Hexadecane	138 ± 4	153 ± 14	192 ± 12	283 ± 65	449 ± 187
Phenanthrene	46 ± 6	62 ± 5	144 ± 11	273 ± 53	482, 515[a]
Decane	66 ± 7	76 ± 6	76 ± 6	158 ± 44	63[b]
Toluene	27 ± 4	59 ± 12	74 ± 25	248 ± 44	125[b]

Radiolabeled hexadecane and phenanthrene were used in Site A experiments and decane and toluene in Site B experiments, dissolved in 50 µl of corresponding free product. Results are presented as the mean of 3 data points ±1 standard deviation except where noted

[a] Data points for two flasks; the third flask did not exhibit mineralization

[b] A single flask of three replicates showed activity; the other two exhibited no mineralization

Results

The indigenous microbial community is not inhibited by 0.1 vol% free product

In initial tests using material from Site A, the general metabolic activity of the indigenous microbial community was demonstrated using ^{14}C-glucose (not shown). Glucose was provided in the presence and absence of 0.1 vol% free product to determine whether the free product was inhibitory to general metabolic activity. The indigenous microbes mineralized the glucose regardless of the presence of free product (achieving 46% vs. 44% $^{14}CO_2$ with and without free product respectively), and at approximately the same rate and extent as the positive control culture (46% $^{14}CO_2$). However, in preliminary experiments (not shown), volumes of 250 or 500 µl (0.25 or 1 vol%) free product resulted in inconsistent or severely reduced rates of ^{14}C-hexadecane mineralization. Therefore, for subsequent experiments we used 50 µl (0.1 vol%) free product as a non-inhibitory volume that still provided sufficient hydrocarbon to form a surface sheen on the groundwater and establish saturated concentrations of hydrocarbons in the aqueous phase. It is typical, in our experience, for only 40–60% of biodegradable radiolabeled substrates to be recovered as $^{14}CO_2$ during a finite incubation time, with the remaining radiolabel presumably converted to biomass, metabolites, or remaining unconverted in the flask.

In addition, no $^{14}CO_2$ was produced in any negative (killed) control flasks from any experiments (data not shown).

Nutrient addition is required for hydrocarbon mineralization

It is well-known that nutrients, particularly nitrogen and phosphorus, are required for efficient hydrocarbon biodegradation, particularly for aliphatics (e.g., Atlas 1981; Foght et al. 1999). Therefore, tests were conducted to determine a suitable nutrient amendment concentration for further experiments. Tests conducted with ^{14}C-hexadecane in Site A soil and groundwater confirmed that nutrient amendment was necessary to achieve significant biodegradation of aliphatic hydrocarbons (Table 1). The 1× N,P amendment regime was deemed suitable for the Site A microbial community because the extent of mineralization decreased with decreasing nutrient concentrations (Table 1). Notably, one of the three replicate flasks with no nutrients mineralized the ^{14}C-hexadecane after a long lag time (ca. 240 h or 10 days), eventually achieving 17% $^{14}CO_2$ by 462 h (19 days), whereas the other two unamended flasks did not show significant mineralization (Table 1). The reason for this delayed and slow mineralization in only one replicate of three is unknown, but is addressed in the Discussion. It was observed in other tests (reported below) that replicate flasks with the greatest stressors showed the most flask-to-flask variability in mineralization.

Nutrient amendment was also necessary for efficient mineralization of the aromatic substrate ^{14}C-phenanthrene. Mineralization at 10°C began rapidly in Site A cultures amended with 1× N,P, showing a similar rate but much shorter lag time than ^{14}C-hexadecane mineralization at 25°C (Table 1). Mineralization of ^{14}C-phenanthrene occurred without a discernable lag in all three replicate flasks without nutrient amendment, but only at low levels, achieving 14% $^{14}CO_2$ by 859 h (5 weeks). Therefore, to ensure efficient aliphatic and aromatic mineralization, 1× N,P nutrient amendment was selected for subsequent tests of nutrient effects.

Temperature affects rate of hydrocarbon mineralization

Some experiments with hexadecane and Site A soil and groundwater included triplicate flasks incubated at 25 or 10°C. The results showed that incubation at 10°C resulted in a longer lag time, a slower rate of mineralization, and a slightly lower extent of mineralization (Table 1). These responses to temperature are as expected for microbial systems. For the majority of subsequent tests, an incubation temperature of 10°C was selected to better represent the in-situ temperatures.

NaCl inhibits general metabolic activity of Site A microbes in the presence of free product

^{14}C-glucose was used to measure the effect of NaCl on the activity of the microbial community in the Site A soil and groundwater. Glucose serves as a carbon source for numerous microbes, including many

hydrocarbon-degraders. Therefore glucose mineralization in the presence of NaCl indicates whether salt effects are specific to the hydrocarbon-degrading community or are more general.

NaCl was inhibitory to general metabolic activity in Site A soil and groundwater at 10°C in the presence of free product (Table 1). Salt concentrations above 1% (w/v) resulted in increased lag times (18 and 101 h for 2.5 and 5% NaCl respectively versus no lag phase at lower NaCl concentrations) and a decrease in mineralization rate with increasing NaCl concentration but a smaller effect on the maximum extent of mineralization (35% at 5% NaCl vs. 51% at 0% NaCl). That is, the microbial community eventually mineralized the same proportion of ^{14}C-glucose regardless of NaCl concentration, but it took longer for the activity to begin and longer for the mineralization to occur when higher concentrations of NaCl were present.

NaCl affects mineralization of hexadecane and phenanthrene by Site A microbes

Radiolabeled hexadecane and phenanthrene were selected as the model substrates for Site A because the former flare pit site was contaminated with aliphatic hydrocarbons from C_{10} to $>C_{20}$, and di- and tri-cyclic aromatic hydrocarbons. Hexadecane (C_{16}) was chosen to represent the aliphatic hydrocarbons and phenanthrene (C_{14}), the polyaromatic hydrocarbons. Both are known from previous experiments to be biodegradable (Foght et al. 1999).

The effects of salt on ^{14}C-hexadecane mineralization were tested at two temperatures, both in the presence of 1× N,P. At 25°C (Fig. 1a), addition of 0.5 or 1% NaCl slightly stimulated mineralization initially, as shown by a shorter lag time than with 0% NaCl. Salt concentrations of 2.5 and 5% appeared to delay the onset of mineralization, and a salt concentration of 5% resulted in triplicate flasks exhibiting very different lag times. The extent of mineralization decreased with increasing NaCl concentrations, from ca. 55% at 0% NaCl to ca. 30% at 5% NaCl. Notably, the rates of $^{14}CO_2$ evolution were similar at all salt concentrations (0.56 ± 0.02% h^{-1}). That is, primarily the lag time and extent of mineralization were affected by varying salt concentrations. At 10°C (Fig. 1b and Table 2), the pattern of

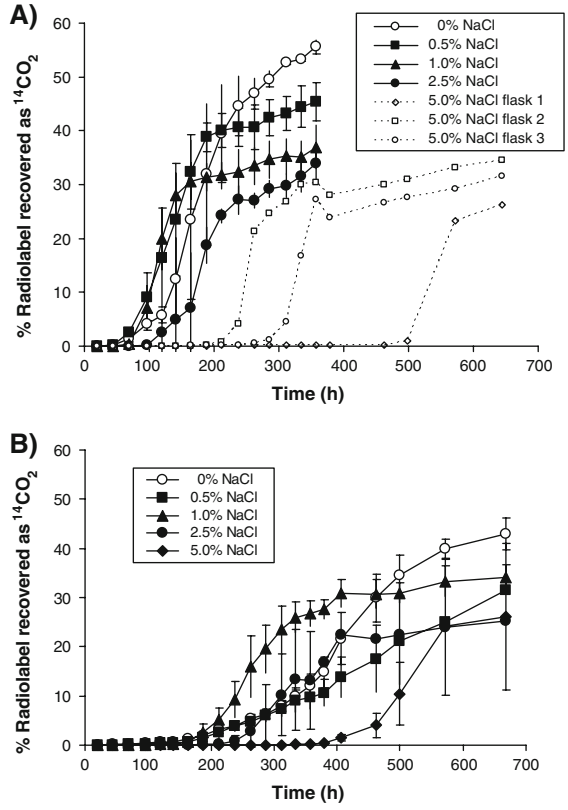

Fig. 1 Effect of NaCl and temperature on mineralization of ^{14}C-hexadecane by soil and groundwater from Site A. All flasks were incubated with 1× N,P nutrient amendment and free product. Upper panel (**a**) incubation at 25°C. Symbols connected with solid lines represent the mean of 3 data points ±1 standard deviation. Small open symbols connected by dotted lines are the data from individual triplicate flasks incubated with 5% NaCl. Lower panel (**b**) incubation at 10°C. Symbols represent the mean of 3 data points ±1 standard deviation

salt effects was more difficult to define. Again, 1% NaCl was stimulatory initially (although 0.5% was not), and 5% NaCl caused the longest lag time. The maximum mineralization rates again were similar at the various salt concentrations, with the exception of a slower rate with 0.5% NaCl (0.13 ± 0.05% h^{-1}). Although the calculated mean maximum extent of mineralization did decrease with increasing NaCl concentration, the variability of the replicates precludes making definitive statements about the extent of mineralization achieved under each NaCl condition. Therefore, the combination of stressors of low temperature plus NaCl and free product resulted in a more complex response.

At both temperatures, the rate of mineralization was not greatly affected by the presence of NaCl (except for the aforementioned anomalously slow rate at 10°C with 0.5% NaCl). However, the lag time significantly increased with NaCl concentration at both temperatures, and the extent of mineralization decreased (Table 2). That is, the general effects of NaCl on rate, lag time and extent of mineralization were consistent at both 10 and 25°C for ^{14}C-hexadecane mineralization by Site A material, and the overall effect was to delay and slightly reduce the extent rather than prevent mineralization.

The effects of salt on phenanthrene mineralization in microcosms with site A soil and groundwater were tested at 10°C, with and without nutrient amendment. In the presence of 1× N,P (Fig. 2a and Table 2), 0.5% NaCl did not affect the lag, rate or extent of mineralization compared to flasks with 0% NaCl. The presence of NaCl at concentrations above 0.5% resulted in greater lag times, but had little effect on either rate or extent of mineralization. At 5% NaCl, only two of three replicates exhibited mineralization, and only after a lengthy lag time of ca. 500 h (3 weeks). The large error bars for the 2.5% NaCl flasks also reflect different lag times of replicates with similar rates.

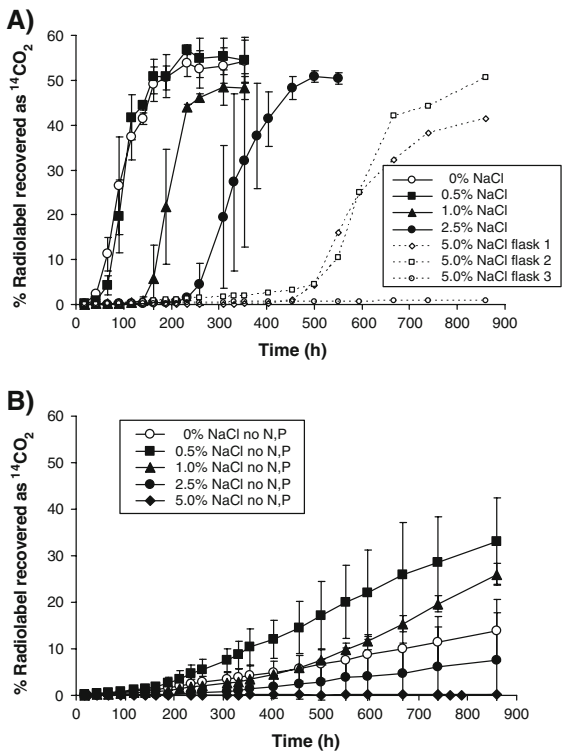

Fig. 2 Effect of NaCl and nutrient amendment on mineralization of ^{14}C-phenanthrene by soil and groundwater from Site A. All flasks were incubated at 10°C. Upper panel (**a**) amendment with 1× N,P. Symbols connected with solid lines represent the mean of 3 data points ±1 standard deviation. Small open symbols connected by dotted lines are the data from individual triplicate flasks incubated with 5% NaCl. Lower panel (**b**) incubation without nutrient amendment (no N,P). Symbols represent the mean of 3 data points ±1 standard deviation

Mineralization of phenanthrene occurred even in the absence of nutrient amendment (Fig. 2b), as has been previously observed (Foght et al. 1999). Most triplicate sets showed considerable variation among the replicates, resulting in large error bars. Even so, the lower two salt concentrations were stimulatory and the higher two concentrations inhibitory, with the 5% NaCl flasks showing no mineralization by 860 h (5 weeks). The pattern of $^{14}CO_2$ release in the absence of N,P was quite different from that in nutrient-amended flasks, with the mineralization progressing in a more linear fashion instead of the classic sigmoidal curve, and no plateau being achieved during the 5-week incubation period.

NaCl affects mineralization of decane and toluene by Site B microbes

Low molecular weight radiolabeled model compounds were chosen for monitoring mineralization by Site B material because the known contaminants were gas condensate-range hydrocarbons. Decane (C_{10}) and toluene (C_7) represent the short-chain aliphatics and monoaromatics, respectively. Both substrates were incorporated into Site B free product, incubated at 10°C with or without N,P amendment, and the effects of NaCl on mineralization were measured.

Mineralization of decane by Site B material was low (Fig. 3 and Table 2). Even with nutrient amendment, $^{14}CO_2$ evolution was less than 10% after >300 h of incubation and showed a linear release of $^{14}CO_2$ (Fig. 3) rather than the sigmoidal curves seen in Fig. 1 with ^{14}C-hexadecane. Analysis of the data showed that, with N,P amendment, increasing NaCl concentrations caused a decreased rate (0.022% h^{-1} at 0% NaCl vs. 0.005% h^{-1} at 2.5% NaCl) and extent of mineralization at 312 h incubation (5% at 0% NaCl vs. 1% at 2.5% NaCl). Only one triplicate sample with nutrients exhibited decane mineralization at 5% NaCl,

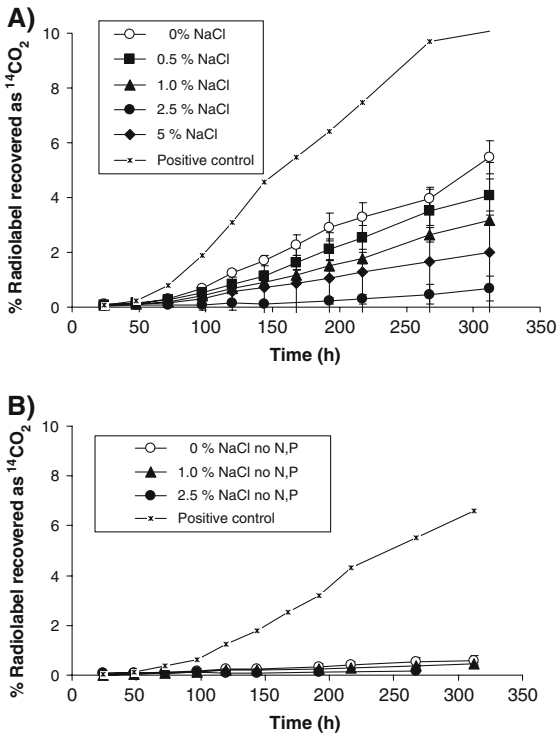

Fig. 3 Effect of NaCl and nutrient amendment on mineralization of ^{14}C-decane by soil and groundwater from Site B. All flasks were incubated at 10°C. Upper panel (**a**) amendment with 1× N,P. Lower panel (**b**) incubation without nutrient amendment (no N,P). Symbols represent the mean of 3 data points ±1 standard deviation, except for single positive control flasks

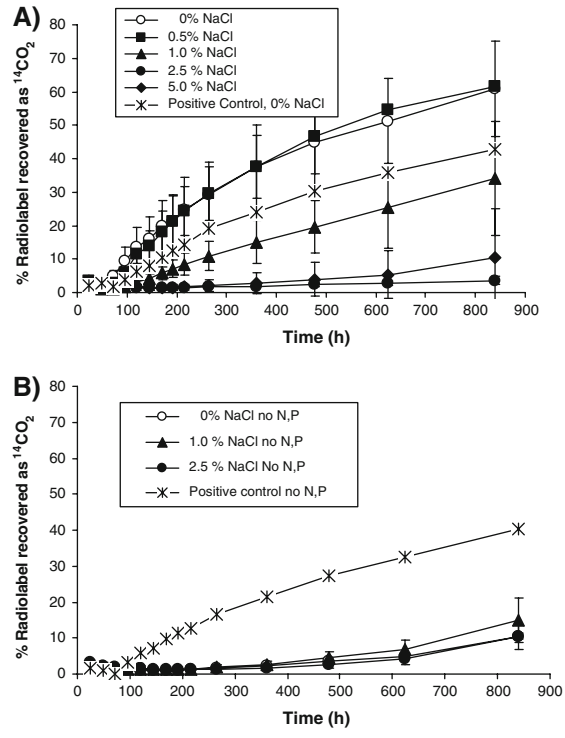

Fig. 4 Effect of NaCl and nutrient amendment on mineralization of ^{14}C-toluene by soil and groundwater from Site B. All flasks were incubated at 10°C. Upper panel (**a**) amendment with 1× N,P. Lower panel (**b**) incubation without nutrient amendment (no N,P). Symbols represent the mean of 3 data points ±1 standard deviation except for single positive control flasks

with a rate (0.023% h^{-1}) and extent (6%) of mineralization at 5% NaCl similar to 0% NaCl values. The effect on lag time is difficult to assess because of the linear shape of the $^{14}CO_2$ evolution curves. In the absence of added N,P, no significant mineralization was observed: the positive control cultures showed better mineralization than the Site B material both with and without nutrients (Fig. 3b).

Although we had expected greater mineralization of decane at Site B, reflecting the carbon range of contaminant that the microbial community presumably had adapted to, it appears that aliphatic mineralization in Site B material is limited even with N,P amendment, because incubation with ^{14}C-hexadecane also showed <10% $^{14}CO_2$ evolution after >450 h (data not shown) compared with >40% at Site A (Fig. 1b). This limitation likely reflects the composition of the microbial community because neither amendment with N,P nor incubation at 25°C resulted in enhanced mineralization.

Mineralization of ^{14}C-toluene by Site B material was rapid when N,P was supplied, but much less without nutrient amendment (Fig. 4). In the presence of 1× N,P (Fig. 4a and Table 2), 0.5% NaCl did not affect the lag, rate or extent of mineralization compared to flasks with 0% NaCl. The presence of NaCl at concentrations >0.5% resulted in greater lag times, slower rates and lower extent of mineralization. The extent of mineralization achieved at 840 h decreased with increasing NaCl, from 61% with 0% NaCl to 4% with 2.5% NaCl (Table 2), as did the rates of $^{14}CO_2$ evolution (Table 2). Again, at 5% NaCl only one flask of three replicates showed activity, which was comparable to cultures at lower NaCl concentrations. Lag time increased ca. 10-fold with increasing NaCl, from 27 h at 0% NaCl to 248 h at 2.5% NaCl, similar to other results reported herein (Table 2). The indigenous microbial community at Site B showed better toluene mineralization at

0 and 0.5% NaCl when N,P was supplied than did the positive control culture.

Discussion and conclusions

To determine the effect of salt on low-temperature, low-nutrient aerobic biodegradation of hydrocarbons, we used indigenous microbes in their native sediment matrix and groundwater to better simulate in-situ conditions. There are limitations to the application of the results from this study to field conditions. It is recognized that the low sediment-to-water ratios do not mimic in-situ conditions. In this study, we also introduced a major change from the in-situ conditions by incubating the microbes under aerobic conditions. While recognizing that anaerobic metabolism likely dominates the microbial activities in the sub-surface, anaerobic testing could require lengthy incubation times (months to years) to observe significant degradation, and monitoring $^{14}CO_2$ evolution might not reflect the extent of partial anaerobic attack on the radiolabeled model substrates. It is also known that various hydrocarbon-degrading facultative anaerobic microbes occur in the sub-surface; although they would utilize different pathways for aerobic and anaerobic attack on hydrocarbons, the effects of salt on the overall metabolic state of these species may be similar under both conditions. The insight gained from these aerobic tests may be used to better design future anaerobic studies.

^{14}C-hexadecane and ^{14}C-phenanthrene were selected as the model radiolabeled substrates for Site A tests to represent, respectively, the biodegradable non-volatile aliphatic and aromatic hydrocarbons present in the contaminant. The substrates were dissolved in free product recovered from the site so that they were presented in the "context" of the contaminant that the microbes have become adapted to in-situ, rather than as pure compounds or dissolved in an arbitrary inert carrier. Petroleum contaminants comprising predominantly low molecular weight compounds (like BTEX monoaromatics; benzene, toluene, ethylbenzene and xylenes) can be inhibitory even to hydrocarbon-degrading microbes. However, free product did not inhibit general metabolic activity (determined by ^{14}C-glucose mineralization) at 0.1 vol% for Site A nor up to 1 vol% for Site B (data not shown) and therefore free product was not likely toxic to the hydrocarbon-degraders at the concentrations tested.

Addition of nutrients was necessary to achieve rapid and significant hydrocarbon mineralization, and more so for the aliphatic substrates (hexadecane, Fig. 1; and decane, Fig. 3) than the aromatic substrates (phenanthrene, Fig. 2; toluene, Fig. 4), as previously observed (Foght et al. 1999). In the in-situ sediment, the microbes may need less nutrient supplement to achieve similar results because nutrient recycling may occur through microbial die-off (Röling et al. 2002; Head and Swannell 1999).

Although aliphatic hydrocarbons are generally considered to be more readily biodegradable than aromatic hydrocarbons, the microbial community in microcosms with site A soil and groundwater mineralized the aromatic substrate (phenanthrene) with a shorter lag time and a slightly faster rate than hexadecane under nutrient-amended conditions. This may be due to differences in substrate solubility and therefore bioavailability, with phenanthrene being about 360-fold more soluble than hexadecane (solubility of 3.6 μg/l). Similarly, toluene was degraded more rapidly and to a greater maximum extent than decane in microcosms with site B soil and groundwater. Analysis of the free product from Site B showed it to consist primarily of C_5 to C_8. Therefore, the in-situ microbial community may be better adapted to degrade aliphatics smaller than the radiolabeled decane. A preference for aromatics has been noted with other natural communities (Foght et al. 1990) and likely reflects the predominant microbial types in-situ adapted to the free product.

Although the subsurface temperature at Site A is cold, fluctuating seasonally between ca. 1 and 7°C, the Site A community was more active at 25°C than at 10°C. This result suggests the presence of a predominantly psychrotolerant rather than psychrophilic community and/or an increase in metabolic activity by the indigenous microbial community. However, even though the optimum temperature for activity was above 10°C, the majority of our experiments were conducted at the more environmentally relevant temperature of 10°C so that the combined effects of free product, low temperature, hydrocarbons and salt could be tested. This temperature effect persisted regardless of the presence of salt (e.g., Fig. 1).

Few reports have been published documenting the effect of salt on hydrocarbon biodegradation in soil

and freshwater (Amatya et al. 2002; Rhykerd et al. 1995; deCarvalho and daFonseca 2005), as opposed to numerous reports on marine or salt marsh environments and even fewer have looked at multiple stressors of temperature, nutrients, salt and free product on terrestrial microbial communities (e.g., Børresen and Rike 2007). Physiological concentrations of salt can be beneficial for microbial activity. For example, "LB broth" commonly used to grow *E. coli* contains 0.5% w/v NaCl. In contrast, high salt concentrations can inhibit the activity of microbes that are not adapted to salt. Possible reasons for this effect include direct inhibition of metabolic activity because of unfavorable high osmotic potential of the microbe's environment (Amatya et al. 2002), and altered solubility or sorption of toxic or essential ions. The tests in the current study do not discriminate between these possible effects, but instead present the sum of inhibitory effects on the total microbial community. The inhibitory salt effects observed included longer lag times and decreased rates and extents of mineralization. Haines et al. (1994) also found that increasing salinity (from 0 to 3% w/v) inhibited crude oil biodegradation by microbes from marine beach sediments in Alaska, resulting in increased lag times and decreased rate and extent of mineralization and O_2 uptake and thus showing that salt concentrations can affect even marine communities. In contrast, in the current study, low levels of salt were sometimes stimulatory (if only transiently). Similarly, Børresen and Rike (2007) found that low levels of NaCl (<0.3% w/w NaCl) slightly stimulated rates of hexadecane mineralization in an Arctic soil. This stimulation may be explained if the salt provides a more ionically balanced medium for the microbes, or a medium which disperses clays and thus provides a larger surface area for attachment of cells, or for access to trace nutrients.

In general, individual stressors resulted in fairly reproducible, predictable patterns of mineralization. For example, either low temperature or low nutrients reduced mineralization. However, the combination of multiple stressors resulted in less predictable, more complex responses that differed depending on the combination of conditions and the substrate being monitored. Based on the experimental results, the microbial community likely contains several types of microbes capable of degrading each of the model substrates. The experimental stressors may shift the community composition, resulting in altered mineralization patterns, as suggested by Børresen and Rike (2007). Although each strain may respond differently to the stressors, the measured mineralization reflects the overall community response to the stressors.

Of particular interest is the observation that certain flasks within a replicate set could experience a long lag time before eventually (and suddenly) beginning to mineralize the substrate at a rate similar to that of less stressed flasks. This lag time may be interpreted as a period of adaptation of the community to the stressors, during which there is enrichment for competent microbes able to endure the conditions, and/or for gene exchange to occur. This inter-replicate variability was clearly observed for flasks amended with 1× N,P and 5% NaCl, where mineralization occurred in only some flasks. These results suggest that under acclimated aerobic conditions intrinsic biodegradation may still proceed at rates similar to non-saline environments, perhaps in microsites or heterogeneous pockets of activity. It bodes well for emerging aerobic or microaerobic in-situ treatment remedial systems.

Further research is necessary to determine whether the effects observed in the laboratory are site- or contaminant-specific, or are applicable more broadly to sub-surface hydrocarbon bioremediation. Further research using more sites, including those previously having been impacted by NaCl, may allow inference of salt tolerance at upstream oil and gas sites. Particularly, the impact of NaCl on anaerobic hydrocarbon degradation should be investigated. Field evidence is sparse with respect to anaerobic biodegradation at salt contaminated upstream oil and gas sites. Before embarking on anaerobic microcosm tests, field evidence of indicators of anaerobic biodegradation including changes in terminal electron acceptors, presence of metabolites, and isotopic analysis would be a reasonable way to initiate the research.

Acknowledgements This project was funded by the Water Research Users Group, Alberta Environment and was part of the Consortium for Research on Natural Attenuation (CORONA, funded by the NSERC Collaborative Research and Development (CRD) program). Summer student funding to KP was received from the NSERC USRA program. In-kind support by Devon Canada Ltd., Conoco-Phillips Canada Ltd., WorleyParsons Komex International Ltd. and Maxxam Analytics is gratefully acknowledged.

References

Amatya PL, Hettiaratchi JPA, Joshi RC (2002) Biotreatment of flare pit waste. J Can Petrol Technol 41:30–36

Atlas RM (1981) Microbial degradation of petroleum hydrocarbons: an environmental perspective. Microbiol Rev 45:180–209

Bartha R, Pramer D (1965) Features of a flask and method for measuring the persistence and biological effects of pesticides in soil. Soil Sci 100:68–70

Børresen MH, Rike AG (2007) Effects of nutrient content, moisture content and salinity on mineralization of hexadecane in an Arctic soil. Cold Regions Sci Technol 48:129–138

deCarvalho CCCR, daFonseca MMR (2005) Degradation of hydrocarbons and alcohols at different temperatures and salinities by *Rhodococcus erythropolis* DCL14. FEMS Microbiol Ecol 51:389–399

Fedorak PM, Westlake DWS (1981) Microbial degradation of aromatics and saturates in Prudhoe Bay crude oil as determined by glass capillary gas chromatography. Can J Microbiol 27:432–443

Foght JM, Fedorak PM, Westlake DWS (1990) Mineralization of [^{14}C]hexadecane and [^{14}C]phenanthrene in crude oil: specificity among bacterial isolates. Can J Microbiol 36:169–175

Foght J, Semple K, Gauthier C, Westlake D, Blenkinsopp S, Sergy G, Wang Z, Fingas M (1999) Effect of nitrogen source on biodegradation of crude oil by a defined bacterial consortium incubated under cold, marine conditions. Environ Technol 20:839–849

Haines JR, Kadkhodayan M, Mocsny DJ, Jones CA, Islam M, Venosa AD (1994) Effect of salinity, oil type, and incubation temperature on oil degradation. In: Hinchee RE, Anderson DB, Metting FB, Sayles GD (eds) Applied biotechnology for site remediation, 2nd international symposium on in situ and on-site bioreclamation, San Diego, USA, 1993. Lewis Publishers, Boca Raton, pp 75–83

Head IM, Swannell RPJ (1999) Bioremediation of petroleum hydrocarbon contaminants in marine habitats. Curr Opin Biotechnol 10:234–239

Kleinsteuber S, Riis V, Fetzer I, Harms H, Müller S (2006) Population dynamics within a microbial consortium during growth on diesel fuel in saline environments. Appl Environ Microbiol 72:3531–3542

Margesin R, Schinner F (2001) Biodegradation and bioremediation of hydrocarbons in extreme environments. Appl Microbiol Biotechnol 56:650–663

Mille G, Almallah M, Bianchi M, van Wambeke F, Bertrand JC (1991) Effect of salinity on petroleum biodegradation. Fresenius J Anal Chem 339:788–791

Nicholson CA, Fathepure BZ (2004) Biodegradation of benzene by halophilic and halotolerant bacteria under aerobic conditions. Appl Environ Microbiol 70:1222–1225

Oren A, Gurevich P, Azachi M, Henis Y (1992) Microbial degradation of pollutants at high salt concentrations. Biodegradation 3:387–398

Pollard SJT, Hrudey SE, Fedorak PM (1994) Bioremediation of petroleum- and creosote-contaminated soils: a review of constraints. Waste Manag Res 12:173–194

Rhykerd RL, Weaver RW, McInnes KJ (1995) Influence of salinity on bioremediation of oil in soil. Environ Pollut 90:127–130

Riis V, Kleinsteuber S, Babel W (2003) Influence of high salinities on the degradation of diesel fuel by bacterial consortia. Can J Microbiol 49:713–721

Röling WFM, Milner MG, Jones DM, Lee K, Daniel F, Swannell RJP, Head IM (2002) Robust hydrocarbon degradation and dynamics of bacterial communities during nutrient-enhanced oil spill bioremediation. Appl Environ Microbiol 68:5537–5548

Ward DM, Brock TD (1978) Hydrocarbon biodegradation in hypersaline environments. Appl Environ Microbiol 35:353–359