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Anaerobic biodegradation of vegetable oil and its metabolic intermediates in oil-enriched freshwater sediments

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Abstract

Anaerobic biodegradation of vegetable oil in freshwater sediments is strongly inhibited by high concentrations of oil, but the presence of ferric hydroxide relieves the inhibition. The effect of ferric hydroxide is not due to physical or chemical interactions with long-chain fatty acids (LCFAs) that are produced as intermediates during metabolism of vegetable-oil triglycerides. The anaerobic biodegradation of canola oil and mixtures of acetic and oleic acids, two important intermediates of vegetable-oil metabolism, were investigated using sediments enriched on canola oil under methanogenic and iron-reducing conditions to determine whether the effect of ferric hydroxide has a biological basis. Sediments enriched under both conditions rapidly and completely converted canola oil to methane when the initial oil concentration was relatively low (1.9 g oil/kg sediments), but the biotransformation was strongly inhibited in sediments enriched under methanogenic conditions when the initial concentration was 19 g/kg ($<30\%$ of the oil-derived electron equivalents were transferred to methane in a 420-day incubation period). Sediments enriched under iron-reducing conditions, however, completely transformed canola oil to methane in about 250 days at this initial oil concentration. The anaerobic biotransformation of mixtures of acetate and oleic acid followed a similar pattern: the rate and extent of conversion of these electron-donor substrates to methane was always higher in sediments enriched under iron-reducing than under methanogenic conditions. These results suggest that enrichment on canola oil in the presence of ferric hydroxide selects a microbial community that is less sensitive to inhibition by LCFAs than the community that develops during enrichment under methanogenic conditions.

Introduction

Accidental spills of vegetable oils and animal fats into coastal and inland water are relatively common (Federal Register 1997). Although vegetable oils lack the acutely toxic components that are present in petroleum and its refined products (e.g., aromatic hydrocarbons), they can cause severe damage to sensitive aquatic organisms and ecosystems nonetheless (Crump-Weisner & Jennings 1975; McKelvey et al. 1980; Mudge 1995), and rapid response is the key to minimizing the harmful environmental impacts of vegetable oil spills (Calanog et al. 1999; Rigger 1997). Because most of the harmful effects are due to oil that is either floating on the water surface or suspended in the water column, a novel response alternative – which involves sedimentation of floating vegetable oil by formation of dense oil-mineral aggregates followed by anaerobic biodegradation of the oil in the sediments – has recently been proposed (Wincele et al. 2004). Although this response alternative is expected to be faster and less expensive than traditional mechanical recovery operations, its feasibility depends on the kinetics of anaerobic biodegradation of the oil. Long-term persistence of the oil in the sediments would make

sedimentation an unacceptable response alternative, and anaerobic biodegradability is a characteristic that distinguishes vegetable oil from petroleum and its refined products.

In the absence of alternative electron acceptors, vegetable oil and other lipids will be transformed to methane and carbon dioxide in freshwater sediments. This transformation is catalyzed by complex microbial consortia involving several physiologically distinct groups of microorganisms. Vegetable oil biodegradation is initiated by enzymatic hydrolysis of triglycerides to glycerol and long-chain fatty acids (LCFAs), which serve as the growth substrates for several members of the microbial consortium. Glycerol is usually fermented to volatile fatty acids and alcohols, such as propionate, acetate, and 1,3-propandiol (Biebl et al. 1998; Schauder & Schink 1989). LCFAs are oxidized to hydrogen, acetate, and shorter chain-length fatty acids by hydrogen-producing acetogens (Mackie et al. 1991; McInerney 1988). LCFAs have long been recognized as being inhibitory to a wide variety of microorganisms, including aerobic heterotrophs (Atlas & Bartha 1973; Bell 1971; Teh 1974), hydrogen-producing acetogens (Hanaki et al. 1981; Lalman & Bagley 2002) and acetoclastic methanogens (Hanaki et al. 1981; Hwu et al. 1996; Koster & Cramer 1987; Lalman & Bagley 2000; Pereira et al. 2001; Rinzema et al. 1994). Instability due to self-inhibition by LCFAs is a chronic problem in the anaerobic treatment of lipid-rich wastes (Angelidaki et al. 1990; Broughton et al. 1998; Hanaki et al. 1981; Pereira et al. 2001).

When microbial growth is limited by the availability of electron-donor substrates, reduction of ferric iron species competes with methane production (Lovley 1991; Lovley & Phillips 1986). When the electron-donor substrate is present in excess, however, the presence of Fe(III) may have more subtle effects on the composition of the microbial community. For example, previous research in our laboratory showed that, in the absence of ferric hydroxide, conversion of high concentrations of canola oil to methane in freshwater sediments was severely inhibited for several months, whereas in the presence of ferric hydroxide, the same concentration of canola oil was transformed to methane without any apparent inhibition (Li et al. 2001). Although the Fe(III) was reduced to Fe(II) with near stoichiometric efficiency in those experiments, more than 80% of the oil-derived electron equivalents were ultimately transferred to methane. The potential for ferric hydroxide to affect canola oil mineralization through a physical or chemical mechanism (e.g., reduced bioavailability of toxic intermediates) was investigated by comparing the effects of ferric hydroxide to those of calcium and clay, which have been shown to reduce the toxicity of LCFAs by precipitation of insoluble salts and adsorption, respectively (Angelidaki et al. 1990; Hanaki et al. 1981). Although ferric hydroxide stimulated the rate of canola oil biotransformation to methane, calcium and clay either inhibited the reaction rate or had no effect, respectively (Li & Wrenn, in press). These results suggested that the effect of ferric hydroxide was likely due to biological factors, such as selecting for microorganisms that were less sensitive to inhibition by LCFAs.

This report describes the results of an investigation of the fatty acid sensitivity of anaerobic freshwater sediment microbial communities that were enriched on a noninhibitory concentration of vegetable oil under methanogenic or iron-reducing conditions. The rates and extents of conversion of canola oil to methane by sediments enriched under these different conditions were compared at two initial oil concentrations: a low, noninhibitory concentration and a higher, inhibitory concentration. In addition, the rates of acetate consumption and methanogenesis by these enriched sediments in the presence of oleic acid, a model LCFA, were compared, because aceticlastic methanogens have been shown to be particularly sensitive to fatty acid inhibition in anaerobic digesters that treat lipid-rich wastes (Hanaki et al. 1981; Hwu et al. 1996; Koster & Cramer 1987; Lalman & Bagley 2000; Pereira et al. 2001; Rinzema et al. 1994). The purpose of these experiments was to determine whether enrichment under iron-reducing conditions affected the fatty acid sensitivity of the microbial community in these sediments.

Methods

Chemicals

Except as specified, all chemicals were ACS reagent-grade and were purchased from Fisher

Scientific (Pittsburgh, PA). Oleic acid, the volatile fatty acid standards, HPLC-grade dimethyl sulfoxide (DMSO), and dimethyl sulfide (DMS) were purchased from Sigma-Aldrich (St. Louis, MO). Canola oil was purchased from a local grocery store. Ultra-pure deionized water was used to prepare the culture medium and the acetic acid stock solution.

Enrichment of sediment microbial communities

Sediments (primarily fine sand and silt with low organic carbon content) were collected from the Illinois River near Grafton, Illinois. The sediments were enriched in two master-culture reactors (MCRs) constructed in 5-l glass bottles: one MCR was operated under methanogenic conditions (i.e., no exogenous ferric hydroxide) and the other was operated under iron-reducing conditions. Each MCR contained 800 g (wet weight) of sediments, 1000 ml nutrient medium, and 3 ml of canola oil (3.5 g oil/kg sediment). In addition, 13 g of amorphous ferric hydroxide was prepared (Lovley & Phillips 1986) and added to the iron-reducing MCR. This represents enough ferric hydroxide to consume about 12% of the electron equivalents added to the MCR as canola oil. The composition of the mineral salts medium was (mg/l): K_2HPO_4 (1206), KH_2PO_4 (420), $MgCl_2{\cdot}6H_2O$ (3150), KCl (336), NaCl (305), $CaCl_2{\cdot}2H_2O$ (148), $MgSO_4{\cdot}7H_2O$ (15), NH_4Cl (10), EDTA (11.6), $MnSO_4{\cdot}2H_2O$ (2.2), $CoSO_4{\cdot}7H_2O$ (0.99), $ZnSO_4{\cdot}7H_2O$ (0.9), $FeCl_3$ (0.3), H_3BO_3 (0.057), Na_2MoO_4 (0.06), $CuSO_4{\cdot}5H_2O$ (0.05), and yeast extract (500). The pH of the medium was adjusted to 7.0, and it was sterilized by autoclaving. The nutrient medium was sparged with filtered nitrogen while cooling to remove dissolved oxygen.

After autoclaving all glass containers and tubing, the MCRs were constructed inside an anaerobic chamber (Coy Laboratory, Grass Lake, MI) containing a mixture of 95% N_2 and 5% H_2 (CeeKay Supply Inc, St. Louis, MO). The sealed reactors were removed from the anaerobic chamber and incubated at room temperature. The volume of methane produced in the MCRs was measured by displacement of 0.1 M sodium hydroxide from a second 5-l carboy, which was connected through a spigot to a 100-ml graduated cylinder. No evidence of inhibition was observed in the methane-production progress curves for either enrichment condition, and biodegradation of the added oil was complete after about 70 days. Electrons added as canola oil were quantitatively (98%) recovered as methane in the methanogenic MCR. Electron recovery was also nearly complete (89%) in the iron-reducing MCR; approximately 80% of the oil-derived electrons were recovered as methane and about 9% were recovered as Fe(II). The ferric hydroxide added to the iron-reducing MCR appears to have been completely reduced during enrichment on canola oil, but the products (e.g., siderite) were probably not efficiently extracted under the relatively mild conditions used in the ferrous iron assay (Heron et al. 1994).

Construction of anaerobic sediment microcosms

Anaerobic sediment microcosms were used to study the biodegradation kinetics of canola oil and mixtures of acetate and oleic acid. The microcosms were constructed in the anaerobic chamber with a 100% nitrogen atmosphere to prevent their contamination with hydrogen. Microcosms consisted of 160-ml serum bottles containing 50 g (wet weight) of oil-enriched sediments and 50 ml of nutrient medium. Microcosms used for investigation of canola oil biodegradation kinetics were provided with either 0.1 ml (1.9 g oil/kg sediment) or 1.0 ml (19 g oil/kg sediment) of canola oil using a 1-ml Gas Tight syringe (Fisher-Hamilton, Reno, NV). Previous research showed that, in the absence of exogenous ferric hydroxide, canola oil biodegradation was inhibited at the higher oil concentration but not at the lower concentration (Li et al. 2001). Microcosms used to study the biodegradation kinetics of acetate and oleic acid were spiked with both substrates to achieve initial concentrations of 10 or 15 mM acetate and 2 or 3 mM oleic acid. Acetate was provided from a filter-sterilized aqueous stock solution (330 mM), and oleic acid was provided from a filter-sterilized stock solution (70 mM) in dimethyl sulfoxide (DMSO).

All treatments were conducted in independent duplicate microcosms. The microcosms were sealed with butyl rubber stoppers and aluminum crimp caps and shaken at 200 rpm for 2 h to homogenize the substrates and sediments. The microcosms were incubated inverted at room temperature (21 °C) without shaking. Microcosms were shaken vigorously prior to sample collection.

Biodegradation of the added substrates was monitored by measuring the concentrations of the products of important terminal electron-accepting processes, including methane, Fe(II), and dimethyl sulfide (DMS), which is formed by reduction of DMSO. The concentrations of aqueous-phase volatile fatty acids (e.g., acetate, propionate, and butyrate) were also measured.

Analytical methods

Methane production was monitored by measuring headspace gas volume and composition. The gas volume was measured at ambient pressure by displacement of the plunger of a water-lubricated syringe (Owen et al. 1979). Gas samples were collected in inverted 2-ml crimp-top vials that were initially filled with 0.05N HCl. The gas samples (about 1.5 ml) displaced an equal volume of HCl, and the vials were stored inverted with the remaining HCl solution in contact with the Teflon-lined septum until the samples were analyzed. The concentrations of hydrogen, methane, and carbon dioxide in the headspace gas samples were measured by isothermal (50 °C) gas chromatography (GC) on a 6' × 1/8'' Chromosorb 102 column (Sigma-Aldrich, St. Louis, MO) with a thermal conductivity detector. The carrier gas was zero-grade nitrogen, and the flow rate was 10 ml/min. The DMS concentration in the headspace gas was measured by isothermal (120 °C) gas chromatography on a 30 m × 0.25 mm DB-17 capillary column (J&W Scientific Inc., Folsom, CA) with a flame photometric detector operated in sulfur mode (Christensen & Reineccius 1992). Ultra-high-purity helium was used as carrier gas at flow rate of 1.0 ml/min with a split ratio of 100:1. The total mass of DMS in each serum bottle was estimated by assuming equilibrium partitioning between the headspace and aqueous phases using the Henry's coefficient reported by Brennan et al. (1998). A 50-μl Gas Tight Sample Lock syringe (Fisher-Hamilton) was used to inject 10-μl samples onto the GC columns.

Volatile fatty acids were measured in aqueous samples (0.1 ml) collected from microcosm supernatant after settling for at least 1 h. The samples were acidified with 0.3 ml of 1% (v/v) formic acid solution and analyzed by gas chromatography using a 30 m × 0.53 mm Nukol column (Supelco, Bellefonte, PA) with a flame ionization detector. The carrier gas was ultra-high-purity helium, and the flow rate was 2.0 ml/min with a 10:1 split ratio. The column temperature program was: 110 °C for 4 min followed by a 10 °C/min ramp to 180 °C, and the final temperature was held at 180 °C for 6 min (total run time was 17 min). One microliter aqueous samples were injected onto the GC column.

Ferrous iron was measured by extracting 0.1-ml samples of sediment slurry into 5 ml of 0.5 M HCl for 1 hr (Lovley & Philips 1986). The concentration of extracted Fe(II) was determined using ferrozine (1 g/l in 50 mM HEPES, pH = 7.5) by measuring the absorbance of the Fe(II)-ferrozine complex at 562 nm. Calibration standards were prepared using ferrous ammonium sulfate.

Results

Canola oil biodegradation kinetics in oil-enriched sediments

Biodegradation of canola oil in oil-enriched Illinois River sediments was investigated at two initial oil concentrations, one of which (19 g oil/kg sediment) had previously been shown to be self inhibitory when sediments were incubated under methanogenic conditions (Li et al. 2001; Li & Wrenn in press). For the experiments described in this report, the sediment microbial communities were enriched by growth on canola oil under either methanogenic or iron-reducing conditions prior to construction of the microcosms. The oil concentration used during enrichment in the MCRs (3.5 g oil/kg sediment) was not inhibitory, regardless of whether or not ferric hydroxide was present. No additional ferric hydroxide was added when the microcosms were assembled. Although extensive reduction of Fe(III) to Fe(II) was observed during enrichment in the iron-reducing MCRs, measurement of Fe(II) in microcosm sediments confirmed that no additional iron reduction occurred during the incubations that are described here.

Methane production from canola oil is shown in Figure 1 for microcosms provided with a low initial oil concentration (1.9 g oil/kg sediment). The aqueous-phase concentrations of acetate, propionate, and butyrate are also shown. At this

initial oil concentration, mineralization of canola oil was almost complete within about 25 days regardless of the sediment-enrichment conditions (Table 1), which is consistent with previous observations (Li et al. 2001). The concentrations of intermediate volatile fatty acids never exceeded 2.5 mM and were undetectable after oil mineralization was completed. Although the differences between enrichment conditions were relatively small, sediments enriched under iron-reducing conditions appeared to convert the added canola oil to methane more rapidly than the sediments enriched under methanogenic conditions (Figure 1a). Biodegradation kinetics at the low initial oil concentration were adequately described using a mixed second-order rate law (first order in substrate and biomass concentrations). The rate coefficients for mineralization of canola oil were 1.07 ± 0.0003 kg sediment/g biomass-day for sediments enriched under methanogenic conditions versus 1.41 ± 0.06 kg sediments/g biomass-day for sediments enriched under iron-reducing conditions ($p = 0.015$). The expected methane-production curves using these rate coefficients are plotted in Figure 1a.

The differences between the sediment-enrichment conditions were more obvious for microcosms provided with a higher initial concentration of canola oil (19 g oil/kg sediment). The rate and extent of methane production from canola oil was very low in microcosms constructed with sediments enriched under methanogenic conditions (0.02 mmol methane/day), but it was much faster in microcosms containing sediments enriched under iron-reducing conditions (0.18 mmol methane/day; Figure 2a). The methane-production rate observed in the high-oil iron-reducing microcosms was similar to the maximum rates observed in the low-oil microcosms (0.34 mmol methane/day and 0.27 mmol methane/day for iron-reducing and methanogenic microcosms, respectively). Differences between the sediment-enrichment conditions were also observable in the volatile fatty acid concentrations: the concentrations of propionate and butyrate were always lower in microcosms containing sediments enriched under iron-reducing conditions (Figure 2c, d). Since the rate of canola oil mineralization was faster in these microcosms, the lower concentrations of propionate and butyrate suggest more balanced interactions among members of the

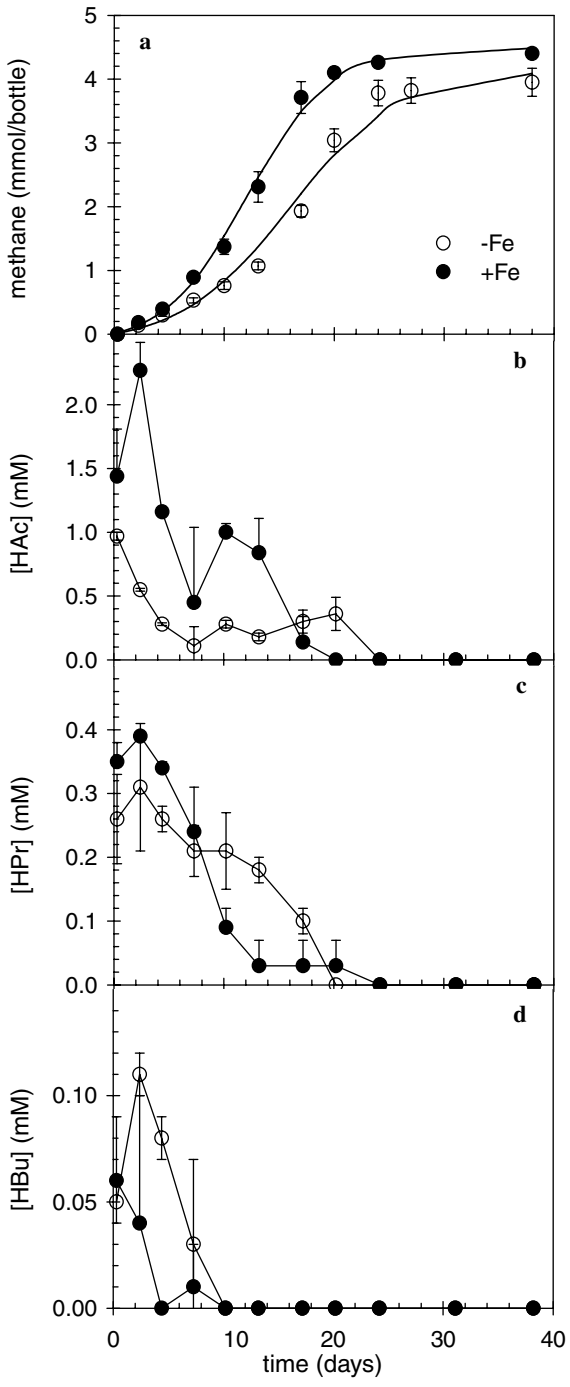

Figure 1. Anaerobic degradation of canola oil at an initial concentration of 1.9 g/kg wet sediment in microcosms containing sediments enriched on canola oil in the presence (+Fe) or absence (−Fe) of ferric hydroxide: (a) headspace methane production; (b) acetate concentration; (c) propionate concentration; and (d) butyrate concentration. Error bars represent one standard deviation of independent duplicate microcosms.

Table 1. Electron balances for anaerobic biodegradation of canola oil in anaerobic microcosms containing sediments enriched on canola oil in the presence (+Fe) or absence (−Fe) of ferric hydroxide

		Electron recovery[a]	
Initial oil concentration (g/kg)	Incubation time (days)	Methanogenic enrichment (−Fe)	Iron-reducing enrichment (+Fe)
1.86	25	90.1 ± 4.69%	101.6 ± 0.73%
	87	98.5 ± 5.44%	107.7 ± 1.09%
18.6	250	12.5 ± 5.34%	98.7 ± 1.39%
	420	27.8 ± 5.46%	102.5 ± 0.45%

[a] All electrons added as canola oil were recovered as methane.

anaerobic microbial consortium. Propionate production was primarily due to fermentation of glycerol, whereas butyrate was produced by incomplete metabolism of oil-derived fatty acids. Further metabolism of both substrates would result in formation of hydrogen and acetate. Acetate accumulated in both types of microcosms during the first week of incubation, but it accumulated more rapidly in microcosms constructed with sediments enriched under iron-reducing conditions (Figure 2b). The equally rapid decrease in acetate concentration that occurred over the next two weeks in microcosms containing sediments enriched under iron-reducing conditions corresponded to a slow increase in propionate and butyrate concentration and a relatively constant rate of methane production. All of these reactions reached a quasi-steady-state after about 4–6 weeks, which persisted until all of the added oil was converted to methane. The relatively high concentrations of propionate and butyrate that were observed in microcosms containing sediments enriched under methanogenic conditions during this time period seems to reflect cessation of metabolic activity of (volatile) fatty-acid-oxidizing syntrophic acetogens, rather than balanced reaction rates among different members of the anaerobic consortium, because methane-production occurred at a very low rate.

In microcosms containing sediments enriched under methanogenic conditions, acetate accumulated over a period of 4–6 weeks after which its concentration was rapidly reduced to less than

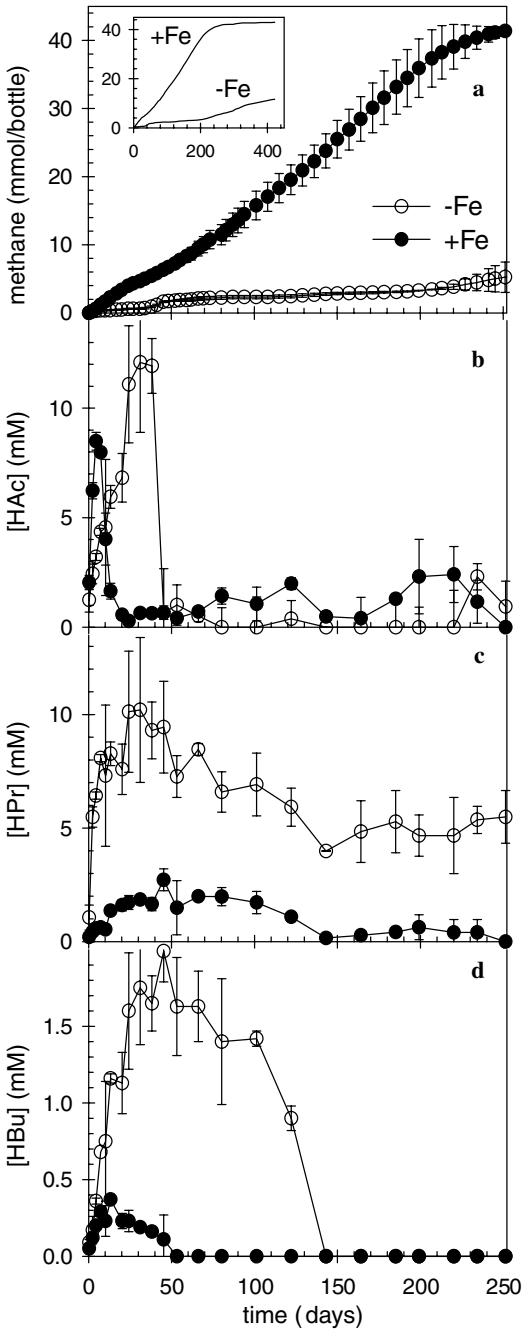

Figure 2. Anaerobic degradation of canola oil at an initial concentration of 19 g/kg wet sediment in microcosms containing sediments enriched on canola oil in the presence (+Fe) or absence (−Fe) of ferric hydroxide: (a) methane production (inset shows methane production over a 420-day incubation period); (b) acetate concentration; (c) propionate concentration; and (d) butyrate concentration. Error bars represent one standard deviation of independent duplicate microcosms.

about 1 mM (Figure 2b). Since very little methane was produced in these microcosms during the period of acetate accumulation, its likely source was fermentation of glycerol. Propionate accumulated to a similar extent over this same time period, suggesting that multiple glycerol fermentation pathways might have been operating simultaneously (Biebl et al. 1998; Schauder & Schink 1989). A brief period of increased methane production coincided with the period of rapid acetate consumption, but it stopped when the acetate concentration approached the method detection limit (Figure 2a). These observations suggest that, although a LCFA-resistant acetate-metabolizing microbial population may have developed after a 5–6-week lag phase, degradation of LCFAs by hydrogen-producing acetogens was inhibited for a longer period of time in microcosms constructed with sediments enriched under methanogenic conditions. This inhibition persisted for over eight months (Figure 2), during which time all electron equivalents added as canola oil had been converted to methane in microcosms containing sediments enriched under iron-reducing conditions (Table 1). In the microcosms constructed with sediments enriched under methanogenic conditions, a second phase of methane production began after about 200 days of incubation (Figure 2a, inset), which corresponded to an increase in the aqueous-phase acetate concentration. Methane-production continued at a low but steady rate for at least another 7 months, but even after more than 1 year of incubation, less than 30% of the added canola oil had been mineralized (Table 1).

Biodegradation of acetate and oleic acid in oil-enriched sediments

The effect of enriching oil-degrading sediment microbial communities under iron-reducing conditions was further investigated using defined mixtures of electron-donor substrates. Acetate and oleic acid were chosen for this purpose, because these compounds represent two of the most important intermediates of anaerobic vegetable oil biodegradation. Oleic acid was selected as a model LCFA, because it constitutes between one-half and two-thirds of the fatty acids in typical canola oil (Patterson 1989). Acetate is the main organic extracellular intermediate product of anaerobic metabolism of LCFAs (Mackie et al. 1991; McInerney 1988), and about two-thirds of the electron equivalents present in the oil are transferred to the terminal electron acceptor through this compound.

Methane production from mixtures of acetate and oleic acid in microcosms containing sediment enriched under methanogenic and iron-reducing conditions is shown in Figures 3a and 4a for two initial substrate concentrations. Whereas methane production began immediately in microcosms containing sediments enriched under iron-reducing conditions at both initial concentrations, a 2–3-week lag phase preceded the onset of methanogenesis in microcosms constructed with sediments enriched under methanogenic conditions. The initial rate of methane production was also faster in microcosms containing sediments enriched under iron-reducing conditions (Table 2). The net result of shorter lag times and faster methane-production rates was that the amount of methane produced in microcosms containing sediments enriched under iron-reducing conditions was 2- to 10-fold higher than in microcosms with sediments enriched under methanogenic conditions over the 7-week incubation period (Table 2). Acetate consumption (Figures 3b and 4b) was consistent with methane production in these microcosms: it began immediately and proceeded rapidly in microcosms containing sediments enriched on canola oil under iron-reducing conditions but was preceded by a lag phase of 2 weeks or more and occurred slowly in microcosms containing sediments enriched under methanogenic conditions.

Although iron reduction was not an important terminal electron-accepting process in these microcosms, reduction of DMSO – which was added as the solvent for oleic acid – was (Figures 3c and 4c). Like methane production, the lag phase that preceded the onset of DMSO reduction was much longer in microcosms containing sediments enriched under methanogenic conditions (17–24 days) than under iron-reducing conditions (< 7 days). Although the lag phase was longer in microcosms containing sediments enriched under methanogenic conditions, the initial rates of DMSO reduction were similar regardless of the sediment-enrichment conditions and were only moderately reduced at the higher initial substrate concentration (Table 2), suggesting that DMSO-reducing microorganisms in these sediments were

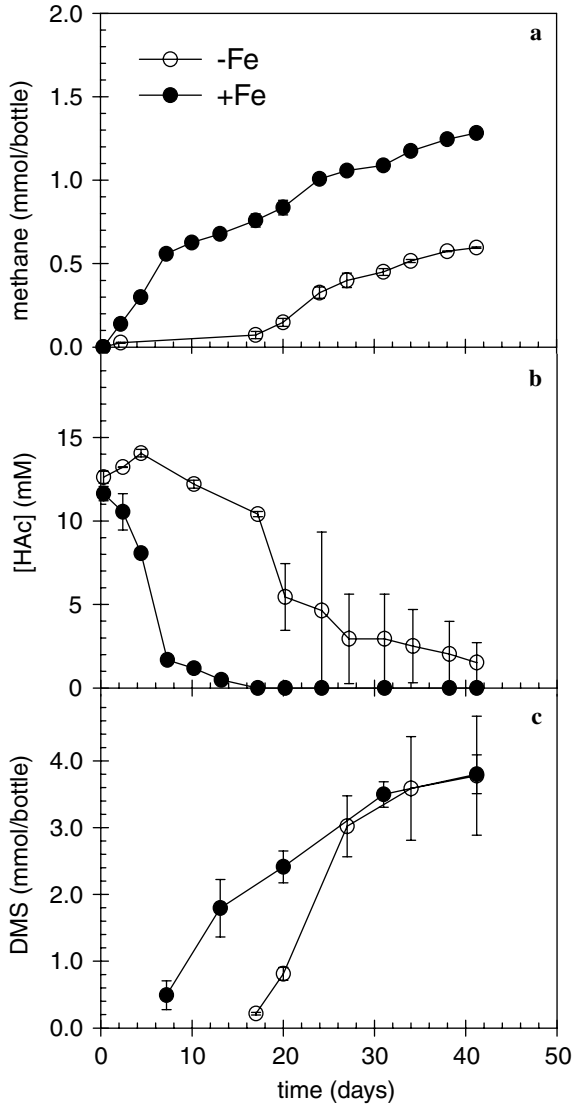

Figure 3. Anaerobic degradation of acetate and oleic acid at initial concentrations of 600 mg/l in microcosms containing sediments enriched on canola oil in the presence (+Fe) or absence (−Fe) of ferric hydroxide: (a) methane production, (b) acetate concentration, and (c) DMS production. Error bars represent one standard deviation of independent duplicate microcosms.

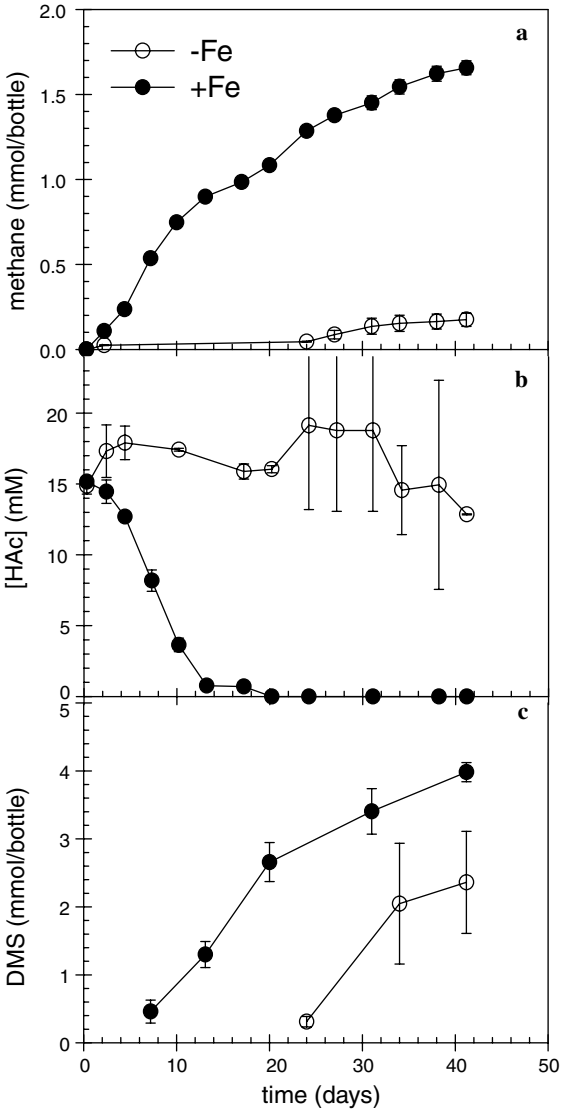

Figure 4. Anaerobic degradation of acetate and oleic acid at initial concentrations of 900 mg/l in microcosms containing sediments enriched on canola oil in the presence (+Fe) or absence (−Fe) of ferric hydroxide: (a) methane production, (b) acetate concentration, and (c) DMS production. Error bars represent one standard deviation of independent duplicate microcosms.

less sensitive to inhibition by LCFAs than were critical members of the oil-degrading microbial community that developed under methanogenic enrichment conditions. Because methanogenesis was inhibited by oleic acid, whereas DMSO reduction was not, a relatively large fraction of electrons were transferred to DMSO in microcosms containing sediments enriched under methanogenic conditions (60–70% of electron equivalents were recovered as DMS versus 30–40% recovered as methane). Microcosms containing sediments enriched under iron-reducing conditions, on the other hand, transferred about 60% of the electrons to methane. With the exception of the methanogenic-enriched sediments

Table 2. Kinetics and products of major terminal electron-accepting processes and electron balances for oil-enriched sediment microcosms provided with acetate (HAc) and oleic acid (OA)

MCR[a]	[HAc] (mM)	[OA] (mM)	t_{lag,CH_4} (days)	r_{o,CH_4}[b] (mmol/day)	Y_{CH_4}[c] (mmol)	$t_{lag,DMS}$ (days)	$r_{o,DMS}$[b] (mmol/day)	Y_{DMS}[c] (mmol)	Electron recovery[d] (%)
−Fe	10	2	16	0.033	0.6	17	0.28	3.8	84
	15	3	24	0.012	0.2	24	0.17	2.0	25
+Fe	10	2	0	0.082	1.3	4	0.22	3.8	121
	15	3	0	0.074	1.7	7	0.17	4.0	97

[a] Sediment-enrichment conditions: −Fe, methanogenic conditions; +Fe, iron-reducing conditions.
[b] Initial rates of methane and dimethyl sulfide (DMS) formation.
[c] Yields of methane and DMS after 42 days of incubation.
[d] Electrons added as acetate and oleic acid that were recovered as methane or DMS after 42 days of incubation.

that were provided with the highest substrate concentration, which were very severely inhibited (Figure 4), electron recovery ranged from 84% to 121% (Table 2), suggesting that the added substrates were completely degraded over the 7-week incubation period.

Discussion

Inhibition of microbial growth by LCFAs has been well documented (Atlas & Bartha 1973; Bell 1971; Hanaki et al. 1981; Hwu et al. 1996; Koster & Cramer 1987; Lalman & Bagley 2002; Pereira et al. 2001; Rinzema et al. 1994; Teh 1974), and the results presented here showing the inhibitory effects of high concentrations of canola oil and oleic acid on methane production by sediments enriched on canola oil under methanogenic conditions are consistent with previous observations. Hanaki et al. (1981), in particular, observed that the presence of long-chain fatty acids caused prolonged lag phases before biodegradation of acetate and butyrate began in batch cultures of anaerobic digester sludge. The duration of these lag phases were roughly proportional to the initial LCFA concentration, but biodegradation proceeded rapidly after it began. Fatty acid concentrations as low as about 500 mg/l were consistently inhibitory to acetoclastic methanogens and hydrogen-producing acetogens, but fermenters and hydrogenotrophic methanogens were generally less sensitive (Hanaki et al. 1981). Other workers have observed similar effects of fatty acids on anaerobic digestion, but the effective concentrations vary greatly from system to system (Hwu et al. 1996; Koster & Cramer 1987; Lalman & Bagley 2000, 2002). In our study, initial oleic acid concentrations of 2 mM (600 mg/l) were sufficient to induce a lag phase of more than two weeks prior to the onset of methane production from acetate (Figure 3), and initial concentrations of 3 mM (900 mg/l) resulted in a more than 3 week lag phase followed by a very low biodegradation rate (Figure 4). Interestingly, canola oil at an initial concentration of 1.9 g/kg sediment, which could potentially result in an equivalent LCFA concentration of 1700 mg/l (6 mM as oleic acid), was not particularly inhibitory, with near complete oil mineralization occurring within 4 weeks (Table 1). Perhaps sequestration of the fatty acids within a separate nonaqueous phase (i.e., the residual vegetable oil) reduced their toxicity to sediment microorganisms. Higher initial oil concentrations, similar to what would be expected to result from sedimentation of floating oil in the proposed spill-response alternative (Wincele et al. 2004), resulted in nearly complete inhibition of biodegradation of canola oil in sediments enriched on canola oil under methanogenic conditions, with only about 13% of the oil-derived electrons being recovered in stable end products after 8 months of incubation (Table 1). The electron recovery increased to about 28% after another 6 months of incubation, which reflected increased microbial activity during a second growth phase (Figure 2a, inset). Fatty acid oxidation by hydrogen-producing acetogens appears to have been more susceptible to long-term inhibition by LCFAs than was acetate metabolism in these microcosms (Figure 2b).

Although the effects of high concentrations of vegetable oil and mixtures of acetate and oleic acid

on methanogenesis in sediments enriched under methanogenic conditions were qualitatively similar to those observed in other methanogenic systems, sediments enriched under iron-reducing conditions were much less sensitive to fatty acid inhibition. Whereas sediments enriched under methanogenic conditions exhibited both long lag phases and low rates of methane production in the presence of high concentrations of oil or oleic acid, sediments enriched under iron-reducing conditions produced methane immediately upon introduction of the substrate, and the rates of methane production were only slightly reduced at higher initial concentrations of oil or oleic acid. This is consistent with previous observations that anaerobic biodegradation of canola oil in freshwater sediments was faster under iron-reducing than methanogenic conditions (Li et al. 2001; Li & Wrenn, in press), but no measurable reduction of Fe(III) took place during the incubations that are described in this report. Thus, active iron reduction was not required for the protective effect that we observed. The effect was previously shown to be unrelated to physical or chemical effects of ferric hydroxide or Fe(II) species on fatty acid bioavailability or toxicity (Li & Wrenn, in press). Instead, growth on vegetable oil coupled to reduction of ferric hydroxide appears to result in enrichment of a microbial population that is less sensitive to inhibition by LCFAs.

Selection for a LCFA-resistant microbial population by growth on vegetable oil under iron-reducing conditions may be related to the nutritional versatility of iron-reducing bacteria. This phylogenetically diverse microbial group can use a wide variety of electron-donor substrates, including carbohydrates, organic acids (including LCFAs, volatile fatty acids, and acetate), and hydrogen, and a similarly diverse array of electron-acceptor substrates (Lovley 2000). In other anaerobic systems, aceticlastic methanogens and hydrogen-producing acetogens were often found to be more sensitive to LCFAs than were hydrogenotrophic methanogens (Hanaki et al. 1981; Lalman & Bagley 2002). Some iron-reducers are able to produce hydrogen as a product of the primary terminal electron-accepting process, and growth on acetate in conjunction with syntrophic hydrogen consumers (not methanogens) has been observed (Cord-Ruwisch et al. 1998). Therefore, enrichment under iron-reducing conditions may have selected microorganisms that shifted the pathway by which vegetable-oil triglycerides were converted to methane, with LCFA-sensitive hydrogen-producing acetogens (e.g., fatty acid oxidizers) and aceticlastic methanogens being replaced by LCFA-resistant iron reducers that were also capable of growing as syntrophic hydrogen producers.

DMSO, which was used as a solvent for oleic acid in microcosms that were amended with acetate and oleic acid, can be reduced as a primary electron acceptor by a wide variety of microorganisms (Bilous & Weiner 1985; Griebler 1997; Jonkers et al. 1996; Lorenzen et al. 1994; Zinder & Brock 1978), including some dissimilatory Fe(III) reducers (Lovley 2000). Reduction of DMSO occurred in microcosms constructed with sediments enriched under methanogenic and iron-reducing conditions, but the lag time preceding its onset was much shorter in the sediments enriched under iron-reducing conditions. In microcosms containing sediments enriched under methanogenic conditions, the lag time before production of DMS began was identical to the lag time that preceded the onset of methane production (Figures 3 and 4, Table 2). It is possible, therefore, that the two processes were related: microorganisms capable of reducing DMSO may have replaced LCFA-sensitive aceticlastic methanogens or hydrogen-producing acetogens in fatty-acid-degrading methanogenic consortia. After the lag phase, DMSO reduction in sediments enriched under methanogenic conditions proceeded at about the same rate as in sediments enriched under iron-reducing conditions, and the effect of higher oleic acid concentration on the DMSO reduction rate was similar in both types of sediments (Table 2). So, similar DMSO-reducing microbial populations may have been present in both systems. The shorter lag phase in sediments enriched under iron-reducing conditions may have been due to co-enrichment of Fe(III) and DMSO reducers in the ferric hydroxide amended MCRs.

Conclusions

Freshwater sediment microbial communities were enriched by growth on canola oil under methanogenic and iron-reducing conditions, and the

kinetics of methane production from canola oil and mixtures of acetate and oleic acid were compared. Biotransformation of low concentrations of canola oil (1.9 g oil/kg sediment) to methane occurred rapidly and completely in sediments enriched under both conditions, but very little biotransformation occurred when sediments enriched under methanogenic conditions were incubated with higher concentrations of oil (19 g oil/kg sediment) for more than one year. Sediments enriched under iron-reducing conditions, however, completely transformed the added canola oil to methane, regardless of the initial oil concentration. Since the higher oil concentration is similar to concentrations that could result from sedimentation of floating oil by formation of dense oil-mineral aggregates (OMAs) in a proposed novel response technology for vegetable-oil spills, incorporation of ferric hydroxide or similar Fe(III) solids into the OMAs may be essential for its successful application.

Similar results were obtained when oil-enriched sediments were amended with mixtures of acetate and oleic acid: sediments enriched under methanogenic conditions were severely inhibited as evidenced by long lag phases, slow methane-production rates, and slow consumption of acetate, whereas sediments enriched under iron-reducing conditions rapidly converted acetate and oleic acid to methane without any discernible lag. Enrichment by growth on canola oil under iron-reducing conditions appears to have selected a microbial community that was less sensitive to inhibition by LCFAs, possibly by replacement of LCFA-sensitive aceticlastic methanogens and/or hydrogen-producing acetogens with iron-reducing bacteria that were also capable of growing as syntrophic hydrogen producers in conjunction with hydrogenotrophic methanogens.

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