Biogeochemistry 17: 23—47, 1992

Fatty acid oxidation in anoxic marine sediments: the importance of hydrogen sensitive reactions

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Received 1 November 1991; accepted 27 May 1992

Key words: anaerobic food chain, fatty acids, hydrogen, inhibitors, sediments, sulfate reduction

Abstract. In anoxic marine sediments fatty acids may be oxidized directly by sulfate reducing bacteria, or may be oxidized by pathways which result in hydrogen production. Some of these latter reactions are quite sensitive to hydrogen concentrations . . . in other words if hydrogen concentrations become elevated, fatty acid oxidation will cease. Thus sulfate reducers may actually play two important roles in the metabolism of fatty acids in marine sediments. The sulfate reducers both can utilize fatty acids directly, and also can oxidize hydrogen and thus control hydrogen partial pressures in the sediments. Therefore sulfate reducers may act indirectly to facilitate fatty acid oxidation by hydrogen-producing pathways. We carried out a series of incubations of slurried salt marsh sediment under high and low hydrogen partial pressures and in the presence and absence of molybdate to investigate the relative importance of sulfate reducers and other bacteria mediating hydrogen-sensitive reactions. Our results suggest that both classes of bacteria contribute significantly to fatty acid turnover in marine sediments. Studies of low molecular weight fatty acid turnover in sediment must explicitly recognize that manipulation of sediment (including addition of molybdate to inhibit sulfate reducers) may have a large impact on hydrogen partial pressures in sediment, and may thus significantly alter the pathways and/or rates of fatty acid turnover.

Introduction

In anoxic sediments, carbon is metabolized by a consortium of microorganisms known as the anaerobic food chain. During the decomposition process, complex biopolymers are hydrolyzed to lower molecular weight compounds which then are fermented to still smaller molecules. Various members of the anaerobic food chain mediate the different steps, and the products of one organism become the substrates of another. The final result of this process can be complete conversion of the starting material to CO_2 and methane (accompanied by production of hydrogen sulfide if sulfate is present) even though no single organism is known to completely metabolize organic polymers anaerobically to CO_2 on its own.

Because of the interdependence of the members of the anaerobic food chain, changes in the constituent organisms or in the chemistry of the environment can have profound effects on rates of reaction and even on the reactions which take place. A well studied example of this is the fact that hydrogen concentrations play a very important role in controlling pathways of metabolism in anaerobic systems (Bryant et al. 1967; Iannotti et al. 1973; Wolin & Miller 1982; Conrad et al. 1986). Hydrogen is produced by many fermentation reactions, but a number of these reactions proceed spontaneously only if the hydrogen concentrations are very low (less than 10s of nM). Therefore these hydrogen sensitive fermentation reactions only occur when a hydrogen consuming organism (such as a sulfate reducer or methanogen) is present. Without the hydrogen consumer, different reactions take place and compounds like lactate and ethanol tend to accumulate (Bryant et al. 1967; Iannotti et al. 1973; McInerney et al. 1979). In this study, we focused on the roles played by terminal food chain members (specifically sulfate reducers) in the cycling of low molecular weight fatty acids (LMWFA) in marine sediments. We examined two distinct roles which sulfate reducers might play in fatty acid oxidation, but which have not been separately studied by previous experimental procedures.

Sulfate reducers such as *Desulfococcus, Desulfobacter, Desulfosarcina, Desulfonema* and some strains of *Desulfovibrio* may completely oxidize fatty acids to CO_2 during sulfate reduction as in reaction 1 (Widdel & Pfennig 1977; Laanbroek & Pfennig 1981; Widdel & Pfennig 1982; Widdel & Pfennig 1984).

$$\begin{aligned} & SO_4^= + CH_3COO^- \rightarrow 2\,HCO_3^- + HS^- \\ & \Delta G^0 = -47.6\ \text{kJ} \end{aligned} \tag{1}$$

Other sulfate reducers may oxidize fatty acids by pathways which result in incomplete oxidation of fatty acids (most *Desulfovibrio, Desulfobulbus, Desulfomonas*; Widdel & Pfennig 1984) as in reaction 3 of Table 1.

If production doesn't change when uptake is inhibited, the rate of accumulation of fatty acids in pore waters of sediments treated with molybdate (a specific inhibitor of sulfate reduction) should reflect the rate of oxidation of fatty acids in undisturbed *in situ* sediments (Smith & Klug 1981; Sørensen et al. 1981; Banat & Nedwell 1983; Christensen 1984; Michelson et al. 1989). However, because sulfate reducers maintain low hydrogen levels in sediments and thus also play an indirect role in fatty acid oxidation by affecting the extent to which hydrogen sensitive fatty

Table 1. Some oxidation reactions for low molecular weight fatty acids.

1. complete oxidation of acetate:	
$CH_3COO^- + 4\,H_2O \rightarrow 2\,HCO_3^- + 4\,H_2 + H^+$	$\Delta G^0 = 144.5$ kJ
2. complete oxidation of propionate:	
$CH_3CH_2COO^- + 7\,H_2O \rightarrow 3\,HCO_3^- + 7\,H_2 + 2H^+$	$\Delta G^0 = 260.8$ kJ
3. incomplete oxidation of propionate:	
$CH_3CH_2COO^- + 3\,H_2O \rightarrow CH_3COO^- + HCO_3^- + 3\,H_2 + H^+$	$\Delta G^0 = 116.4$ kJ
4. incomplete oxidation of butyrate:	
$CH_3CH_2CH_2COO^- + 2\,H_2O \rightarrow 2\,CH_3COO^- + 2\,H_2 + H^+$	$\Delta G^0 = 88.2$ kJ

acid oxidation reactions occur, this approach is overly simplified. For example, reaction 2 is thermodynamically unfavorable at standard molar concentrations of reactants and products.

$$\begin{gathered} CH_3COO^- + 4\,H_2O \rightarrow 2\,HCO_3^- + 4\,H_2 + H^+ \\ \Delta G^0 = +144.5\ \text{kJ} \end{gathered} \tag{2}$$

If hydrogen concentrations are low enough, and if bacteria with the corresponding fermentation capacity are present, the fermentation reaction proceeds readily (Wolin & Miller 1982). However, when sediments are treated with molydbate, the hydrogen uptake by sulfate reducers stops, and then the hydrogen concentrations in the pore waters increase. This, in turn, makes the hydrogen sensitive fatty acid fermentations thermodynamically unfavored, and fatty acid turnover by these pathways may decrease. Also, since some fatty acids (e.g. acetate) can be products of the fermentation of other fatty acids (e.g. propionate), they may accumulate at a different rate in samples to which molybdate has been added than the rate at which were being consumed in the original, undisturbed sediment.

In previous experiments, Banat et al. (1981) and Banat & Nedwell (1983) found that addition of large amounts of H_2 (80% by volume in headspace) had no noticeable effect on LMWFA oxidation in slurries of salt marsh sediment, while addition of molybdate resulted in cessation of acid oxidation. Banat & Newell (1983) suggested these results demonstrated that LMWFA were oxidized directly by sulfate reducers, and that hydrogen-sensitive fatty acid oxidations were relatively unimportant. However such studies may not be simple to interpret because sulfate reducers may create microenvironments low in hydrogen, in which fermentation of fatty acids by hydrogen sensitive pathways may occur. Thus elevated hydrogen concentrations in bulk pore water might not mean that hydrogen concentrations within microenvironments was also elevated. Addition of molybdate would stop both the hydrogen consuming and fatty acid consuming roles of sulfate reducers at the same time. Conrad et al. (1985) and Conrad et al. (1986) have proposed the existence of microenvironments in freshwater sediments in which there is efficient transfer of hydrogen between producers and consumers and in which microenvironment hydrogen concentrations are lower than in bulk sediment.

In our work we examined fatty acid oxidations in stirred slurried salt marsh sediments under both high and low hydrogen headspace concentrations. Under high hydrogen concentrations we would not expect hydrogen-sensitive reactions to occur. We also compared oxidation under different hydrogen concentrations in samples with and without added molybdate to look at rate constants with and without sulfate reduction. Using our results we were able to divide the fatty acid oxidizing populations in the sediment conceptually into 'sulfate reducers which directly oxidize fatty acids', 'hydrogen sensitive fatty acid oxidizers' and 'other oxidizers' (which include hydrogen-insensitive non-sulfate-reducing fatty acid oxidizers). The same organisms may be active in one or more groups depending on environmental conditions. By comparing rate constants for fatty acid oxidation in systems in which different combinations of these groups are present, we can assess the relative importance of direct versus indirect effects by sulfate reducers on fatty acid oxidation.

Methods

Sediment for our experiments was collected from Flax Pond, a salt marsh on the north shore of Long Island, NY, using hand-held 9 cm diameter core tubes. The major organic input to the sediment is in the form of *Spartina alterniflora* and macroalgal mats (Ranwell 1972; Brinkhuis 1976; Long & Mason 1983) and sedimentary organic carbon contents are between 2.5 and 3.5% (Jacobson et al. 1987). Oxygen penetrates only a few millimeters into the sediment (Swider & Mackin 1989). In summer, sediment was collected directly from the marsh, while in winter, sediment was maintained in a sea-table in the Flax Pond laboratory and subsampled there. All cores were maintained at *in situ* temperatures in the dark until processed, and all sediment sectioning and sample preparation was done in a nitrogen-flushed glove bag. For all the experiments reported in this paper, the 4—8 cm interval of the sediment column was used, as this depth range is characterized by rapid sulfate reduction and rapid turnover of organics (Novelli 1987; Novelli et al. 1988; Michelson et al. 1989; Swider & Mackin 1989). Previous experiments have shown that sulfate reduction rates (Swider & Mackin 1989) and acetate oxidation rates (Michelson et al. 1989) for Flax Pond are comparable, although no direct measurements were made of sulfate reduction during our study.

Fatty acid oxidations were measured in sediment slurries (5 ml added pore water/10 ml whole wet sediment) at *in situ* temperatures under high and low hydrogen concentrations to alter the importance of hydrogen sensitive reactions, and both in the presence (20 mM final concentration) and absence of molybdate. Pore water (collected from sediments at the same depth as the experimental sediment) was used as the slurrying fluid to maintain metabolite concentrations close to *in situ* levels. All slurries were flushed with an O_2-free N_2/CO_2 mixture for 5 minutes and preincubated at *in situ* temperature for 12 hours. The ratio of nitrogen to carbon dioxide in the stripping gas was adjusted so that the CO_2 concentration of the pore water would remain at approximately *in situ* levels. Preincubation was necessary to ensure that sulfate reduction was fully suppressed in molybdate treated slurries (Novelli 1987). Slurries were stirred with stirring bars as the headspaces were flushed to enhance gas exchange and to break up microenvironments.

Hydrogen concentrations in pore waters were varied using a gas proportioner to regulate hydrogen partial pressures in the headspace gas. Total gas flow was kept constant at about 100 ml/min. To obtain 'low' hydrogen concentrations, gas was passed through a column of heated hopcalite. To give elevated hydrogen levels, a H_2/N_2 standard was mixed in. The mixed gas was stripped of oxygen by passage through an oxygen scrubber, and bubbled through distilled water to reduce evaporation in the slurried samples. Then the gas was split to pass through several slurry vials.

Uniformly ^{14}C-labeled acetate (54 mCi/mmol), 1-^{14}C propionate (50 mCi/mmol) and 1-^{14}C butyrate (46 mCi/mmol) were added to determine rates of fatty acid oxidation. Concentrations of the spike were chosen to be less than 1% of the measured fatty acid pool size, or if the pool size was below the detection limit, to be less than 6% of the detection limit. Incubation times were usually 1—2 hours or less. Slurries were equilibrated for 30 min with the $H_2/N_2/CO_2$ gas mixture flowing through the incubation line and then were spiked with a ^{14}C-labeled compound. The length of time of incubation was dependent on the oxidation rate of the compound, with shorter times used for higher rates. Gas exiting each flask was bubbled through two Hungate vials, each containing 10 ml of Carbosorb, a CO_2 absorber. The evolved $^{14}CO_2$ was usually trapped completely in the first vial, but this depended on the total carbon dioxide flow. $^{14}CO_2$ was determined by counting 1 ml of the trapping Carbosorb in 10 ml of Permafluor (a scintillant for Carbosorb). Trapping efficiency for CO_2 was 89 ± 9%. Corrections for counting efficiency and quenching were made using external standards. Acid killed controls indicated 'abiotic' CO_2 production was always very low, although it was important that the spiking solution be purged of CO_2 prior to use. Data presented here represent 'biotic' carbon dioxide production. The amount of label remaining in the slurry pore water at the end of each experiment was determined by centrifuging 3 ml of slurry at 3000 rpm for 20 minutes and then adding 0.1 ml of the supernatant of Optifluor. Recovery of ^{14}C was monitored in each slurry by determining both the total label trapped in Carbosorb, and the amount of ^{14}C remaining in the pore water at the end of each experiment.

The effect of substrate concentration on oxidation rates was examined by adding various amounts of unlabeled acetate or propionate to slurries prior to the addition of radioactive spike. Slurries receiving substrate additions had final concentrations of about 270 and 1020 μM acetate or 50 and 100 μM propionate. Oxidation rates of the respective fatty acids were then determined under high and low partial pressures (and thus concentrations) of hydrogen.

To evaluate the effect of slurrying on LMWFA oxidation rate constants, in one experiment ^{14}C-propionate oxidation also was measured in whole and slurried sediments in sealed vials (off the incubation line). Neither whole nor slurried sediments were stirred, and slurries were prepared as described earlier. Following a 2 hour incubation, $^{14}CO_2$ was removed from sealed vials by acidification followed by stirring and flushing with a CO_2/N_2 gas mixture. $^{14}CO_2$ was trapped in Carbosorb, which was counted as described above.

Fatty acid oxidation rates were determined under 'high' and 'low' partial pressures of hydrogen. The 'high' partial pressure (300—4000 ppm) was chosen so that, at equilibrium, it would result in an aqueous phase concentration approximately 10 to 100 times higher than the concentration thermodynamic calculations predict would inhibit the reactions in Table 1. The 'low' hydrogen partial pressures (less than 0.2 ppm) were in equilibrium with pore water concentrations a factor of 10 to 100 below inhibitory levels.

Turnover rate constants, k, for each fatty acid were determined using the integrated form of the equation for a single step, first order reaction (Capellos & Bielski 1972).

$$k = \frac{\ln(A_0/(A_0 - b))}{t} \tag{3}$$

where: k = turnover rate constant

A_0 = activity of added labeled substrate

b = activity of product, $^{14}CO_2$, recovered at the end of the experiment,

t = incubation time.

Preliminary experiments showed that plots of $\ln(A_0/(A_0 - b))$ vs. time were linear over the incubation period (Fig. 1), so that subsequent incubations were sampled at a single time point. Using *in situ* concentrations and the turnover rate constant, turnover rate can be calculated as $k * C$.

Low molecular weight fatty acids (C_2—C_5) were measured in pore water samples obtained from sectioned cores or from sediments in sealed vials. Pore waters were collected by centrifugation (3000 rpm for 20 min at 5 °C) and then filtered through 0.22 μm Nuclepore membranes in a syringe press. Filtered samples either were placed in acid washed glass scintillation vials (storage less than 1 week) or were sealed in ampules (for longer storage). In both cases samples were frozen at −15 °C until analysis. Fatty acid concentrations were measured by a modification (Monetti 1991) of the method described by Christensen & Blackburn (1982) and Michelson et al. (1989) which involves vacuum distillation (to remove salts) followed by gas chromatography. Both standards and samples were distilled before analysis and one microliter was injected onto a Nukol capillary column (15 m × 0.53 μm i.d.) in a Hewlett Packard 5890A dual FID gas chromatograph. The carrier gas was O_2-free, UHP helium at a flow rate of 20 ml/min. The Nukol column did not require formic acid injections to prevent ghosting as did the columns used by Christensen & Blackburn (1982) and Michelson et al. (1989). Injector and detector temperatures were 135 °C and 175 °C respectively. The column oven was temperature programmed (93 °C for 0.5 min; 93—95 °C @ 2 °C min^{-1}; 95 °C for 3 min; 95—110 °C @ 5 °C min^{-1}; 110 °C for 3 min). The detection limit for acetate was 5 μM, for propionate was 2 μM, and for butyrate was 1 μM. The detection limit was primarily controlled by the size of the blank.

Hydrogen was determined using both syringe and loop injections and a Reduction Gas Detector (RGD-2) as described by Scranton et al. (1984). Briefly, a gas sample is dried over $MgClO_4$ and then is carried by the air carrier gas through a silver oxide trap (to remove CO). Hydrogen is separated from other reduced gases present using a 1 m × 1/8″ o.d.

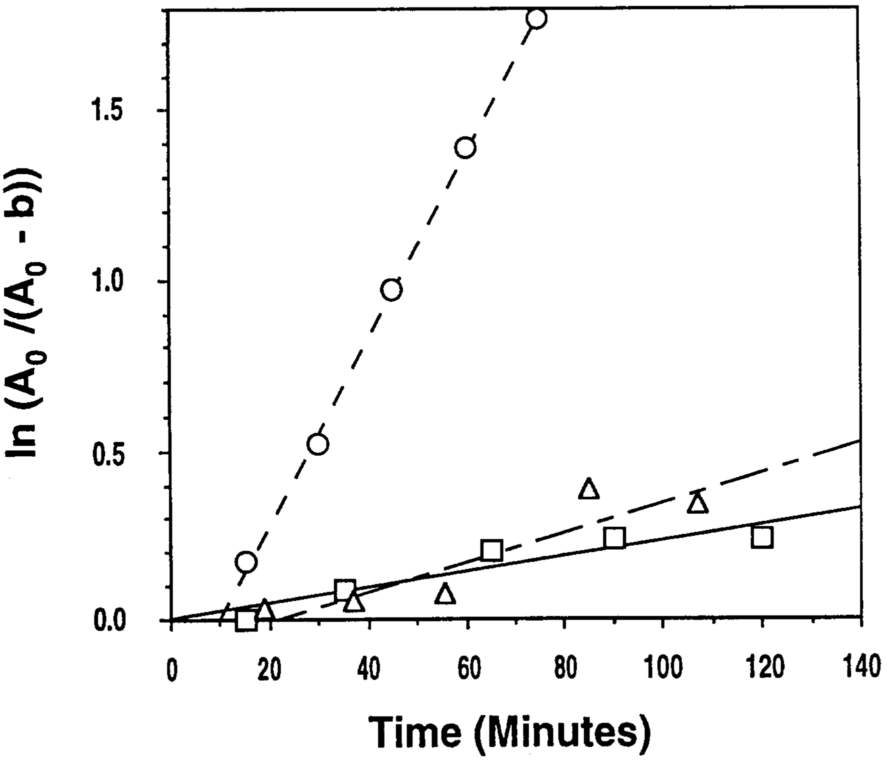

Fig. 1. Acetate (△), propionate (○) and butyrate (□ × 10) oxidation as a function of time. Data are from experiments in which replicate samples were spiked with tracer and then sacrificed at the indicated times. Rate constants were 0.26 h^{-1} for acetate, 1.6 h^{-1} for propionate and 0.014 h^{-1} for butyrate respectively and are equal to the slopes of the lines.

molecular sieve 5A column at 35 °C. Detection is by the mercuric oxide reduction method (Herr & Barger 1978; Seiler et al. 1980). Samples were compared to hydrogen in nitrogen gas standards, purchased from Scott Specialty Gas and calibrated in our laboratory.

ΣCO_2 was determined on samples from duplicate cores for the 4—8 cm depth interval at which slurry incubation samples were collected. Sediment was centrifuged (3000 rpm for 20 minutes at 5 °C). Then pore water was removed with a glass syringe (which had been flushed to eliminate bubbles) and was filtered through an 0.22 μM Nuclepore filter into an empty syringe (also flushed to eliminate bubbles). One milliliter of each sample was injected into a helium-flushed crimp-sealed 13 ml serum vial. Standards were prepared from stock Na_2CO_3 (in 1 mM NH_4OH) by diluting with 1 mM NH_4OH. To determine ΣCO_2 in these samples, 0.1 ml of 0.5 M H_3PO_4 was added with a syringe and the vials were vortexed for 30 seconds. Then an 0.5 ml aliquot of the vial headspace was injected into a Perkin Elmer Sigma 2B gas chromatograph equipped with a thermal conductivity detector and dual 1.5 m × 1/8″ o.d. Porapak R. columns. Standard curves were linear to approximately 0.8 μmoles injected CO_2 (equivalent to 20 mM in the pore water). A correction was made for differences in the solubility of CO_2 between samples and standards using the equations of Weiss (1974) and the salinity and temperature of the samples. CO_2 partial pressure and HCO_3^- concentration were calculated using pH, CO_2, in-situ temperature, and salinity and the equations presented in Skirrow (1975).

In situ sediment temperature was measured by inserting a thermometer into the sediment. Salinity was determined with a hand-held refractometer. Porosity was measured using 10 ml weighed and homogenized sediment which was then dried at 65 °C for three days and reweighed. pH was determined in dedicated replicate cores taken before each experiment. Readings were recorded as a pH electrode was slowly pushed into the sediment (from the surface down). An average *in situ* pH value was calculated for each depth interval sampled for dissolved species.

Results

In situ temperatures at 6 cm in the sediment at the times sampled ranged from 11 °C—20.5 °C and varied as expected from previous studies of seasonal variations in Flax Pond (Swider & Mackin 1989). Salinities in the 4—8 cm interval ranged from 22.1—25.0 o/oo. Porosity was 71—78%. Total carbon dioxide concentrations in both sea table and Flax Pond sediment increased almost linearly with depth over the top 10 cm. For depths of 4—8 cm, *in situ* ΣCO_2 concentrations were typically 8—13 mM in Flax Pond sediment. pH values in overlying waters were about 7.7, while values within the sediment ranged from slightly more than 6 near the sediment water interface to about 6.9 at depth in the core. A subsurface minimum in pH was seen in all cores sampled.

We assumed that, on the incubation line, gaseous equilibrium was reached between pore waters and headspace. We calculated concentrations of hydrogen and carbon dioxide in pore waters for vials on the incubation line from solubility data (Weiss 1974; Wiesenburg & Guinasso 1979) and the partial pressures of H_2 and CO_2 in the gas flushed through the slurry vials prior to each run. Although we cannot prove that no microenvironments containing elevated or depressed H_2 levels were present, unpublished data indicate that elevated concentrations of hydrogen and methane in pore waters are rapidly flushed from vials in experiments similar to those done here (Monetti 1991). In addition, if low hydrogen microenvironments were important, one would predict that measured hydrogen production rates would be lower in sediments equilibrated with a headspace when compared with vigorously mixed and degassed sediments. Hydrogen production rates measured in sealed vials (sediment equilibrated with a large head space but not mixed and degassed) and in vigorously purged vials (sediment stirred and the headspace purged with hydrogen free gas and evolved hydrogen trapped) were equal suggesting that gas phase equilibration was not a significant problem (Monetti 1991).

The effects of slurrying sediments was examined using ^{14}C-propionate. Oxidation rate constants were measured in both slurried (10 ml homogenized sediment: 5 ml pore water) and unslurried homogenized sediments to which 0.1 ml of a solution containing ^{14}C-propionate was added, followed by vortexing. The rate constants for the two treatments were similar (Monetti 1991) so it was assumed that the rate constants for fatty acid oxidations were not significantly affected by slurrying.

Plots of $\ln(A_0/(A_0 - b))$ as a function of time were used to calculate rate constants for acetate, propionate and butyrate oxidation and seemed to be linear (see Figs. 1a—c). Therefore first order rate constants were calculated using equation 3. The propionate time course was determined by monitoring the production of $^{14}CO_2$ for a single sample, while for acetate and butyrate, results were obtained using 5 replicate slurry samples sacrificed at different times. The correlation coefficient (r^2) for propionate (0.999) is better than for acetate and butyrate (0.832 and 0.867 respectively), perhaps as a result of variability between aliquots of sediment. In subsequent experiments, we assumed that the oxidation rate constant was independent of incubation time. Rate constants were obtained using a single time point (usually 60—120 minutes). Measurements of label recovery indicated that adsorption onto particles and/or incorporation into bacteria was minimal (less than 7%).

For our measurements of propionate cycling we used 1-^{14}C propionate. Some syntrophic and sulfate reducing bacteria may use the succinate pathway for propionate oxidation (Boone 1984; Houwen et al. 1987; Gottschalk 1986), and during this reaction, there is an interconversion between propionate and succinate which may result in release of either carboxyl group of succinate. Therefore if this were the dominant pathway of metabolism of propionate in marine sediments, significant amounts of CO_2 could have been released with no net change in propionate concentration. This effect has been observed for syntrophic cocultures of propionate oxidizers and methanogens (Boone 1984; Houwen et al. 1987). Therefore it is possible that our measured propionate oxidation rate constant are too high. In Table 2 it can be seen that the propionate

Table 2. Rate constants (hour^{-1}) calculated for different groups of bacteria.

Experiment date	$k_1 + k_2 + k_3$ or K_A	k_1		k_2		k_3 or K_D
		$K_A - K_B$	$K_C - K_D$	$K_A - K_C$	$K_B - K_D$	
Acetate						
6/1	0.066	0.052	0.024	0.038	Similar	0.0048
6/15	0.14	0.11	0.042	0.088	Similar	0.0082
7/19	0.26	0.24	0.092	0.15	Similar	0.0036
Corrected 7/19[1]	0.056	0.039	0.026	0.026	0.013	0.0036
Propionate						
3/18	0.65	Similar	Similar	0.59	0.49	0.021
4/11	0.038	0.020	0.013	0.019	0.012	0.006
7/19	0.63	0.61	0.69	Similar	Similar	0
Corrected 7/19[1]	0.107	0.083	0.110	Similar	0.024	0
Butyrate						
6/9	0.012	0.0107	0.0057	0.0037	Similar	0.0026

[1] The rate constants for experimental conditions A and C were recalculated to values expected at a higher substrate concentration using Michaelis-Menton kinetics.

rate constants are considerably more variable from date to date than are the rate constants for acetate and butyrate oxidation (acids for which this problem would not exist). However, as noted in the introduction, many sulfate reducers oxidize propionate completely to CO_2 (Widdel & Pfennig 1984). Since we see no evidence in Table 2 that relative patterns of propionate oxidation by sulfate reducing bacteria as compared to hydrogen sensitive bacteria are different from patterns of acetate or butyrate oxidation, we do not believe our choice of 1-^{14}C-propionate has affected the conclusions we make below.

Although the rate constants did not seem to be affected by slurrying, experimental manipulations (slurrying, homogenization, stirring, etc.) did change the fatty acid concentrations of the sediments. Fatty acid concentrations in slurried sediments were higher than *in situ* values for all fatty acids measured and for all experiments (Monetti 1991). Fatty acid concentrations measured at the end of the experiment in slurries which did not receive a molybdate addition ranged from <5—239 μM for acetate, from <2—20 μM for propionate, from <1—6.3 μM for isobutyrate, and from <1 to 11.3 μM for butyrate. For molybdate-treated slurries, concentrations ranged from 107—824 μM for acetate, from 10.1—170 μM for propionate, from 3.7—39.2 μM for isobutyrate and from 4.0—

43.0 μM for butyrate. Even simple homogenization (without slurrying) of the sediment resulted in an increase in fatty acid concentration. The increase in fatty acid concentration appeared to be a transitory one in uninhibited sediments as LMWFA concentrations were below the detection limit 90 hours after slurrying. This effect was quite different than the affect of molybdate addition which resulted in a steady trend of increasing acid concentration with time for all acids and for all experiments.

Prior to 8 May 1989, fatty acid concentrations used to calculate turnover rates for an experiment were measured in unspiked slurries run in parallel on the incubation line with the slurries being assayed for oxidation rate. After that date, to permit more on-line vials to be treated with a ^{14}C-spike, acid concentrations were measured in sealed (unflushed) vials, incubated off line. Later experiments showed that concentrations in flushed, stirred vials were always different than those measured in sealed vials. The effect was greatest for propionate, with 10-fold higher concentrations found in on-line vials than were present in sealed vials for two cases of samples incubated without molybdate. For other LMWFAs, changes ranged from a 3-fold decrease to a 2-fold increase for samples incubated on-line. In most cases, use of fatty acid concentrations measured for sealed vials underestimates the fatty concentrations in the incubation line vials.

During one experiment (19 July 1989), rate constants were measured under different LMWFA concentrations and hydrogen concentrations. The relationship among rates, rate constants and LMWFA concentrations are examined in Table 3. Rate constants (k) decreased with increasing LMWFA concentration. Calculated reaction rates (kC) showed the opposite trend. Because the reaction rates are so strongly influenced by the concentrations used in the calculation, and given the uncertainty in the appropriate LMWFA concentration to use with a particular rate constant, our further discussion will be based upon the reaction rate constants alone.

pH values were also affected by experimental manipulations. Typically, pH increased by 0.3 units after slurrying, probably due to the loss of carbon dioxide. The pH of slurries in flushed vials was lower than that in sealed vials by 0.1 pH units. For both types of slurry incubations, pH was relatively constant with time.

Figure 2 shows the oxidation rate constant of propionate as a function of hydrogen partial pressure. In both the molybdate-inhibited and uninhibited slurries, the oxidation rate constant decreased as hydrogen partial pressures increased. Above the calculated inhibitory hydrogen value, the oxidation rate constant leveled off and remained approximately constant.

Table 3. Acetate and propionate oxidation rates measured in sediment slurries with different substrate concentrations on 7/19/89.

Fatty acid	Substrate concentration	Rate constant		Oxidation rate	
		Low H_2 (0.1 ppm)	High H_2 (3,800 ppm)	Low H_2 (0.1 ppm)	High H_2 (3,800 ppm)
Acetate	< 0.1 μM	0.25/hour	0.096/hour	< 0.25 μM/hour	1.4 μM/hour[1]
	270 μM	0.062 ± 0.0078/hour	0.036 ± 0.0014/hour	17 ± 2.2 μM/hour	9.8 ± 0.4 μM/hour
	1020 μM	0.036 ± 0.0014/hour	0.019 ± 0.0042/hour	37 ± 1.4 μM/hour	19 ± 4.4 μM/hour
Propionate	< 0.1 μM	0.63/hour	0.69/hour	< 0.063 μM/hour	< 0.069 μM/hour
	50 μM	0.11 ± 0/hour	0.076 ± 0.0035/hour	5.5 ± 0 μM/hour	3.8 ± 0.2 μM/hour
	100 μM	0.074 ± 0.11/hour	0.050 ± 0.030/hour	7.3 ± 1.1 μM/hour	4.9 ± 2.9 μM/hour

[1] Actual substrate concentration was 14.6, not < 0.1 μM.

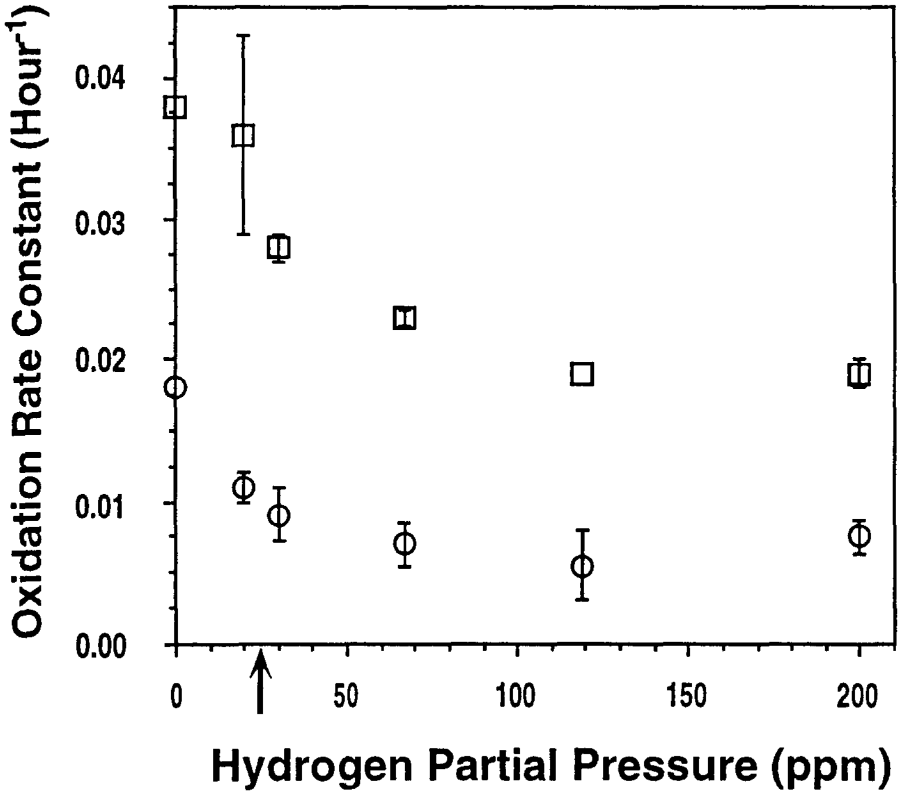

Fig. 2. Propionate oxidation rate constants measured in sediment slurries with (○) and without molybdate (□) as a function of hydrogen partial pressure. The rate constants are the means of rate constants measured on duplicate slurries and the error bar gives the range of the values. The arrow indicates the hydrogen partial pressure at which the hydrogen-producing oxidation of propionate should become thermodynamically inhibited.

Discussion

Many fatty acid oxidation reactions are hydrogen sensitive. In other words, they are thermodynamically unfavorable (and thus cannot proceed spontaneously) unless the product hydrogen is consumed. In a study of lake sediment and sewage sludge, Conrad et al. (1986) calculated that H_2 partial pressures in the systems they examined must be less than 30 to 70 ppmv for propionate oxidation, and less than 20 to 120 ppmv for butyrate oxidation to occur. These same workers measured hydrogen partial pressures within pore waters which were higher than the calculated values, even though they confirmed fatty acid oxidation was occurring. Therefore they hypothesized that the hydrogen-sensitive reactions were taking place in microenvironments where actual hydrogen concentrations were lower than those measured in the bulk pore waters. Similar phenomena also seem to occur in marine (sulfidic) systems (Novelli 1987). In sediments studied in this series of experiments, we calculated, using the equations in Table 1 and measured concentrations of the various chemical species, that hydrogen partial pressures exceeding 20 ppm (equivalent to about 14 nM for seawater at 20 °C) would inhibit hydrogen-sensitive oxidations of acetate and propionate, while more than 300 ppm hydrogen would be required to inhibit butyrate oxidation.

In our experiments we used slurries to examine the sensitivity of various fatty acid oxidations to hydrogen concentrations. The principle rationale for this was that the number and size of microenvironments in the slurries would be considerably lower than for whole sediment, and thus the measurement of hydrogen in the headspaces of the experimental vials would reflect the pore water hydrogen concentration. Slurrying has been shown to reduce the rates of microbial processes in some cases (Jørgensen 1978; Alperin & Reeburgh 1985; Conrad & Babbel 1989; Burdige 1989) while in other cases, rates have increased (McNichol 1986). Several workers (Alperin & Reeburgh 1985; Conrad & Babbel, 1988; Burdige 1989) have suggested that the mechanisms of microbially mediated processes are less likely to change due to slurrying than are the reaction rates. We were interested in studying mechanisms rather than in measuring *in situ* rates, and our data indicate that slurrying did not affect the rate constant measured for propionate oxidation (Monetti 1991). We attempted to minimize the effect of dilution of the pore waters which occurs during slurrying (Burdige 1989) by using pore water collected from the same sediment depth as the sample, as well as by using a low slurry ratio (1 volume pore water/2 volume sediment).

Our treatment of the data is based on the hypothesis that there are several groups of microorganisms which can be actively involved in LMWFA oxidation. Each microbial group is assumed to oxidize fatty acids with a particular first order rate constant. If k, k_1, k_2, and k_3 are the oxidation rate constants for the whole sediment population, sulfate reducers which are directly oxidizing fatty acids, hydrogen-sensitive fatty acid-oxidizing bacteria, and 'other (hydrogen insensitive)' bacteria respectively, the rate of fatty acid oxidation at a given fatty acid concentration will be

$$V = k\,[FA] = k_1\,[FA] + k_2\,[FA] + k_3\,[FA]. \tag{4}$$

In the different experimental treatments, different combinations of these groups of bacteria are active, so we can solve for the individual values of k in the above equation. For example, in treatments with low H_2 and without $MoO_4^=$, all bacterial groups will be actively oxidzing LMWFAs. If hydrogen concentrations are low but molybdate is present, hydrogen sensitive and/or 'other' bacteria will dominate sulfate oxidation. If hydrogen is high, but molybdate is absent, fatty acid oxidizing sulfate reducers and 'other' bacteria will be most important. And if hydrogen concentrations are high and molybdate is present, only 'other' bacteria need be considered. (Previous experiments have shown that methanogenesis is undetectable in untreated sediments (Michelson, personal communication 1987; Novelli 1987) and is stimulated in molybdate treated sediments only after 36 hours (Kiene 1986).)

Figure 3 presents a schematic example of the way in which rate constant data were treated. The rate constant which describes LMWFA oxidation occurring under low hydrogen partial pressures and without $MoO_4^=$ (bar A) is an overall rate constant which includes the effects of all three groups of bacteria ($k_A = k_1 + k_2 + k_3$). The rate constant measured for low hydrogen but in the presence of molybdate (bar B) represents oxidation by hydrogen sensitive and other bacteria, but not by sulfate reducers which are inhibited by the molybdate ($k_B = k_2 + k_3$). Under high hydrogen concentrations but without molybdate (bar C), oxidation is caused by sulfate reducers and 'other' bacteria ($k_C = k_1 + k_3$). Finally, if both high concentrations of hydrogen and molybdate are present, fatty acid oxidation will be carried out by 'other' bacteria only (condition D; $k_D = k_3$). Rate constants for individual processes can be calculated from the measured rate constants as follows:

$$\begin{aligned} &k_1 = k_A - k_B \text{ and } k_1 = k_C - k_D \\ &k_2 = k_A - k_C \text{ and } k_2 = k_B - k_D \qquad (5) \\ &k_3 = k_D. \end{aligned}$$

Before constants were compared, we used the Duncan's multiple range test with a 95% confidence interval (Ott 1984) to determine whether the rate constants were statistically similar. This test is a procedure used to make pairwise comparisons among a number of sample means, and is a powerful method because the samples are ranked and the protection level decreases as the means become further apart. In Table 2, dissimilar rate constants are presented, while 'similar' indicates that the two experimental rate constants being compared were found to be statistically similar. Using the rate constants presented in Table 2, we calculated the percentage of the total fatty acid oxidation due to each bacterial group (Table 4). The data clearly show that *both* fatty acid oxidizing sulfate-reducing bacteria and hydrogen sensitive fatty acid oxidizing bacteria can play important

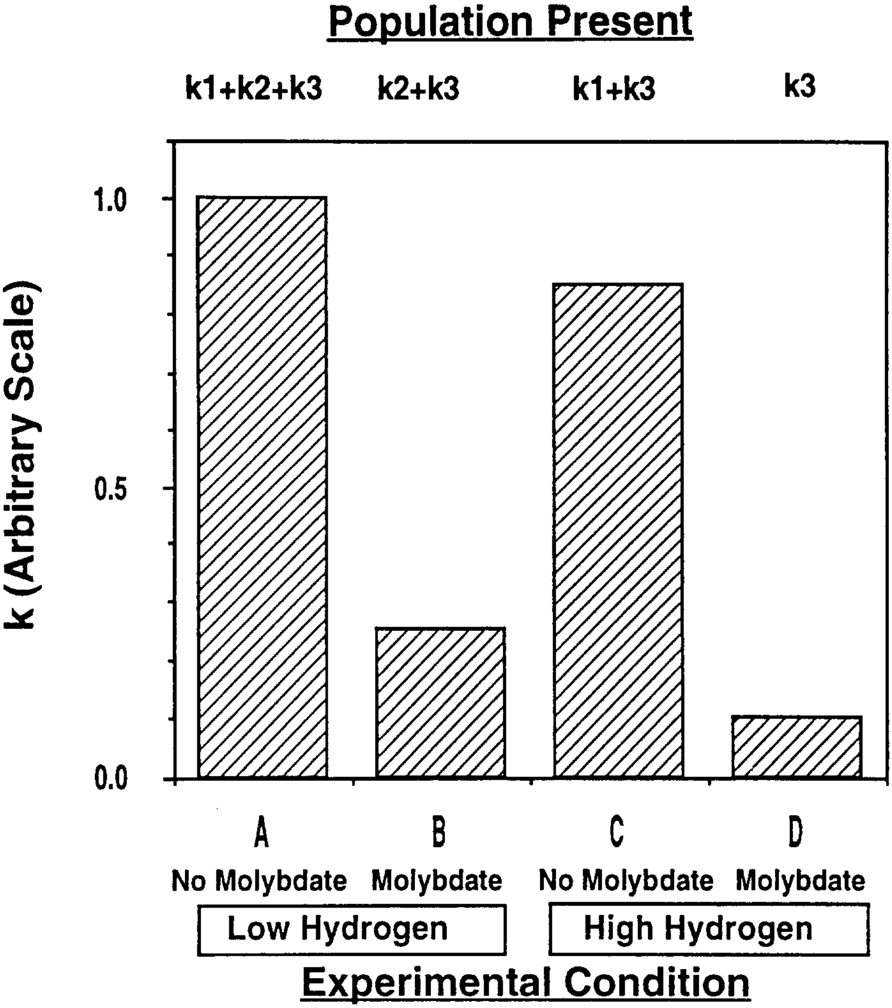

Fig. 3. Paradigm used to compare the fatty acid oxidation rate constants of slurries exposed to the four different experimental conditions. k_1, k_2 and k_3 represent the oxidation rate constants of sulfate reducers, hydrogen-sensitive bacteria and 'other' VFA oxidizing bacteria respectively. The values represented in this figure are illustrative and do not refer to a particular experiment.

roles in fatty acid oxidation in sediments. 'Other' bacteria also may play a small role, and might include bacteria which could use oxidants such as elemental sulfur or thiosulfate rather than sulfate. However it is also possible that this fraction represents a small amount of hydrogen-sensitive fatty acid oxidizing activity occurring within microenvironments which were not broken up by our techniques, and which were therefore not

Table 4. The percentage of fatty acid oxidation due to three different groups of bacteria for each experiment.

	Percent of fatty acid oxidation due to[1]:				
Experiment date	Sulfate reducers[2]		Hydrogen sensitive[2] bacteria		Other bacteria
	$K_A - K_B$	$K_C - K_D$	$K_A - K_C$	$K_B - K_D$	
Acetate					
6/1	79	36	58	—[3]	7
6/15	80	30	64	—	6
7/19	92	35	59	—	1
Corrected 7/19	70	46	46	23	6
Propionate					
3/18	—	—	91	75	3
4/11	53	34	50	32	16
7/19	97	100+	—	—	0
Corrected 7/19	78	100+	—	22	0
Butyrate					
6/9	89	48	31	—	22

[1] The percentage is estimated from the ratio of the oxidation rate constant of each group to the oxidation rate constant of the total bacterial population using values from Table 3.

[2] Two values are reported for the oxidation due to sulfate reducers and hydrogen sensitive bacteria since two independent oxidation rate constants were calculated for these bacteria.

[3] A dash in the table indicates two constants found to be statistically similar were compared.

exposed to elevated hydrogen levels. We believe that this latter possibility is less likely than the former since in condition D, both hydrogen and molybdate were added to the sediment and we assume that the processes responsible for removing hydrogen in microenvironments (i.e., sulfate reduction) were suppressed.

There were three potential problems with the experimental approach used here to evaluate the relative importance of several bacterial populations in VFA oxidation. First, during our experiments we were concerned with the possibility that sulfate reducers might alter their preference for substrates as conditions change. In particular, the sulfate reducers might shift from utilizing fatty acids to using hydrogen under a high hydrogen atmosphere. Our data (Table 2) do indicate that slurries without molybdate additions always had higher fatty acid oxidation rate constants when hydrogen concentations were low than when they were high (conditions A and C), but this was also the case for molybdate treated samples (conditions B and D). Since sulfate reducers were presumably not active in the molybdate treatments, the fact that both $k_A > k_C$ and $k_B > k_D$ suggests the importance of the hydrogen sensitive fatty acid oxidizers.

We were also concerned about the effect of elevated H_2 in sediments treated with molybdate. The loss of a H_2 consuming bacterial group (sulfate reducers) results in the accumulation of hydrogen in anoxic marine sediment (Sørensen et al. 1981; Novelli et al. 1988). At higher pore water hydrogen partial pressures, the rate of fatty acid oxidation should decrease if the role of H_2 sensitive fatty acid oxidizers is significant. In fact the hydrogen production rate was such that, in molybdate inhibited sediment slurries, the hydrogen partial pressures would be expected to increase to inhibitory values in about 1 hour in sealed vials. In micro-environments surrounding the H_2 producing bacteria, hydrogen partial pressures would be expected to increase even more rapidly. In our experiments in which fatty acid oxidation rate constants were determined, the headspace of the incubation vials was constantly purged, and the sediment slurries were constantly stirred. Therefore hydrogen should not have accumulated in the pore waters. In molybdate treated samples, it is possible that hydrogen levels could have increased within the micro-environments, underestimating the oxidation activity due to hydrogen sensitive bacteria.

An additional problem in our approach is related to the fact that, for logistical reasons, fatty acid concentrations were not measured in slurries incubated on the incubation line but were determined on slurries in sealed vials incubated at the same time and at the same temperature. Direct comparisons of fatty acid concentrations measured in slurries in sealed vials with concentrations measured in slurries from on-line vials indicated that the concentrations were often markedly lower in sealed vials than in vials incubated on the oxidation line. Therefore our calculated oxidation rate constants may be lower than *in situ* values.

We can compare acetate oxidation rate constants obtained using our technique with acetate oxidation rate constants obtained by Michelson et al. (1989) for the same system (Flax Pond) in 1986. Our homogenization and slurry method gave values of about 0.07 to 0.26 h^{-1}, which are not too different from the value of 0.34 obtained by Michelson et al. (1989) in homogenized (but not slurried) sediment. Considering the fact that our sediment manipulations were more drastic than manipulations used by Michelson et al. (1989), and the fact that seasonal and spatial variablity in salt marsh sediments is high, agreement is fairly good.

Our goal in this experiment was to use fatty acid oxidation rate constants measured under different experimental conditions to determine the rate constants for the different groups of bacteria present. However, oxidation rate constants may vary with substrate concentration (Table 3), and fatty acid concentrations were very different in the molybdate treated slurries from the concentrations in the untreated samples.

Because we found that reaction rate constants varied with VFA concentration, in order to compare the oxidation rate constants, we should correct for concentration effects. We assume that Michaelis-Menten kinetics describe microbially mediated processes better than first order kinetics (Parsons & Strickland 1962; Wright & Hobbie 1965). Michaelis-Menten kinetics have been successfully applied to fatty acid oxidation in other studies (Kaspar & Wuhrmann 1978; Heyes & Hall 1983; Ahring & Westermann 1987). Here we made reciprocal plot transformations of the Michaelis-Menten equation (Smith et al. 1983) for the acetate and propionate data obtained on 19 July 1989. Values of K_m and V_{max} can be calculated from these plots, and then can be substituted into the Michaelis-Menten equation to determine the overall oxidation rate, V.

$$V = \frac{V_{max} \times [FA]}{K_m + [FA]} \tag{7}$$

where: V = fatty acid oxidation rate
V_{max} = maximum rate of fatty acid oxidation
$[FA]$ = fatty acid concentration
K_m = substrate concentration at which oxidation rate is 0.5 V_{max}.

Figures 4a and 4b show the experimental data plotted together with curves generated using the above equation. The fit to the curve at low fatty acid concentrations is uncertain because only a few fatty acid concentrations were used. We have used all results to correct measured fatty acid rate constants. Future studies should do more detailed, and more frequent, experiments in which substrate levels are varied.

Experimental values of V plotted in Figs. 4a and b were obtained using measured fatty acid concentrations and first order rate constants (Table 3). In theory, the first order rate equation 5 should be used only over the lower concentration ranges where the rate constant is independent of fatty acid concentration. As fatty acid concentrations increase, the oxidation rate approaches V_{max} and is zeroth order (see equation 7). However, as long as the fraction of initial ^{14}C-spike oxidized is low, fatty oxidation rates calculated using both first order and zeroth order kinetic equations are very similar (see discussion in Monetti 1991). Therefore, we have presented first order oxidation rate constants throughout our discussion.

If we equate the oxidation rate (V) calculated using equations 6 and 7,

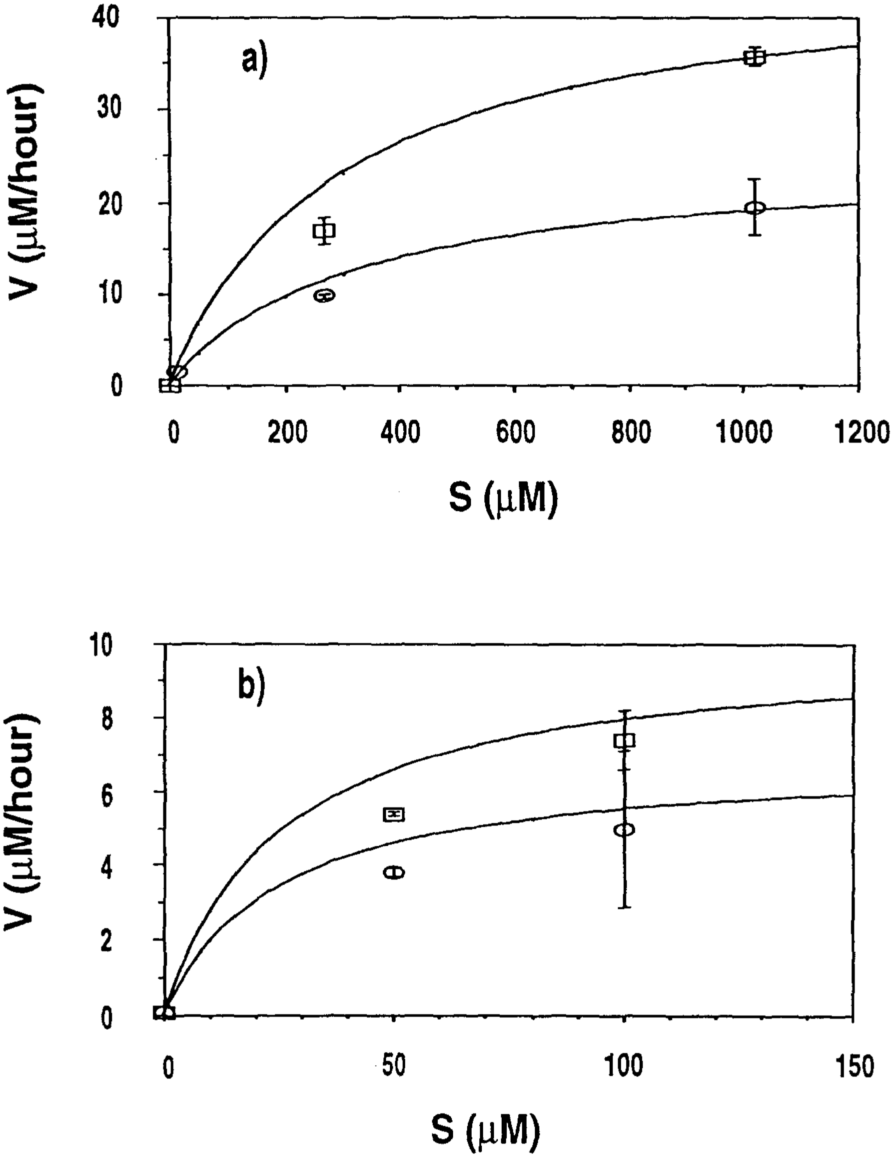

Fig. 4. Michaelis-Menten plots for acetate (a) and propionate (b) oxidation under high (□) and low (○) hydrogen partial pressure from 19 July 1989. The range of values given by two slurries is given by the error bars. Calculated values of V_{max} and K_m were 46 μM h^{-1} and 300 μM respectively for acetate under low H_2, 25 μM h^{-1} and 320 μM respectively for acetate under high H_2, 10 μM h^{-1} and 26 μM respectively for propionate under low H_2, and 6.9 μM h^{-1} and 25 μM respectively for propionate under high H_2.

we can obtain a relationship between the fatty acid concentration and the oxidation rate constant in the experimental slurries.

$$\frac{V_{max}\,[FA]}{K_m + [FA]} = k\,[FA] \tag{8}$$

or

$$k = \frac{V_{max}}{K_m + [FA]} \tag{9}$$

This equation was used to correct the oxidation rate constants of slurries which had not been treated with molybdate so that they could be compared to rate constants measured in slurries inhibited with molybdate. (V_{max} and K_m were determined only for untreated slurries.) Following this correction, rate constants for all four experimental conditions can be compared again (Table 3; corrected 7/19). Duncan's multiple range test (with a 95% confidence interval) was used as before to test the extent to which rate constants were statistically similar. Comparisons between dissimilar values are presented in Table 4.

Even after correction of the rate constants on July 19, the basic interpretation of the results remains the same. Acetate and propionate are oxidized by three pathways (by sulfate reducers, by hydrogen sensitive fatty acid oxidizers, and by 'other' processes), as was shown using the uncorrected rate constants. The relative importance of the bacterial groups does appears to be slightly different following the corrections of the rate constants, but the change is probably not significant. The most noticeable effect of correcting the rate constants is that the sulfate reducers may be less important. The relative importance of hydrogen sensitive pathways remains roughly the same, while 'other' bacteria are more important. The two independent calculations of the rate constants for sulfate reducers and for hydrogen sensitive bacteria were more similar using the corrections (Table 4).

Conclusions

Our study has shown that fatty acid oxidations in anoxic marine sediments are independently inhibited by the presence of molybdate and of elevated hydrogen partial pressures. This indicates that both hydrogen-sensitive fatty acid-oxidizing bacteria and sulfate reducing bacteria can have important roles in the cycling of LMWFAs in anoxic marine sediments. Sulfate reducers can utilize fatty acids directly, and can also control hydrogen concentrations in pore waters, so these organisms may affect fatty acid distributions and turnover rates both directly and indirectly. Addition of molybdate can result in increases of hydrogen as well as of LMWFA in sediments. The hydrogen can accumulate rapidly to levels which inhibit the hydrogen sensitive fatty acid oxidation pathways. Therefore treatment of sediment with molybdate in sealed containers may cause an inhibition of LMWFA oxidation by affecting *both* the direct and indirect roles of sulfate reducers. Addition of an inhibitory partial pressure of hydrogen to sediment slurries will result in inhibition of hydrogen sensitive fatty acid oxidations only if the hydrogen sensitive bacteria experience the elevated hydrogen concentration, and are not present in microenvironments where hydrogen levels are kept low by hydrogen consumers.

Thus, studies of low molecular weight fatty acids in marine sediments must explicitly recognize the influence of experimental design on hydrogen concentrations if a true understanding of the *in situ* concentrations and turnover rates is to be obtained.

Acknowledgements

We wish to thank Dr. Cindy Lee and Dr. James Mackin for advice and Dr. Bruce Brownawell and Dr. Paul Novelli for valuable comments on earlier versions of the manuscript. This work was supported by NSF grant OCE 85-00270 and OCE 87-19049. This is contribution number 859 from the Marine Sciences Research Center.

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