Biodegradation (2009) 20:499–510
DOI 10.1007/s10532-008-9239-6

ORIGINAL PAPER

Anaerobic degradation of citrate under sulfate reducing and methanogenic conditions

Victor M. Gámez · Reyes Sierra-Alvarez · Rebecca J. Waltz · James A. Field

Received: 10 July 2008 / Accepted: 3 December 2008 / Published online: 17 December 2008

Abstract Citrate is an important component of metal processing effluents such as chemical mechanical planarization wastewaters of the semiconductor industry. Citrate can serve as an electron donor for sulfate reduction applied to promote the removal of metals, and it can also potentially be used by methanogens that coexist in anaerobic biofilms. The objective of this study was to evaluate the degradation of citrate with sulfate-reducing and methanogenic biofilms. During batch bioassays, the citrate, acetate, methane and sulfide concentrations were monitored. The results indicate that independent of the biofilm or incubation conditions used, citrate was rapidly fermented with specific rates ranging from 566 to 720 mg chemical oxygen demand (COD) consumed per gram volatile suspended solids per day. Acetate was found to be the main fermentation product of citrate degradation, which was later degraded completely under either methanogenic or sulfate reducing conditions. However, if either sulfate reduction or methanogenesis was infeasible due to specific inhibitors (2-bromoethane sulfonate), absence of sulfate or lack of adequate microorganisms in the biofilm, acetate accumulated to levels accounting for 90–100% of the citrate-COD consumed. Based on carbon balances measured in phosphate buffered bioassays, acetate, CO_2 and hydrogen are the main products of citrate fermentation, with a molar ratio of 2:2:1 per mol of citrate, respectively. In bicarbonate buffered bioassays, acetogenesis of H_2 and CO_2 increased the yield of acetate. The results taken as a whole suggest that in anaerobic biofilm systems, citrate is metabolized via the formation of acetate as the main metabolic intermediate prior to methanogenesis or sulfate reduction. Sulfate reducing consortia must be enriched to utilize acetate as an electron donor in order to utilize the majority of the electron-equivalents in citrate.

Keywords Citric acid · Anaerobic biodegradation · Acetate · Methanogenesis · Sulfate reduction

V. M. Gámez · R. Sierra-Alvarez · R. J. Waltz · J. A. Field (✉)
Department of Chemical and Environmental Engineering, University of Arizona, P.O. Box 21001, Tucson, AZ 85721, USA
e-mail: jimfield@email.arizona.edu

Introduction

Citric acid ($C_6H_8O_7$) is a common metabolite of living cells and is also a ubiquitous compound abundant in nature; therefore, it is not surprising to find a large variety of bacteria capable of citrate degradation. Citrate is mainly used in the food industry and it was originally synthesized from citrus fruits, which have high contents of this compound (Antranikian and Giffhorn 1987). Citrate is also a naturally occurring chelating agent that forms soluble multidentate complexes with metals. The ability of

citrate to bind to metals is of special interest to metal processing industries like the metallurgical, electroplating, nuclear, and the semiconductor industry (Francis et al. 1992; Golden et al. 2000; Thomas et al. 2000; Juang et al. 2006; Chen and Huang 2007). In the semiconductor industry citrate is mainly used in the chemical mechanical planarization (CMP) step of the fabrication, and is an important component of the wastewaters generated by this process (Golden et al. 2000). In the nuclear industry, as well as in the electroplating industry, it is generally found in the waste stream chelating metals of interest such as uranium, copper, and nickel (Francis et al. 1992; Juang et al. 2006; Chen and Huang 2007). Citrate has also been applied to the remediation of heavy metal-laden soils, by mobilization and concentration of the metals (Thomas et al. 2000).

Knowledge of the mechanism of citrate degradation is of importance for the biological treatment of metal processing wastewaters. Citrate can potentially serve as an electron donor for sulfate reduction applied to promote the removal of metals (Foucher et al. 2001; Hulshoff Pol et al. 2001; Quan et al. 2003; Tabak et al. 2003), and it can also potentially be used by methanogens that coexist in anaerobic biofilms. Complete degradation of citrate to CO_2 by aerobic bacteria is known to proceed via the tricarboxylic acid (TCA) cycle (Antranikian and Giffhorn 1987). Under anaerobic conditions, bacteria cannot fully oxidize citrate with the TCA cycle due to the limited possibilities of recycling reduced electron carriers. In order to degrade citric acid without elemental oxygen, bacteria have developed unique fermentation pathways such as the production of pyruvate, acetate and carbon dioxide, referred to as the "citrate fermentation" pathway (Antranikian and Giffhorn 1987; Antranikian and Gottschalk 1989; Bott et al. 1995). The pathway is initiated by citrate lyase that converts citrate to oxaloacetate and acetate. Subsequently, oxaloacetate is converted to pyruvate and CO_2 by oxaloacetate decarboxylase (Antranikian and Giffhorn 1987).

The objective of this study is to evaluate the degradation of citrate under sulfate-reducing and methanogenic conditions. Two microbial consortia were studied. One was a sulfate-reducing granular sludge (SRS) obtained from a laboratory-scale citrate-fed bioreactor, which had the ability to utilize acetate as an electron-donor for sulfate reduction. The other consortium was a methanogenic granular sludge (MS) obtained from a full-scale bioreactor treating recycled paper effluent. The time course of citrate utilization and the formation of intermediates and end-products (i.e., acetate, methane, sulfide) was monitored in the batch bioassays conducted under sulfate-reducing and methanogenic conditions. Additional studies were performed to study the carbon and chemical oxygen demand (COD) balances.

Materials and methods

Microorganisms

Methanogenic granular sludge (MS) and the sulfate-reducing granular sludge (SRS) were obtained from an industrial upflow granular sludge bed (UASB) methanogenic bioreactor treating recycle paper wastewater (Eerbeek, The Netherlands), and from a laboratory-scale expanded granular sludge blanket (EGSB) bioreactor treating citrate for the removal of copper, respectively. The latter bioreactor was operated for approximately 750 days with citric acid (2.1–6.1 g l^{-1}) and non-limiting concentrations of sulfate (COD/sulfate = 0.56), at a temperature of 30°C, and hydraulic retention times ranging from 8 to 18 h. The content of volatile suspended solids (VSS) in MS and SRS was 13.7 and 6.1% (wet weight), respectively. The microbial cultures were elutriated to remove the fines from biofilm granules and stored under nitrogen gas at 4°C.

Basal media

The basic anaerobic basal mineral medium used in the all the experiments contained (in mg l^{-1}): NH_4Cl (280), KCl (270), K_2HPO_4 (169), $CaCl_2 \cdot 2H_2O$ (10), $MgCl_2 \cdot 6H_2O$ (150), yeast extract (20), and 1 ml l^{-1} of trace element solution. The trace element solution contained (in mg l^{-1}): H_3BO_3 (50), $FeCl_2 \cdot 4H_2O$ (2,000), $ZnCl_2$ (50), $MnCl_2 \cdot 4H_2O$ (50), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (50), $AlCl_3 \cdot 6H_2O$ (90), $CoCl_2 \cdot 6H_2O$ (2,000), $NiCl_2 \cdot 6H_2O$ (50), $CuCl_2 \cdot 2H_2O$ (30), $NaSeO_3 \cdot 5H_2O$ (100), EDTA (1,000), resazurin (200) and 36% HCl (1 ml l^{-1}).

For all the studies the pH of the medium was adjusted to a value of 7.2. Potassium phosphate

dibasic was used as a buffer at a concentration of 8,700 mg l^{-1} for the carbon distribution studies. Sodium bicarbonate was used as a buffer at a concentration of 5,000 mg l^{-1} for all the other experiments.

Biodegradation batch bioassays

The degradation of citrate (1,800 mg l^{-1}) was studied in the presence and absence of sulfate (1,334 mg l^{-1}). Some assays were supplied with 2-bromoethane sulfonate (BES, 6,300 mg l^{-1}), a methanogenic inhibitor, to study the degradation of citrate by sulfate-reducing bacteria only. Background controls were not supplied with citrate to determine the endogenous production of sulfide, acetate, and methane.

The experiments were performed in 330 ml serum flasks sealed with rubber septa. The anaerobic sludge was previously acclimated for 24 h under the conditions of the experiment and then transferred to flasks (1.5 g VSS l^{-1}) containing 200 ml of the mineral medium. The headspace of the serum flasks was flushed with N_2/CO_2 (80/20, v/v) for 5 min and then flasks were incubated at 30 ± 2°C. Methane, sulfide, and acetate production, as well as citrate consumption, were measured one to three times a day, depending on the activity observed for each experiment. Methane production was calculated from the volume of the headspace and the methane percentage composition in the biogas as determined with a gas-lock syringe. Net cumulative methane, sulfide, and acetate production were calculated by subtracting background production in the endogenous control to obtain the net production in the test flask.

The maximum production and degradation rates for methane, acetate, sulfide, and citrate were calculated from the slope of the cumulative consumption or production for each case (mg COD) versus time (days) and the biomass concentration at the end of the assays, as the mean value of duplicate assays.

Acetogenic assays

An experiment was performed to study the autotrophic acetogenic activity of the utilized sludge using only H_2 as electron donor. No sulfate was provided, and methanogenesis was inhibited by adding BES to the mineral medium. The anaerobic basal mineral medium (pH 7.2) used in acetogenic bioassays contained (in mg l^{-1}): NH_4Cl (280), KCl (270), K_2HPO_4 (169), $CaCl_2 \cdot 2H_2O$ (10), $MgCl_2 \cdot 6H_2O$ (150), yeast extract (20), and 1 ml l^{-1} of trace element solution. The medium contained 5,000 mg l^{-1} of sodium bicarbonate as buffer and carbon source.

The experiments were performed in 160 ml serum flasks with rubber septa. The sludge was previously acclimated for 24 h under the conditions of the experiment and then transferred to flasks (3 g VSS l^{-1}) containing 50 ml of the mineral medium. The headspace of the flasks was then flushed with H_2/CO_2 (80/20, v/v) for 10 min to assure that the atmosphere was completely replaced. The flasks were then incubated at 30 ± 2°C. Acetate production was then measured and the maximum specific acetogenic activities were calculated from the slope of the cumulative acetate production (mg) versus time (days) and the biomass concentration at the end of the assays, as the mean value of triplicate assays.

Carbon distribution experiments

In order to study the distribution of the citrate's carbon on the different metabolites and products, a study was conducted using a sulfate-free medium. Methanogenesis was inhibited using BES. The medium contained citrate and BES with a concentration of 2,752 and 6,330 mg l^{-1}, respectively.

The experiments were performed in 330 ml serum flasks sealed with rubber septa. The anaerobic sludge was previously acclimated for 24 h under the conditions of the experiment and then transferred to flasks (1.5 g VSS l^{-1}) containing 150 ml of the mineral medium. The headspace of the serum flasks was flushed with N_2 gas for 5 min and then flasks were incubated at 30 ± 2°C. Acetate and CO_2 production, as well as citrate consumption, were measured one to three times a day, depending on the activity observed for each experiment. CO_2 production was calculated from the volume of the headspace and the CO_2 percentage composition in the headspace as determined with a gas-lock syringe. Then this concentration was converted to total CO_2 using the following expressions:

$$\text{Tot}\,CO_2 = \frac{P_{CO_2}}{RT\left(\frac{1}{\alpha_0 \times H} + F\right)}$$

$$\alpha_0 = \frac{1}{1 + \frac{Ka_1}{[H^+]} + \frac{Ka_1 \times Ka_2}{[H^+]^2}}$$

where: Tot CO_2 = Total inorganic carbon in system (mmol/l_{liquid}); P_{CO_2} = Partial pressure of CO_2 (atm); $F = V_H/V_L$; V_H = Volume of headspace (l); V_L = Volume of liquid (l); T = Temperature (°K); R = Universal gas constant (atm l (mol K)$^{-1}$); H = Dimensionless Henry's constant (1.2); α_0 = Molar fraction of H_2CO_3 of dissolved inorganic carbon species; Ka_1, Ka_2 = Acidity constants for CO_2 ($10^{-6.35}$ and $10^{-10.33}$, respectively).

Net cumulative acetate and CO_2 production were calculated by subtracting background production in the endogenous control to obtain the net production in the test flask. The concentrations of acetate, CO_2 and citric acid were plotted in a concentration (mM) versus time (days) graph. The CO_2 was plotted as mmoles of total inorganic carbon per liter of liquid.

Analytical methods

The acetate concentration in liquid samples and the methane concentration in the flask headspace for the batch bioassays were determined by gas chromatography (GC) using an HP5290 Series II system (Agilent Technologies, Palo Alto, CA) equipped with a flame ionization detector and a Nukol fused silica capillary column (30 m length × 0.53 mm ID, Supelco, St Louis, MO). The carrier gas was helium at a flow rate of 11 ml min^{-1} and a split flow of 84 ml min^{-1}. For acetate measurements the temperatures of the column, injector port, and detector were 140, 180 and 275°C, respectively. For methane the temperatures of the column, injector port, and the detector were 140, 180, and 250°C, respectively. CO_2 was analyzed by gas chromatography using an HP5290 Series II system equipped with a thermal conductivity detector and a Carboxen 1010 Plot column (30 m length × 0.32 mm I.D., Supelco, St. Louis, MO). Helium was used as carrier gas at a flow rate of 18.2 ml min^{-1} and a split flow of 33.5 ml min^{-1}. The temperature of the column was 250°C, with a temperature for the injector port and the detector of 230°C.

Sulfide was analyzed colorimetrically by the methylene blue method (Trüper and Schlegel 1964). Citrate was analyzed by ion chromatography with suppressed conductivity using a DIONEX DX-500 system equipped with an AS11-HC4 column (Dionex, Sunnydale, CA). The mobile phase had a gradient of 0.67 mM KOH min^{-1} going from 20 to 30 mM at a flow rate of 1.2 ml min^{-1}. The column was maintained at room temperature. The injection volume was 25 µl. The VSS content in sludge samples was determined according to *Standard Methods for Examination of Water and Wastewater* (APHA 1998). The pH was determined before and after each experiment with an Orion model 310 PerpHecT pH-meter with a PerpHecT ROSS glass combination electrode.

Chemicals

All chemicals used were reagent grade or better. Sodium citrate tribasic, dihydrate ($C_6H_5Na_3O_7 \cdot 2H_2O$, >99%), sodium sulfate (Na_2SO_4, >99%), and 2-bromoethane sulfonate (98%) were obtained from Sigma–Aldrich Corp. (St Louis, MO, USA).

Results

Methanogenic and sulfate-reducing activities of the anaerobic consortia

The SRS consortium used in the study had low methanogenic activities tested with H_2 and acetate as electron donors (Table 1). The methanogenic activities observed for the MS consortium were considerably higher. The sulfidogenic activities of the SRS indicated low activities with H_2 and formate, but high activity with acetate as electron donor. The results confirm that the SRS primarily links the oxidation of acetate to sulfate reduction.

Citrate degradation in the presence of sulfate

The MS and SRS were fed with citrate (1,000 mg citrate-COD l^{-1}) to study its degradation under non-sulfate-limiting conditions (1.8 g SO_4^{2-} g^{-1} COD) in the presence and absence of the methanogenic inhibitor BES. Figures 1 and 2 show that citrate was rapidly depleted from the medium after only

Table 1 Methanogenic and sulfidogenic activities for the different inocula

Sludge	Electron donor	Methanogenic activity (mg CH_4-COD/gVSS/day)	Sulfidogenic activity (mg H_2S-COD/gVSS/day)	Acetogenic activity (mg acetate-COD/gVSS/day)
Sulfate reducing	Acetate	8.3 ± 1.1	520.9 ± 6.8	NA
	H_2	7.1 ± 0.4	87.3 ± 25.6	115.8 ± 2.44
	Formate	19.5 ± 1.8	89.5 ± 4.46	NM
Methanogenic	Acetate	174.3 ± 0.8	1.1 ± 0.1	NA
	H_2	125.9 ± 34.7	3.8 ± 0.2	12.1 ± 0.80

NM not measured; *NA* not applicable

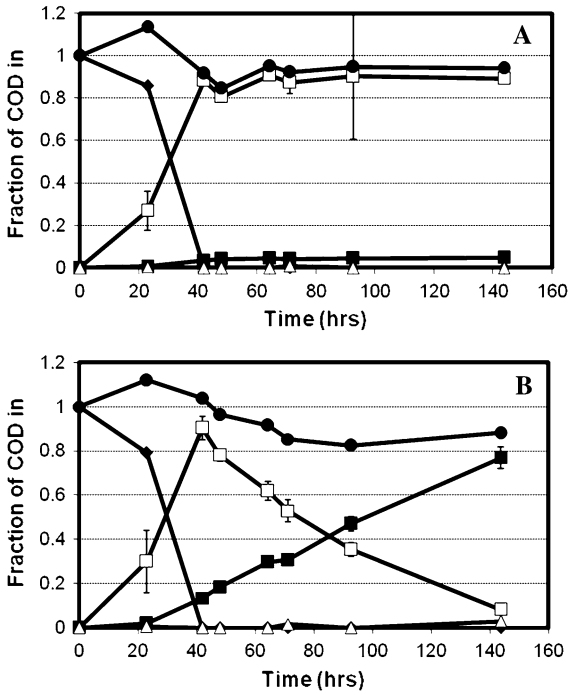

Fig. 1 Time course for the degradation of citrate by methanogenic sludge in assays supplied with sulfate, in the presence of BES (**a**), and absence of BES (**b**). ◆ Citrate, □ acetate, Δ sulfide, ■ methane, ● total COD

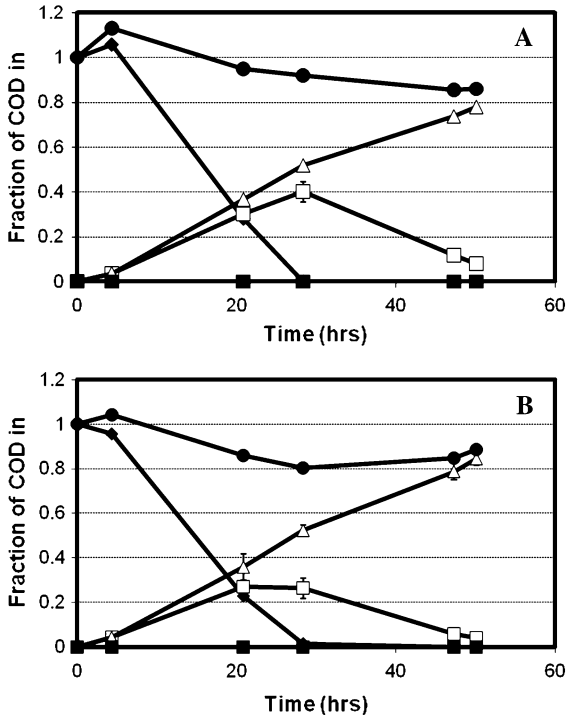

Fig. 2 Time course for the degradation of citrate by sulfate-reducing sludge in assays supplied with sulfate, in the presence of BES (**a**), and absence of BES (**b**). ◆ Citrate, □ acetate, Δ sulfide, ■ methane, ● total COD

40 h of incubation in all the assays. However, the microbial consortia behaved differently with respect to the final fate of the reducing equivalents in citrate.

An initial accumulation of acetate was observed in the SRS independent of the presence or absence of BES (Fig. 2). However, the acetate concentration steadily decreased after approximately 30 h, and this decrease coincided with an increase in the sulfide levels. The final concentration of sulfide corresponded to approximately 80% of the citrate-COD consumed. By the end of the experiment, there was no detectable residual of acetate. The results indicate conversion of citrate to acetate and subsequent use of acetate for sulfate reduction. Methanogenic conversion of the acetate was not evident in this consortium since no methane formation occurred in the absence of BES.

When MS was used, the pattern was distinct. In this case, the final fate of citrate depended on the presence or absence of BES. In the absence of BES, citrate was converted to acetate to a large extent at the beginning of the experiment, but after 40 h, the acetate was transformed stoichiometrically to methane (Fig. 1). In the presence of BES, citrate

was stoichiometrically converted into acetate, a metabolite that accumulated in the medium. A little bit of methane (up to 5% of the citrate-COD) was also observed since BES did not inhibit methanogenesis completely. No sulfide production was observed under any of the conditions studied with MS even though sulfate was supplied in excess. The results indicate conversion of citrate to acetate and subsequent conversion to methane. If methanogenesis was blocked by BES there was accumulation of acetate. The methanogenic consortium was incapable of degrading acetate by sulfate reduction (or any other electron donor).

Citrate degradation in the absence of sulfate

The degradation of citrate by the two microbial consortia was also studied under methanogenic and fermentative conditions in the absence of SO_4^{2-} as an external electron acceptor. Figures 3 and 4 show the results obtained during these experiments, indicating

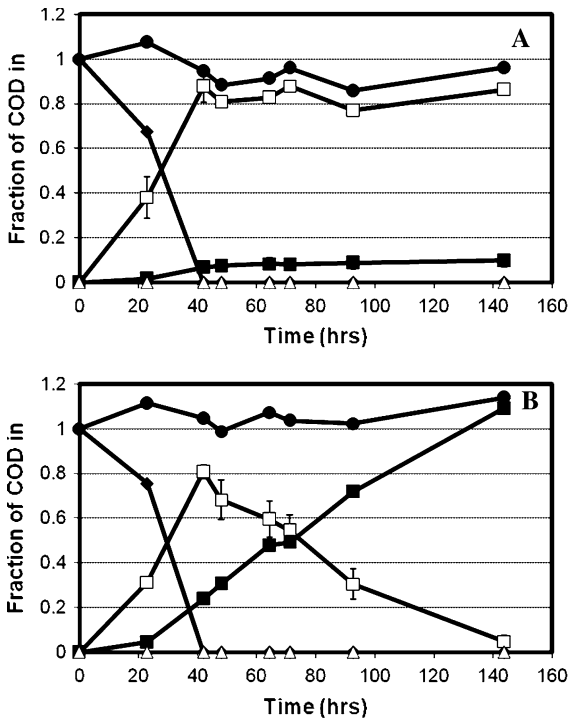

Fig. 3 Time course for the degradation of citrate by methanogenic sludge in assays lacking sulfate, in the presence of BES (**a**), and absence of BES (**b**). ◆ Citrate, □ acetate, Δ sulfide, ■ methane, ● total COD

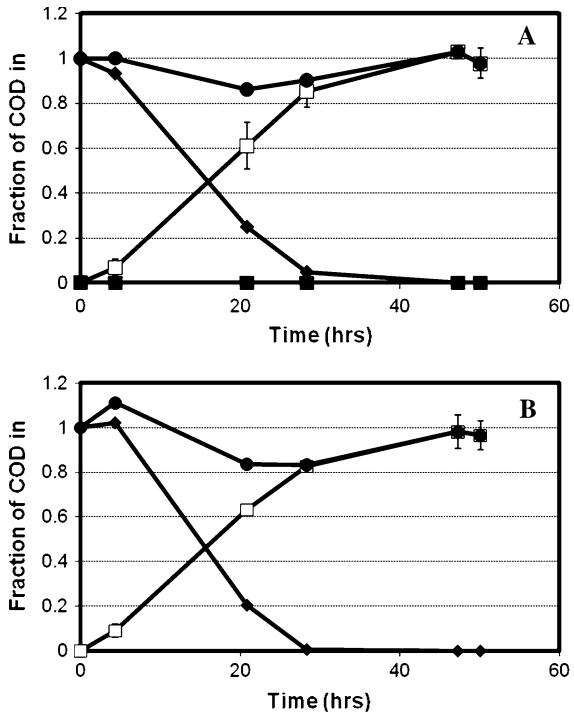

Fig. 4 Time course for the degradation of citrate by sulfate-reducing sludge in assays lacking sulfate, in the presence of BES (**a**), and absence of BES (**b**). ◆ Citrate, □ acetate, Δ sulfide, ■ methane, ● total COD

rapid citrate degradation in the absence of sulfate. Again differences between the two types of consortia were observed with respect to the final fate of the electron-equivalents in citrate. The MS followed a similar trend to that observed under conditions in which sulfate was provided. The determining factor in the fate of citrate was again found to be the presence or absence of BES. When BES was provided, the final metabolite of citrate degradation was acetate, which was recovered in near stoichiometric yields. However, when methanogenesis was not inhibited, the final product was methane, after temporal accumulation of acetate, reaching a maximum concentration around 40 h after the initiation of the experiment.

The SRS only converted citrate to acetate, which was not degraded further in the absence of sulfate due to the low methanogenic activity in the consortium. Consequently, BES had no impact on the outcome. Without SO_4^{2-}, citrate was converted completely to acetate, which accounted for 100% of the citrate-COD consumed.

Carbon distribution experiments

In order to better determine the stoichiometry of citrate fermentation, an experiment was performed to measure the CO_2 production from citrate while selectively inhibiting methanogenesis. For this study, bicarbonate was removed as the selected buffer and phosphate was used instead. This was done with the objective of avoiding any interference a high background of bicarbonate might cause in measuring net production of CO_2. Likewise, exogenous inorganic C should be avoided to prevent acetogenesis of any H_2.

The results obtained in assays with MS and SRS show a similar trend where both, acetate as well as CO_2, are generated from citrate consumption (Fig. 5). Table 2 shows the carbon balances for the different sets of experiments at three different times. At the end of the experiment, citrate is almost completely consumed in the case of SRS and completely removed in the case of MS. The corresponding average acetate and CO_2 concentrations are 51.5 and 26.2 mM of carbon for SRS, and 57.9 and 29.9 mM of carbon for the MS, respectively. These results indicate that the carbon derived from citrate is distributed between acetate and CO_2 in a molar ratio of 2:1, and that these are the only two metabolites containing carbon from citrate fermentation, since they account for the initial carbon concentration added in the form of citrate. The data at the final time point in Table 2 corresponds to a molar ratio of 1.96 and 1.99 mol acetate and CO_2 per mol citrate consumed, respectively, for the SRS; and a molar ratio of 2.01 and 2.06 mol acetate and CO_2 per mol citrate consumed, respectively, for the MS.

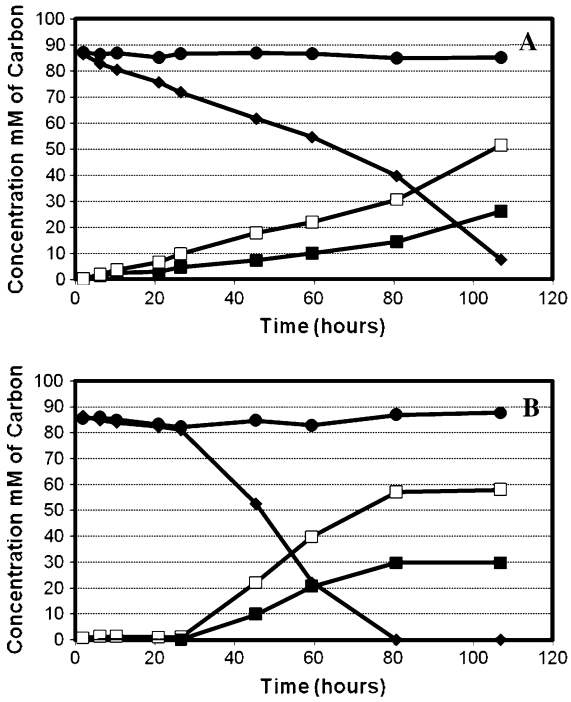

Fig. 5 Time course for carbon distribution. **a** Sulfate-reducing sludge. **b** Methanogenic sludge. ◆ Citrate, □ acetate, ■ carbon dioxide, ● total carbon

Rates of citrate and metabolite degradation and formation

Maximum specific activities observed during the degradation of citrate are summarized in Table 3. Citrate degradation rates were similar in all the experiments without any dependence on the type of sludge used or whether sulfate was present or absent. Likewise the rates were not impacted by the presence or absence of BES. In all cases, citrate was rapidly fermented with specific rates ranging from 566 to 720 mg COD consumed g^{-1} VSS d^{-1}. Acetate accumulated as the major end-product in four bioassays (MS spiked with BES in the presence and absence of SO_4^{2-}; SRS without SO_4^{2-} in the presence and absence of BES). In those assays, the acetate production rates (Table 3) accounted for 72 to 87% of the COD flux of citrate consumption. The lowest accumulations of acetate occurred in the bioassays with the SRS and SO_4^{2-} which is consistent with the fact that the acetate production rate was nearly equal to the rate of sulfate reduction production (which primarily used acetate as the electron). The acetate accumulations were higher in the bioassays with the MS due to the fact that the methanogenic activity was lower than the acetate production rate. The rates of acetate consumption were in the same order of magnitude as sulfate reduction and methanogenesis in the SRS and MS, respectively. These finding are in agreement with the observation that acetate was the main electron donor for sulfide and methane formation, respectively.

Table 4 summarizes the rates of acetate-C and CO_2-C formation in the phosphate buffered bioassays. The ratio of the acetate-C to CO_2-C formation rates is almost exactly equally to two. The fluxes are therefore consistent with the stochiometries which

Table 2 Carbon distribution for the different conditions at three different times (beginning, middle and end of experiment)

Time (h)	Concentration (mM of carbon)				
	Citrate-C	Acetate-C	CO_2-C	Total carbon	Ratio Acetate-C/CO_2-C
Sulfate-reducing sludge					
2.0	86.55 ± 0.55	0.26 ± 0.15	0.34 ± 0.79	87.16	NA
59.5	54.65 ± 2.53	22.00 ± 1.09	10.04 ± 1.25	86.69	2.19
107.0	7.54 ± 5.51	51.50 ± 2.89	26.15 ± 1.44	85.19	1.97
Methanogenic sludge					
2.0	86.37 ± 3.48	0.91 ± 0.22	0.04 ± 0.02	87.33	NA
59.5	22.27 ± 18.32	39.93 ± 7.03	20.61 ± 7.50	82.80	1.94
107.0	0.00 ± 0.00	57.88 ± 0.13	29.87 ± 0.86	87.74	1.94

Table 3 Maximum production–consumption rates for citrate, acetate, sulfide and methane under different conditions

Experiment	Maximum rate of production or consumption (in mg COD/gVSS/day)				
	Citrate	Acetate		CH_4 production	H_2S production
	Consumption	Production	Consumption		
Sulfate-reducing sludge					
Sulfate					
BES	712.3	245.7	238.0	–	252.4
No BES	638.2	219.7	167.0	–	274.9
No sulfate					
BES	601.3	524.0	–	–	–
No BES	694.0	524.4	–	–	–
Methanogenic sludge					
Sulfate					
BES	719.8	515.6	–	12.6	–
No BES	665.1	506.6	124.2	99.5	–
No sulfate					
BES	566.0	416.2	–	25.6	–
No BES	633.2	414.6	116.4	137.8	–

indicated 2 mol of acetate and 2 mol of CO_2 formed per mol of citrate.

Discussion

In this study, rapid degradation of citrate was observed independent of the sludge used, or incubation conditions. Similar rates were evident in all cases (Table 3). This behavior might be explained by the fact that citrate fermenters in the methanogenic and sulfate-reducing consortia were similar, having fast growth rates which were enriched rapidly upon exposure to citrate. For example, *Clostridium sphenoides* was consistently enriched in pasteurized mud samples with citrate as the sole carbon source (Walther et al. 1977). The capacity to degrade citrate in the absence of O_2 is found among lactic acid bacteria (Hugenholtz 1993), enterobacteria (Bott 1997), selected clostridia (Walther et al. 1977) and the phototrophic bacterium, *Rhodopseudomonas gelatinosa* (Schaab et al. 1972). These organisms differ in the strategies used to degrade citrate (Antranikian and Giffhorn 1987).

Table 4 Maximum rates of production and consumption for the different carbon distribution studies

	Maximum rates of production–consumption (mM of C/day)			
	Consumption	Production		Ratio Acetate/CO_2
	Citrate	Acetate	CO_2	
Sulfate-reducing sludge	16.56	10.90	5.30	2.06
Methanogenic sludge	36.79	25.10	13.45	1.87

Citrate degradation pathway

In methanogenic and sulfate-reducing sludge, acetate was found to be the main product of citrate metabolism. In bioassays where the further catabolism of acetate was blocked (absence of sulfate in SRS or the specific methanogenic inhibitor BES in MS), acetate was recovered in nearly stoichiometric yields. In such cases, acetate accounted for 90–100% the COD of the citrate metabolized. Furthermore, carbon distribution experiments with BES (to block methanogenesis), showed that acetate and CO_2 were the only carbon-containing products derived from citric acid (Fig. 5). The stoichiometry indicated by the carbon-balance experiments reveals a production of 2 mol of acetate and 2 mol of CO_2 from each mol of citrate as follows in Eq. 1:

$$C_6H_5O_7^{3-} + H_2O + H^+ \rightarrow 2C_2H_3O_2^- + 2CO_2 + H_2 \quad (1)$$

Based on the carbon balance, only around 88.9% of the initial COD (or electron equivalence) can be accounted for by acetate. The remainder should be accounted for by H_2 based on the assumption that the citrate fermentation pathway was involved, yielding pyruvate (Antranikian and Giffhorn 1987; Bott 1997). The further metabolism of pyruvate has to involve the formation of H_2 to be consistent with the stoichiometry in Eq. 1, since all carbon in pyruvate is accounted for by acetate and CO_2. Molecular H_2 has been detected during citrate metabolism by *Clostridium sphenoides* (Walther et al. 1977) and *trans*-aconitate metabolism via citrate in *Acidaminococcus fermentans* (Hartel and Buckel 1996). Molecular H_2 formation from the anaerobic metabolism of pyruvate may be accounted for by intermediate formation of formate (Gorrell and Uffen 1977; Liu 2003). Additional evidence for acetate as the main product of citrate fermentation is obtained from the specific activities of the SRS cultivated on citrate (Table 1). The SRS had very high sulfate reducing activity with acetate as an electron donor; whereas by comparison the activity was sixfold lower on either hydrogen or formate. This unusual preference of electron donors for a mesophilic sulfate-reducing biomass is most likely a reflection of the fact that acetate was the main intermediate from citrate fermentation available to support sulfate reduction. The high yield of acetate observed in this study is unprecedented. In most previous studies, the reducing equivalents generated from citrate fermentation are used to form reduced organic fermentation products such as ethanol, butyrate or lactic acid in addition to acetate (Bott 1997; Hartel and Buckel 1996; Hugenholtz 1993; Walther et al. 1977).

An overview of the citrate degradation pathway observed in this study is provided in Fig. 6. The figure shows the most likely patterns of citrate fermentation and the three possible fates of the fermentation products: acetogenesis, sulfate reduction and methanogenesis.

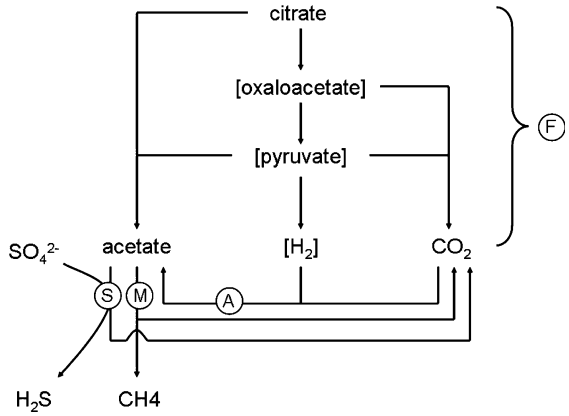

Fig. 6 Overview of the main pathways of citrate degradation occurring in SRS and MS. *Pathway F*: Citrate fermentation. *Pathway A*: Acetogenesis from H_2. *Pathway M*: Acetoclastic methanogenesis. *Pathway S*: Sulfate reduction with acetate as electron donor. The compounds between brackets were not measured in this study; their presence is inferred from the cited literature

The different inocula used and incubation conditions determined the fate of acetate, either accumulating or being metabolized further.

Acetogenesis

The COD balance appears to be in disagreement with the carbon balance since acetate consistently accounted for 100% of the COD with the SRS consortium when sulfate reduction was blocked; and acetate accounted from 90 to 96% of the COD with the MS consortium when methanogenesis was blocked. These values do not agree with 89% maximum acetate yield according to the carbon balance. However, the discrepancy has a simple explanation. The COD balance studies were performed in bicarbonate buffered assays; whereas, the carbon balance studies were performed in phosphate buffered assays. With the large excess of inorganic C in the bicarbonate buffered assays and with methanogenesis and sulfate reduction blocked, acetogenesis from H_2/CO_2 would be promoted (Breznak and Kane 1990; Dolfing 1988). Moreover, acetogenic activity was demonstrated in SRS and MS (Table 1). The activity was highest in SRS which had the highest yield of acetate. By contrast in the phosphate buffered systems, the endogenous CO_2 production was probably too low to support significant acetate production via acetogenesis.

Sulfate reduction

The sulfate-reducing consortium had no appreciable methanogenic activity; therefore, the main product of citrate degradation was acetate and H_2. However, when sulfate was provided as final electron acceptor, acetate which had initially accumulated was subsequently degraded. This was feasible since the consortium had the capacity to couple sulfate reduction with acetate degradation. The COD equivalents in the acetate were recovered almost completely in sulfides formed. Moreover, the rate of acetate consumption was only slightly lower than the rate of sulfide production in COD equivalence (Table 3). The differences being due to the fact that aside from acetate, H_2 probably also contributed as an electron donor for sulfate reduction. Taken as a whole, the data indicates that mostly acetate and to a small extent H_2 but not citrate itself were the electron donors for sulfate reduction.

Some species of sulfate-reducing bacteria are known to utilize acetate for sulfate reduction, i.e., members of the genus *Desulfobacter* (Madigan et al. 2003). The SRS originated from a sulfate-reducing bioreactor treating citrate. Since acetate is the main intermediate it is therefore safe to assume that sulfate reducers that can link acetate to sulfate reduction had enriched in the sludge. The sulfate-reducing sludge had no significant methane producing activity under any condition, not even in the absence of BES and sulfate; this could be explained by the fact that methanogens were out competed in the sulfate-reducing bioreactor.

The ability of mesophilic anaerobic sludge to utilize acetate as an electron donor for sulfate reduction is not common, and is only observed after prolonged bioreactor operation favoring sulfate reduction (Visser et al. 1993). Most species of sulfate-reducing bacteria are not capable of acetate degradation to support sulfate reduction (Kaksonen 2004; Lens et al. 2003; Madigan et al. 2003). This is of special importance when considering a biotreatment technology such as sulfate reduction for wastewaters containing citric acid, like those from the semiconductor industry. The degradation of citric acid under anaerobic conditions leads primarily to acetate production (88.8% of the initial COD), which may not be degraded further by most sulfate reducing bacteria. Therefore, to properly utilize most of the electron equivalence in citrate, the sulfate reducing consortium must be enriched to utilize acetate as an electron donor for sulfate reduction.

Methanogenesis

The main pathway of anaerobic citrate degradation in MS was also initiated by the formation of acetate. The main product of citrate degradation was either methane or acetate depending on whether the system was inhibited by BES or not. The presence or absence of sulfate did not alter these fates due to the inability of MS to perform sulfate reduction with acetate as an electron donor. The link between methanogenesis and acetate degradation is evident on Table 3 where the degradation rate for acetate is comparable to the production rate for methane, which was similar to

the methanogenic activity found for that sludge (Table 1).

The final products from the anaerobic degradation of citric acid will depend on the competition between methanogens and sulfate reducers for acetate. The outcome of this competition varies in different studies. In some studies sulfate-reducers out compete methanogens (Bhattacharya et al. 1996; Colleran et al. 1995; Oude Elferink et al. 1994). In other studies methanogens out competed sulfate-reducers (Lens et al. 1998) sometimes in conditions where sulfate is non-limiting. The competition between sulfate-reducers and methanogens for acetate utilization appears to depend on several factors like pH, COD to sulfate ratio, loading rates, acetate concentrations, etc. Among those parameters, the COD to sulfate ratio appears to be particularly important in determining the fate of acetate in sulfate reducing environments. Bhattacharya et al. (1996) showed that when the influent acetate-COD/SO_4^{2-} ratios are 0.71 g g^{-1} or lower, sulfate-reducers out competed methanogens, while for ratios exceeding 3.56 g g^{-1} methanogens out competed sulfate reducers. So if anaerobic treatment is the remediation technology selected for citric acid containing wastewaters, the competition between these two processes is of utmost importance on the outcome and success of the selected technology.

Acknowledgments This study was conducted with financial support from the Semiconductor Research Corporation/Sematech Engineering Research Center for Environmentally Benign Semiconductor Manufacturing and by a fellowship (V.G.) from the Mexican Consejo Nacional de Investigación y Ciencia (CONACyT).

References

Antranikian G, Giffhorn F (1987) Citrate metabolism in anaerobic bacteria. FEMS Microbiol Rev 46:175–198. doi:10.1111/j.1574-6968.1987.tb02458.x

Antranikian G, Gottschalk G (1989) Phosphorylation of citrate lyase ligase in *Clostridium sphenoides* and regulation of anaerobic citrate metabolism in other bacteria. Biochimie 71:1029–1037. doi:10.1016/0300-9084(89)90107-7

APHA (1998) In: Clesceri LS, Greenberg AE, Eaton AD (eds) Standard methods for the examination of water and wastewater, 20th edn. American Public Health Association, Washington DC

Bhattacharya SK, Uberoi V, Dronamraju MM (1996) Interaction between acetate fed sulfate reducers and methanogens. Water Res 30:2239–2246. doi:10.1016/0043-1354(95)00238-3

Bott M (1997) Anaerobic citrate metabolism and its regulation in enterobacteria. Arch Microbiol 167:78–88. doi:10.1007/s002030050419

Bott M, Meyer M, Dimroth P (1995) Regulation of anaerobic citrate metabolism in *Klebsiella pneumoniae*. Mol Microbiol 18:533–546. doi:10.1111/j.1365-2958.1995.mmi_18030533.x

Breznak JA, Kane MD (1990) Microbial H_2/CO_2 acetogenesis in animal guts—nature and nutritional significance. FEMS Microbiol Rev 87:309–313. doi:10.1111/j.1574-6968.1990.tb04929.x

Chen JH, Huang CE (2007) Selective separation of Cu and Zn in the citric acid leachate of industrial printed wiring board sludge by D(2)EHPA-modified Amberlite XAD-4 resin. Ind Eng Chem Res 46:7231–7238. doi:10.1021/ie0616242

Colleran E, Finnegan S, Lens P (1995) Anaerobic treatment of sulfate-containing waste streams. Anton Leeuwen 67:29–46. doi:10.1007/BF00872194

Dolfing J (1988) Acetogenesis. In: Zehnder AJB (ed) Biology of anaerobic microorganisms. Wiley, New York, pp 417–468

Foucher S, Battaglia-Brunet F, Ignatiadis I, Morin D (2001) Treatment by sulfate-reducing bacteria of Chessy acid-mine drainage and metals recovery. Chem Eng Sci 56:1639–1645. doi:10.1016/S0009-2509(00)00392-4

Francis AJ, Dodge CJ, Gillow JB (1992) Biodegradation of metal citrate complexes and implications for toxic-metal mobility. Nature 356:140–142. doi:10.1038/356140a0

Golden JH, Small R, Pagan L, Shang C, Raghavan S (2000) Evaluating and treating CMP wastewater. Semicond Int 23:85–98

Gorrell TE, Uffen RL (1977) Fermentative metabolism of pyruvate by *Rhodospirillum rubrum* after anaerobic growth in darkness. J Bacteriol 131:533–543

Hartel U, Buckel W (1996) Fermentation of *trans*-aconitate via citrate, oxaloacetate, and pyruvate by *Acidaminococcus fermentans*. Arch Microbiol 166:342–349. doi:10.1007/s002030050393

Hugenholtz J (1993) Citrate metabolism in lactic-acid bacteria. FEMS Microbiol Rev 12:165–178. doi:10.1111/j.1574-6976.1993.tb00017.x

Hulshoff Pol LWH, Lens PNL, Weijma J, Stams AJM (2001) New developments in reactor and process technology for sulfate reduction. Water Sci Technol 44:67–76

Juang RS, Kao HC, Chen W (2006) Column removal of Ni(II) from synthetic electroplating waste water using a strong-acid resin. Separ Purif Technol 49:36–42. doi:10.1016/j.seppur.2005.08.003

Kaksonen AH (2004) The performance, kinetics and microbiology of sulfidogenic fluidized-bed reactors treating acidic metal- and sulfate-containing wastewater. PhD dissertation, Tampere University of Technology, Tampere

Lens PNL, Visser A, Janssen AJH, Hulshoff Pol LW, Lettinga G (1998) Biotechnological treatment of sulfate-rich wastewaters. Crit Rev Environ Sci Technol 28:41–88. doi:10.1080/10643389891254160

Lens P, Vallero M, Esposito G, Zandvoort M (2003) Perspectives of sulfate reducing bioreactors in environmental biotechnology. Rev Environ Sci Biotechnol 1:311–325

Liu SQ (2003) Practical implications of lactate and pyruvate metabolism by lactic acid bacteria in food and beverage fermentations. Int J Food Microbiol 83:115–131. doi: 10.1016/S0168-1605(02)00366-5

Madigan MT, Martinko JM, Parker J (2003) Sulfate- and sulfur-reducing Proteobacteria. In: Madigan MT, Martinko JM, Parker J (eds) Brock biology of microorganisms. Prentice Hall, Englewood Cliffs, pp 396–399

Oude Elferink S, Visser A, Hulshoff Pol LW, Stams AJM (1994) Sulfate reduction in methanogenic bioreactors. FEMS Microbiol Rev 15:119–136

Quan ZX, La HJ, Cho YG, Hwang MH, Kim IS, Lee ST (2003) Treatment of metal-contaminated water and vertical distribution of metal precipitates in an upflow anaerobic bioreactor. Environ Technol 24:369–376

Schaab C, Schobert S, Gottscha G, Pfennig N, Giffhorn F (1972) Phototropic growth of *Rhodopseudomonas gelatinosa* on citrate. Accumulation and subsequent utilization of cleavage products. Z Naturforsch 27:962–967

Tabak HH, Scharp R, Burckle J, Kawahara FK, Govind R (2003) Advances in biotreatment of acid mine drainage and biorecovery of metals. 1. Metal precipitation for recovery and recycle. Biodegradation 14:423–436. doi: 10.1023/A:1027332902740

Thomas RAP, Beswick AJ, Basnakova G, Moller R, Macaskie LE (2000) Growth of naturally occurring microbial isolates in metal-citrate medium and bioremediation of metal-citrate wastes. J Chem Technol Biotechnol 75:187–195. doi:10.1002/(SICI)1097-4660(200003)75:3<187::AID-JCTB206>3.0.CO;2-I

Trüper HG, Schlegel HG (1964) Sulphur metabolism in Thiorhodaceae. 1. Quantitative measurements on growing cells of *Chromatium okenii*. Anton Van Lee J M S 30:225–238. doi:10.1007/BF02046728

Visser A, Beeksma I, Vanderzee F, Stams AJM, Lettinga G (1993) Anaerobic degradation of volatile fatty-acids at different sulfate concentrations. Appl Microbiol Biotechnol 40:549–556

Walther R, Hippe H, Gottschalk G (1977) Citrate, a specific substrate for isolation of *Clostridium sphenoides*. Appl Environ Microbiol 33:955–962