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Characterization of Thermophilic Consortia from Two Souring Oil Reservoirs

ROBERT F. MUELLER* AND PER H. NIELSEN†

National Science Foundation Engineering Research Center for Biofilm Engineering, Montana State University, Bozeman, Montana 59717

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The microbial consortia from produced water at two different oil fields in Alaska (Kuparuk) and the North Sea (Ninian) were investigated for sulfate-reducing and methanogenic activity over a range of temperatures and for a variety of substrates. The consortia were sampled on site, and samples were either incubated on site at 60°C with various substrates or frozen for later incubation and analyses. Temperature influenced the rates of sulfate reduction, hydrogen sulfide production, and substrate oxidation, as well as the cell morphology. The highest rates of sulfate reduction and substrate oxidation were found between 50 and 60°C. Formate and *n*-butyrate were the most favorable electron donors at any tested temperature. Acetate was utilized at 35°C but not at 50 or 70°C and was produced at 60°C. This indicates that the high levels of acetate found in produced water from souring oil formations are due mainly to an incomplete oxidation of volatile fatty acids to acetate. The cell size distribution of the microbial consortium indicated a nonuniform microbial composition in the original sample from the Kuparuk field. At different temperatures, different microbial morphologies and physiologies were observed. Methane-producing activity at thermophilic temperatures (60°C) was found only for the Kuparuk consortium when hydrogen and carbon dioxide were present. No methane production from acetate was observed. Suppression of methanogenic activity in the presence of sulfate indicated a competition with sulfate-reducing bacteria for hydrogen.

Injection of seawater into oil field reservoirs for secondary oil recovery is common practice in Norwegian and British sectors of the North Sea as well as the coastal United States. Seawater contains sulfate (approximately 32 mM) and other substances essential for bacterial growth. The produced water is separated from the oil phase and either reinjected with additional seawater into the formation or discharged. When produced water is reinjected, the injection water is rich in organic carbon (3). Eventually, the growth requirements of sulfate-reducing bacteria (SRB) are met, resulting in the production of hydrogen sulfide (37).

The total cost related to SRB-mediated corrosion is estimated to be $1 billion to 2 billion per year in the United States (20). Additionally, precipitation of amorphous ferrous sulfide and production of exopolymer by SRB or other microorganisms in the formation cause plugging and diminish the effect of water injection (10, 19). Contamination of crude oil (3) and fuel gas (28) with hydrogen sulfide decreases the value of the product and increases refining costs.

In North Sea reservoirs, pressures range from 2×10^4 to 5×10^4 kPa and temperatures vary from 60 to 100°C (31). The initial reservoir conditions prior to well flooding at the Ninian North Sea oil field were 110 to 120°C and 4.5×10^4 kPa (7). Areas close to the injection well bores are subjected to a cooling effect, and temperatures rarely exceed 40°C. For ARCO's Prudhoe Bay oil reservoir, temperatures range from less than 20°C near seawater injection areas to 100°C at the production wells (2). In the Kuparuk reservoir, the maximum temperature is approximately 75°C (3).

The current understanding of souring is that it occurs at a mixing zone, where the injection water, the formation water, and the oil mix and nutrients for the growth of SRB are present (3, 11, 12, 21). Mesophilic SRB may be active in close vicinity to the injection well, where temperatures of 20 to 45°C are common and thermophilic and extremely thermophilic SRB may be active at the water/oil mixing zone at temperatures between 45 and 100°C (35). Abiotic reactions are not considered of major importance in the generation of hydrogen sulfide (9). They are important, however, in scavenging of H_2S, since many iron-containing minerals are capable of reacting with H_2S to form iron sulfide, pyrite, or pyrrhotite (8, 25).

The time course of souring was simulated by Chen et al. in an anaerobic porous-medium upflow column with a pure culture of *Desulfovibrio desulfuricans* and a microbial consortium from produced water from ARCO's Kuparuk oil production field (4, 5).

In this paper, results on substrate characterization for microbial consortia from two oil field reservoirs at a temperature range of 35 to 75°C are presented. The competition of SRB and other groups of organisms occurring in oil reservoirs is discussed. The following specific questions were addressed. (i) What carbon and energy sources are present in a souring reservoir? (ii) Which metabolic groups of microorganisms are present in a souring oil reservoir? (iii) What carbon and energy sources are utilized by these organisms? (iv) How does temperature affect the rate of sulfate reduction and substrate utilization?

MATERIALS AND METHODS

Selection of the sampling sites. ARCO's Kuparuk field on Alaska's North Slope and Chevron's Ninian offshore field in the North Sea are presently producing hydrogen sulfide and were chosen for sampling of microbial consortia. The Kuparuk field was H_2S free from startup in 1981 until seawater flooding started in 1985. The produced water is separated from the oil phase and rein-

* Corresponding author. Present address: Department of Geochemistry at Montana Tech of the University of Montana, 1300 West Park St., Butte, MT 59701-8997. Phone: (406) 496-4334. Fax: (406) 496-4334. Electronic mail address: RMUELLER@MTVMS2.MTECH.EDU.

† Present address: Environmental Engineering Laboratory, University of Aalborg, DK-9000 Aalborg, Denmark.

TABLE 1. Chemical composition of produced water sampled from ARCO's Kuparuk oil field and Chevron's Ninian oil field

Parameter	Value for:	
	Kuparuk (n = 4)	Ninian (n = 1)
Temp (°C)	68	74
Acetate concn (mM)	8.3–14.7	0.8
Propionate concn (mM)	0.8–1.2	0.1
Butyrate concn (mM)	0.07–0.12	ND[a]
Sulfate-S concn (mM)	77–123	32.8
Hydrogen sulfide-S concn (mM)	0.2–0.7	0.06
pH	7.7–7.9	7.2
Chloride concn (mM)	340–417	451

[a] ND, not detected by the method used for analysis.

jected into the reservoir along with fresh seawater. Hydrogen sulfide was first detected in 1986 in five wells at a maximum concentration of 1.3 mM (3). After 1988, all wells with significant seawater breakthrough produced H_2S. At the Ninian field, seawater flooding was used from the beginning of the operation and was accompanied by the production of hydrogen sulfide. The produced water is discharged after separation from the oil phase.

Sampling procedure. The sampling was performed on site for each oil field. The produced water was sampled prior to entering the surface facilities. The produced oil-water mixture was concentrated by approximately a factor of 100 with a membrane filter device (pelicon). A prefilter (pore size, 15 μm) eliminated oil and other particulates that could clog the filter membrane. All sampling was performed by anaerobic techniques, including positive N_2 pressure on all containers used. Half of the final sample concentrate was introduced into 1.6-ml vials and was shock frozen in isopropanol-dry ice. All liquid transfers were carried out in an anaerobic glove box in a N_2-H_2-CO_2 (85:10:5) gas mixture. The frozen samples were transported in dry ice; upon arrival in the laboratory, the frozen samples were stored at −70°C.

Medium preparation. The medium used was suggested for marine-type SRB (29). The sulfate concentration in the medium was 5.625 mmol liter^{-1}, the salinity was 2% sodium chloride, and the pH was adjusted to 7.2. When methanogenic activity was tested, no sulfate was added to the medium.

Testing for potential substrates. A 1-ml portion of the sample concentrate was injected on site into 60-ml glass serum vials sealed with thick butyl rubber stoppers to test for utilization of various potential substrates. SRB medium (30 ml) was transferred into the oxygen-free vials. The vials were pressurized with N_2 (2×10^2 kPa). Different electron donors, each at an initial concentration of 200 mg liter^{-1}, were added to the medium (see Table 3). Gases such as methane, hydrogen, and carbon dioxide were added as a volumetric addition of 10 ml of normalized gas volume to the headspace of each serum vial. For each test, four replicate vials plus a control without inoculation were used. The vials were incubated at 60°C for 31 days. Redox potential, pH, total cell number (obtained by acridine orange direct counts [AODC]), sulfate, hydrogen sulfide, formate, acetate, propionate, isobutyrate, *n*-butyrate, and lactate concentrations in the aquous phase and methane concentration in the vial headspace were monitored.

The test was defined as positive for microbial sulfate reduction if there was significant disappearance of sulfate (>0.6 mmol liter^{-1} compared with an uninoculated control), production of hydrogen sulfide, disappearance of substrate, and production of cellular material. The test was positive for microbial methane production if a significant amount of methane (>1% in the vial headspace, compared with an uninoculated control) and cellular material was produced.

Test of the effect of temperature on microbial sulfate reduction and substrate utilization. Test vials were incubated with a microbial consortium from the Kuparuk field at 35, 50, 60, and 75°C. Hydrogen sulfide, sulfate, and volatile fatty acid concentrations were monitored over time for each test temperature. The experiment was carried out in triplicate for each temperature. A mixture of volatile fatty acids (C_1 to C_4), each at an initial concentration of 200 mg liter^{-1} (total weight for each of the volatile fatty acids present), was added as the substrate. Frozen stock culture (1 ml) plus 1 ml from an enrichment culture growing in the log phase at 60°C was injected into each vial. Abiotic controls identical to the test vials were prepared.

Microbial analysis. Total cell counts were performed by the AODC method (15). Image analysis was used for enumeration of the cells as well as for a statistical cell size analysis (26). The most-probable-number method (five-tube assay) was used to differentiate between SRB, methanogenic bacteria, and general anaerobic bacteria. The media used were Postgate B with acetate, lactate, propionate, and butyrate as carbon sources; a broad methanogenic medium including hydrogen and carbon dioxide (30, 36); and fluid thioglycolate (Difco), respectively.

Chemical analysis. A Dionex ion chromatograph equipped with an AS4A-SC 2-mm column and 5 mM sodium bicarbonate as the eluent was used for sulfate analysis. Volatile fatty acids were separated on an AS10 4-mm column with 3.5 mM potassium tetraborate as the eluent and 100 mM potassium tetraborate to regenerate the column. A pulsed electrochemical conductivity detector was used for sulfate and volatile fatty acid detection. Hydrogen sulfide was analyzed by the methylene blue method (6). Lactate was detected with a Sigma enzymatic assay kit. Methane in the headspace of the test vials was measured with a Perkin-Elmer gas chromatograph equipped with A chromosorb 100/80-mesh stainless steel column and a flame ionization detector.

RESULTS

Produced-water composition. The water chemistry was determined for produced water from ARCO's Kuparuk field and from Chevron's Ninian field. The water chemistry varied significantly between these two fields. The produced water from the Kuparuk field exhibited a ratio of acetate to propionate to butyrate of approximately 100:10:1 (Table 1). The produced water from the Ninian field contained acetate and propionate at much lower concentrations. The average concentration of sulfate was 4.7 mM in the produced water from the Kuparuk field and 32.8 mM in that from the Ninian field. The low sulfide concentration measured for both fields might result from precipitation in the formation and transfer of hydrogen sulfide in the water phase to the oil and gas phase. The pH was slightly higher in the Kuparuk field.

Consortium collection. At both fields, the microbial consortia consisted of several microbial groups, including SRB, methanogenic bacteria, and general anaerobic bacteria. The total cell concentration in the produced water was considerably higher at Kuparuk than at the Ninian field. Viable mesophilic anaerobic bacteria (general anaerobic bacterium most-probable-number tests) in the produced water from the Kuparuk field were detected at about one-third of the total number of bacteria measured by the acridine orange direct count method (Table 2). At 35°C, the numbers of methanogens were significantly larger than the numbers obtained for mesophilic SRB. The numbers of thermophilic methanogens were similar to the numbers of thermophilic SRB. Approximately 4% of the total number of bacteria present was detected as viable thermophilic SRB.

Statistical analysis (t test) of the cell size distribution of the Kuparuk consortium indicated a distinction between three morphologically different populations (Fig. 1). The popula-

TABLE 2. Microbial composition of produced water from the Kuparuk oil field

Parameter	Value
Total cell no. (cells/ml)[a]	$(4.5 \pm 0.4) \times 10^4$
Cell size distribution (μm^2)	
Population 1	0.28 ± 0.08
Population 2	0.87 ± 0.18
Population 3	12.45 ± 1.4
No. of mesophilic bacteria (35°C) (cells/ml)[b]	
General anaerobic bacteria	1.4×10^4
SRB	1.2×10^2
Methanogenic bacteria	2.4×10^3
No. of thermophilic bacteria (60°C) (cells/ml)[b]	
General anaerobic bacteria	1.1×10^4
SRB	1.6×10^3
Methanogenic bacteria	0.9×10^3

[a] Determined by acridine orange direct counts.
[b] Determined by the most-probable-number technique.

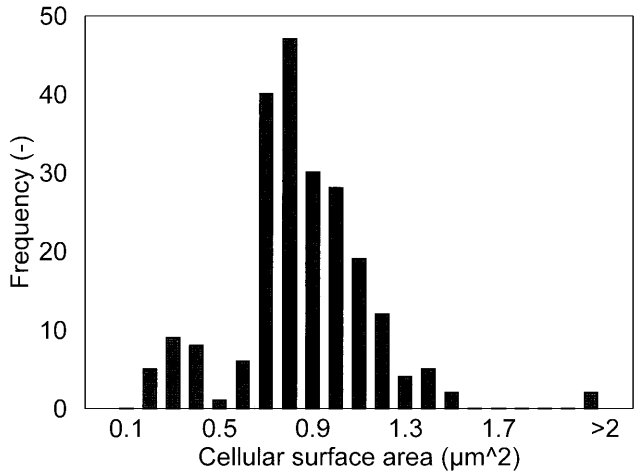

FIG. 1. Histogram of the cell size distribution, using the acridine orange direct count method and image analysis. The sample measured was taken from produced water at ARCO's Kuparuk river field in Alaska.

tions are listed in order of size in Table 2; they contributed 6.4, 92.8, and 0.8%, respectively, to the total cell numbers.

Characterization of potential substrates supporting microbial activity. When single substrates were tested for microbial sulfate reduction and methanogenesis at 60°C, similar results were obtained for consortia from both tested fields. Microbial growth correlated with sulfate reduction, hydrogen sulfide production, and the disappearance of the electron donor, i.e., hydrogen plus carbon dioxide, formate, propionate, butyrate, or lactate (Table 3). When lactate, propionate, or butyrate was utilized, acetate was produced. Acetate was not oxidized for sulfate reduction at the temperature and for the time measured. Saturated fatty acids such as caprolate, stearate, and palmitate were utilized for sulfate reduction. Unsaturated long-chain fatty acids such as oleate and hydrocarbons such as hexadecane were not utilized for sulfate reduction.

Sulfate reduction was correlated with an increase in pH and a decrease in redox potential. The pH in the medium prior to any microbial activity was adjusted to pH 7.2, and the redox potential (E_h) was measured between −70 and −100 mV. After sulfate was depleted in the test vials, the pH was measured between 8.6 and 9.2 and the redox potential was measured between −340 and −400 mV.

Samples from the Kuparuk reservoir tested for methanogenic activity indicated the presence of small amounts of methane (<1% in the headspace) in all vials inoculated directly on site. When sulfate was present, methane production from hydrogen and carbon dioxide was measured at 5 to 10% methane in the headspace (Table 3). When no sulfate was present, the methane production from H_2 plus CO_2 increased to 72% in the headspace. Butyrate or lactate also supported methanogenesis in the absence of sulfate (5 and 8% methane in the headspace, respectively). Acetate or formate did not support methane production at 60°C. In contrast to the Kuparuk consortium, no methanogenesis was observed on any tested substrates for the Ninian field consortium.

The test for potential substrates was repeated after 3 months with frozen samples from the Kuparuk field (Table 3). Similar results were observed for sulfate reduction with all tested substrates. Methanogenesis with a frozen inoculum was observed only when hydrogen and CO_2 were present.

Effect of temperature on microbial sulfate reduction. The effect of temperature on sulfate reduction was tested with the Kuparuk consortium. Simultaneous sulfate reduction, hydrogen sulfide production, and volatile fatty acid uptake were observed in all inoculated test vials and for all tested temperatures. Abiotic controls at 75°C showed no conversions. The maximum rate for sulfate reduction was observed between 50 and 60°C (Fig. 2). At lower or higher temperatures, sulfate reduction occurred at lower rates and with a longer lag phase.

The rate of formate, acetate, and *n*-butyrate utilization varied with temperature, whereas the rates of propionate and isobutyrate oxidation were relatively constant at all tested temperatures (Fig. 3). The maximum rates of formate and *n*-butyrate utilization were the highest at all tested temperatures, being 23.4 and 11.5 μmol liter^{-1} h^{-1}, respectively, at 50°C. Acetate was utilized at 3.5 μmol liter^{-1} h^{-1} at 35°C but was produced at 13.4 μmol liter^{-1} h^{-1} at 60°C. At 50 and 75°C, the acetate concentration did not change significantly.

The morphology of the microbial consortium appeared significantly different at each temperature. At 35°C, rods 0.3 to 0.6 μm in diameter and 1.5 to 3.0 μm long dominated, whereas at 50°C, primarily filamentous organisms 5 to 50 μm long were dominant. More extracellular material was observed at 50°C than at any other tested temperature. At 60°C, the dominant organisms were 2 to 4 μm long and 0.3 to 0.7 μm in diameter. The dominant organisms at 75°C were small cocci with a diameter of 0.2 to 0.5 μm. All cell dimensions were based on 95% confidence intervals.

DISCUSSION

The microbial consortia from produced-water sampled from both oil fields were capable of sulfate reduction over a wide range of temperatures and for a variety of substrates. However, to extrapolate our results from the laboratory tests directly to oil reservoirs, we must also remember that differences in pressure, temperature, etc., may affect the rate and processes of native microbial consortia. The sampling and storage procedures used in this study seemed to be sufficient for sampling, characterizing, and storing SRB and methanogenic bacteria. It seemed that the fermentative and syntrophic organisms may have been damaged by the freezing process, and on-site incu-

TABLE 3. Comparison of the on-site tested potential microbial activity at 60°C with respect to various substrates

Carbon and energy source	Kuparuk[a]				Ninian[a]	
	SO_4^{2-} reduction		CH_4 production		SO_4^{2-} reduction (fresh)	CH_4 production (fresh)
	Fresh	Frozen	Fresh	Frozen		
Produced water	0	NT	+	NT	0	0
Formate	+	+	0	0	+	0
Acetate	0	0	0	0	0	0
Propionate	+	+	0	0	+	0
n-Butyrate	+	+	0	0	+	0
Isobutyrate	+	+	+	0	+	0
Lactate	+	+	+	0	+	0
Caprolate	+	NT	0	NT	NT	NT
Hexadecane	0	NT	0	NT	NT	NT
Oleate	0	NT	NT	NT	NT	NT
Stearate	+	NT	NT	NT	NT	NT
Palmitate	+	NT	NT	NT	NT	NT
H_2-CO_2	+	+	+	+	+	0

[a] NT, substrate not tested; 0, very little or no activity; +, substrate activates sulfate reduction or methanogenesis, respectively. Fresh and frozen refer to the culture type used.

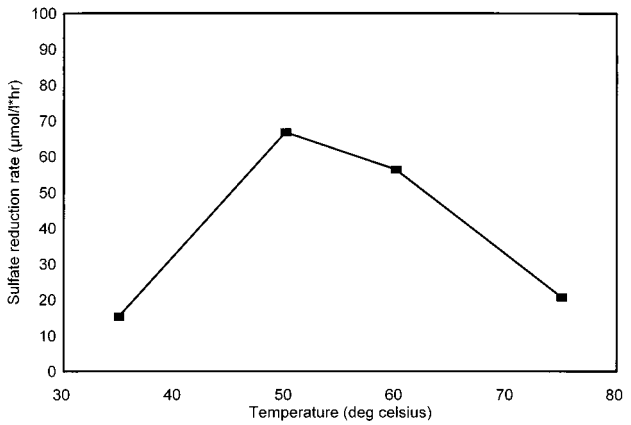

FIG. 2. Influence of temperature on the maximum observed rate of microbial sulfate reduction. The maximum sulfate reduction rate is plotted against the temperature of inoculation. Each datum point represents the mean rate for maximum sulfate reduction determined from three replicative experiments. The tested temperatures were 35, 50, 60, and 75°C.

bations (without freezing) were required for some type of experiments.

At 60°C, sulfate reduction was observed for both consortia with hydrogen plus carbon dioxide, lactate, formate, butyrate, and propionate substrates. Saturated (stearic acid and palmitic acid) and unsaturated (oleic acid) long-chain fatty acids might be associated with the crude oil and dissolve in formation water as a result of the water/oil partitioning at the mixing zone. Fermentative and syntrophic acetate-oxidizing bacteria may play an important role in the utilization of these compounds. In contrast to the results of one study of mesophilic SRB (1), a hydrocarbon substrate (hexadecane) did not initiate sulfate reduction within the time used for testing.

Most thermophilic SRB that were isolated from natural environments are capable of utilizing substrates similar to those found in our experiments (16, 32, 35). The most favorable electron donors for thermophilic sulfate reduction are reported to be hydrogen, lactate, formate, propionate, butyrate, and in some cases even longer-chain fatty acids up to C_7 (32). In the Kuparuk consortium, formate and *n*-butyrate seemed to be the most favorable substrates and were consumed prior to any other volatile fatty acids offered. Acetate was utilized for sulfate reduction at 35°C. No growth was observed with acetate as the sole substrate above 50°C. The oxidation of butyrate and propionate was associated with the production of acetate at 60°C, most probably by an incomplete oxidation to acetate and carbon dioxide. Although some thermophilic SRB, such as *Desulfotomaculum kuznetsovii* (27) and *Desulfotomaculum thermoacidooxidans* (24), were reported to oxidize acetate, SRB isolated from North Sea and Alaska oil formations were not able to grow on acetate under reservoir conditions (32, 35). This observation is important since several authors have suggested that acetate is one of the main substrates for SRB in the formation as a result of the high acetate concentrations found in most produced water at souring oil fields (3, 7).

The high acetate concentration measured in the produced water from both tested oil fields might be a product of the incomplete oxidation of longer-chain volatile fatty acids by thermophilic SRB in the reservoir. When longer-chain volatile fatty acids were oxidized, we observed a stoichiometry similar to that suggested by others (37, 38). The high acetate concentration at the Kuparuk field was associated with low sulfate and butyrate concentrations and intermediate propionate concentration, thus promoting our hypothesis. The high level of volatile fatty acids associated with a low sulfate concentration in the Kuparuk field, which has been souring for many years, may indicate that souring can cause an increased level of volatile fatty acids, in particular acetate.

The consortium from the Kuparuk field was capable of methane production from hydrogen and carbon dioxide at mesophilic and thermophilic temperatures; however, we were unable to activate methanogenesis in samples from the Ninian field. One possible explanation is the high sulfate concentration (32.8 mol m^{-3}) measured in the Ninian produced water, thus suppressing methanogenesis. As a result of high SRB activity within the Kuparuk reservoir, the produced water had a low sulfate concentration (2.4 to 7 mM). A complex system such as an underground oil reservoir represents a heterogeneous system, and some of the high SRB activity could be localized. At these locations, sulfate might be depleted or may be present at levels low enough to enable methanogens to become active. The Ninian reservoir is saturated with a high sulfate level throughout the entire field, suppressing methanogenesis. This was confirmed by the observation of suppression of methane production in the presence of sulfate, as demonstrated with the Kuparuk consortium. This result is supported by other investigations in mesophilic environments (18, 22, 34). However, other factors such as sulfide concentration (14) or pH (23) will also determine the outcome of the competition. In mesophilic environments, acetate and hydrogen are the most important substrates for methane formation in natural systems (17), but little information exists about the substrates under thermophilic reservoir conditions. In our experiments, no methane was formed from acetate at 60°C. Hydrogen was the only substrate for the methanogens, despite the large quantities of acetate available throughout the reservoir.

Microbial sulfate reduction was observed over the temperature range from 35 to 75°C. The temperature affected the rate of microbial sulfate reduction as well as the substrate specificity and the morphology of the microorganisms present. The highest rate of specific sulfate reduction was found at 50 to 60°C. Mesophilic SRB were commonly detected in seawater that is used as injection water for oil formations (3, 12, 13). It was speculated that thermophilic SRB may even be present in very small numbers in seawater (33), and the finding of ther-

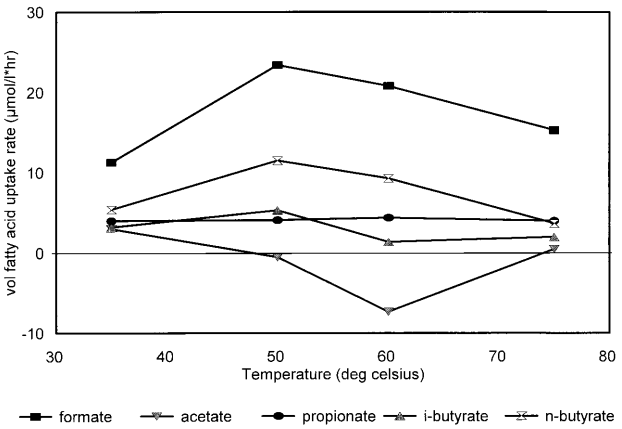

FIG. 3. Maximum observed rate of substrate oxidation as a function of temperature. A mixture of volatile fatty acids such as formate, acetate, propionate, isobutyrate, and *n*-butyrate were offered to the consortium from the produced water from the Kuparuk field. The maximum consumption rates for each of these volatile fatty acids were temperature dependent.

mophilic SRB in large numbers in sediments, even in Arctic areas (16), supports this hypothesis.

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