APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 1986, p. 572–579
0099-2240/86/030572-08$02.00/0

Vol. 51, No. 3

Sulfate Reduction Relative to Methane Production in High-Rate Anaerobic Digestion: Technical Aspects

ZAID ISA, STÉPHANE GRUSENMEYER, AND WILLY VERSTRAETE*

Laboratory of Microbial Ecology, State University of Ghent, Coupure L 653, B-9000 Ghent, Belgium

Received 5 August 1985/Accepted 8 December 1985

The effect of different substrates and different levels of sulfate and sulfide on methane production relative to sulfate reduction in high-rate anaerobic digestion was evaluated. Reactors could be acclimated so that sulfate up to a concentration of 5 g of sulfate S per liter did not significantly affect methanogenesis. Higher levels gave inhibition because of salt toxicity. Sulfate reduction was optimal at a relatively low level of sulfate, i.e., 0.5 g of sulfate S per liter, but was also not significantly affected by higher levels. Both acetoclastic and hydrogenotrophic methane-producing bacteria adapted to much higher levels of free H_2S than the values reported in the literature (50% inhibition occurred only at free H_2S levels of more than 1,000 mg/liter). High levels of free H_2S affected the sulfate-reducing bacteria only slightly. Formate and acetate supported the sulfate-reducing bacteria very poorly. In the high-rate reactors studied, intensive H_2S formation occurred only when H_2 gas or an H_2 precursor such as ethanol was supplied.

The inhibition of methanogenesis owing to the presence of sulfate has been reported for marine and freshwater sediments (5, 20–22, 31) and also for anaerobic digesters (2, 14, 19, 26). For sediments, the lowest level of sulfate reported to affect methanogenesis was about 6 mg of sulfate S per liter (0.2 mM), whereas complete inhibition occurred at a sulfate concentration of about 320 mg of sulfate S per liter (10 mM) (31).

Often, the inhibition of methanogenesis is interpreted in relation to the levels of sulfide produced by the microbial reduction of sulfate. Speece and Parkin (26) found that methane production from an unacclimated batch digester was inhibited by a sulfide level as low as 50 mg of S^{2-} S per liter (1.6 mM). However, with a submerged anaerobic filter, they noticed that sulfide levels up to 400 mg of S^{2-} S per liter had no significant effect on methane production. At 800 mg of S^{2-} S per liter methane production was only reduced by about 30%. Kroiss and Wabnegg (14) have related methanogenesis inhibition to the level of free H_2S in solution which, according to them, is toxic to methane-producing bacteria (MPB). The sulfide produced by the microbial reduction of sulfate is distributed between H_2S, HS^-, and S^{2-} in solution and H_2S in biogas. At pH 7.5, ca. 20% of the total sulfide (H_2S, HS^-, S^{2-}) present in solution exists as free H_2S. Kroiss and Wabnegg (14) found that a free H_2S level of 50 mg/liter inhibits acetoclastic MPB by about 50%, while complete inhibition occurred at a free H_2S level of ca. 200 mg/liter.

In anaerobic digestion, sulfate reduction is undesirable for several reasons. The biogas produced will contain a high level of H_2S. H_2S is a very toxic and corrosive gas, and its removal from the biogas is quite expensive. According to Butlin et al. (2), the addition of 5% (wt/vol) calcium sulfate to sewage sludge treated in interconnected fermentations, in which gas from a methane fermentation swept the H_2S from a sulfide fermentation, yielded about 5 to 10% H_2S in the biogas. Kroiss and Wabnegg (14) found that about 4% H_2S was present in the biogas obtained from the anaerobic treatment of citric acid factory wastewater which contained about 600 mg of sulfate S per liter. The level of H_2S in biogas from anaerobic treatment systems in the Netherlands varied from 0.01 to 1.1% (9). The difference in the level of H_2S in the biogas was mainly due to the difference in the level of sulfate in the wastewaters.

The presence of sulfate and the subsequent formation of sulfide can also induce the precipitation of nonalkali metals in the digester and thus severely reduce their availability for the microorganisms (3). This will affect the growth of these microorganisms, which could result in a drop in biogas production from the digester.

However, the presence of sulfate can also have beneficial effects on the anaerobic treatment of wastewaters. Sulfide produced from the microbial reduction of sulfate can precipitate toxic heavy metals such as Co, Cu, Ni, Pb, and Zn (15, 18). Capestany et al. (4) reported that the addition of sulfate as Na_2SO_4 at the biological oxygen demand/N/P/S ratio of 1,000/5/5/5 to phenolic wastewater containing 1,000 mg of unsubstituted phenol per liter decreased the phenol level to about 0.2 mg/liter as compared with 450 mg/liter when sulfate was not added. In their study on the anaerobic digestion of cellulose to methane, Khan and Trottier (13) showed that sulfate stimulated degradation at concentrations up to 25 mg of sulfate S per liter (0.8 mM).

The work reported here examines the effect of sulfate reduction on methane production from the anaerobic digestion of synthetic media. The experiments were performed with upflow reactors containing polyurethane sponges as colonization matrixes (8, 10).

MATERIALS AND METHODS

High-rate anaerobic reactor. A bench-scale reactor of 1-liter capacity (Fig. 1) was used in this study. A series of three reactors in parallel was set up. Each reactor was filled with reticulated polyurethane sponges as carrier matrixes for the bacteria. Initially, each reactor was seeded with 200 ml of well-digested anaerobic sludge from a similar reactor which treated distillery wastewater. The influent was pumped continuously from the feed reservoir with a membrane pump. The effluent from the reactor was recycled to the feed reservoir at a recycle ratio of ca. 110. The temperature was kept constant at 35°C.

Media. Synthetic media containing acetate (medium A),

* Corresponding author.

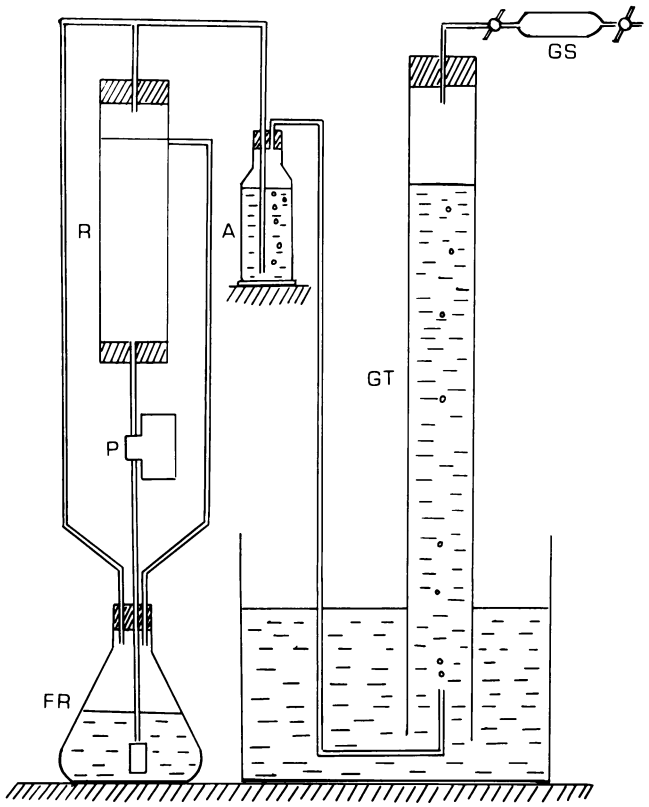

FIG. 1. Laboratory-scale high-rate anaerobic reactor. Abbreviations: R, reactor; P, membrane pump; FR, feed reservoir containing synthetic medium; A, flask containing zinc acetate solution to capture H_2S; GT, gas collection tube containing acidified water; GS, gas sampler.

acetate plus ethanol (medium AE), or formate (medium F) were used as the influents. The compositions of these media are given in Table 1. Each of these media has a chemical oxygen demand (COD) concentration of about 5,000 mg/liter.

Source of sulfate. A stock solution containing 10,000 mg of sulfate S per liter was prepared by diluting a concentrated sulfuric acid solution (d = 1.84) in distilled water. A sufficient volume of this solution was added to the influent to give the required level of sulfate. After adding this solution, the pH of the influent was adjusted to 7.0 with a 50% (wt/vol) KOH solution. At higher sulfate-to-COD ratios (more than 10 g of sulfate S per 100 g of COD), sulfate was added in the form of Na_2SO_4.

Source of sulfide. Sulfide was added to the influent by dissolving $Na_2S \cdot 9H_2O$. The concentration of sulfide in the influent was immediately determined after sulfide addition.

Digestion procedure. The reactor was started up with synthetic medium as the influent at a volumetric loading rate of 2.5 g of COD per liter of reactor per day. After 24 h of digestion, the mixed liquor in the feed reservoir was replaced with new influent of similar composition and concentration. The influent and the liquor after 24 h of digestion (effluent) were sampled daily for analysis. The biogas collected was also sampled and analyzed for its composition.

During the start-up period, the loading rate was increased gradually until a steady-state condition was reached at a volumetric loading rate of 10 g of COD per liter of reactor per day as indicated by a constant daily gas production. The results obtained during the steady-state condition at this volumetric loading rate were taken as control values.

After reaching the steady-state condition at the volumetric loading rate of 10 g of COD per liter of reactor per day, the reactors were fed with synthetic media supplemented with different levels of sulfate up to 20 g of sulfate S per liter. The level of sulfate supplemented was changed after the steady-state condition was reached for each sulfate level.

In the experiment on the effect of sulfide on methane production and sulfate reduction, the media were supplemented with 0.5 g of sulfate S per liter and different levels of sulfide and were digested in a similar reactor at a volumetric loading rate of 10 g of COD per liter of reactor per day. Similarly, the level of sulfide added was changed only after a steady-state condition was reached for a particular sulfide level.

Gas analysis. The volume of biogas produced was measured by the liquid displacement technique (Fig. 1). The volume was normalized to standard temperature (273 K) and pressure (1 atm [101.29 kPa]). An Intersmat gas chromatograph equipped with Hewlett-Packard 3390 A integrator was employed for the determination of methane, carbon dioxide, and hydrogen (more than 1,000 ppm [1,000 μl/liter]). For a hydrogen concentration of less than 1,000 ppm, the GMI Exhaled Hydrogen Monitor was used. The percentage of H_2S in the biogas was estimated by passing the gas through a solution of zinc acetate and analyzing titrimetrically as described in the American Public Health Association *Standard Methods* (1).

Chemical analysis. Sulfate was analyzed by a modified turbidimetric method (6), and sulfide was analyzed by a titrimetric method (1). The COD was determined by the standard dichromate method (1). The MAIHAK TOC analyzer was used for the determination of total organic carbon. Since sulfide can interfere in the COD determination, the COD at higher sulfide levels was calculated from the total organic carbon value.

Sulfur balance. In anaerobic digestion, sulfate is reduced to sulfide which is distributed between H_2S in the gas phase, H_2S, HS^-, and S^{2-} in solution, and insoluble metallic sulfides. The equilibrium between H_2S in the gas phase and free H_2S in solution is governed by Henry's law (19):

$$[H_2S]_s = \alpha[H_2S]_g$$

TABLE 1. Composition of media

Composition	Medium A	Medium AE	Medium F
CH_3COONa (g)	6	3	
HCOONa (g)			21.3
KH_2PO_4 (g)	3	3	3
K_2HPO_4 (g)	1	1	1
$CaCl_2 \cdot 2H_2O$ (g)	0.3	0.3	0.3
NH_4Cl (g)	1	1	1
$FeCl_3 \cdot 6H_2O$ (g)	0.1	0.1	0.1
$MgCl_2 \cdot 6H_2O$ (g)	0.1	0.1	0.1
Sucrose (g)	0.1	0.1	0.1
Tryptic soy (g)	0.2	0.2	0.2
Yeast extract (g)	0.2	0.2	0.2
Trace elements solution (ml)[a]	1.0	1.0	1.0
Ethanol (ml)		1.5	
Tap water (liter)	1.0	1.0	1.0
COD concn (g/liter)	5.0	5.0	5.0

[a] Composition of trace elements solution (mg): $NiSO_4 \cdot 4H_2O$, 500; $MnCl_2 \cdot 4H_2O$, 500; $FeSO_4 \cdot 7H_2O$, 500; $ZnSO_4 \cdot 7H_2O$, 100; H_3BO_3, 100; $Na_2MoO_4 \cdot 2H_2O$, 50; $CoCl_2 \cdot 5H_2O$, 50; $CuSO_4 \cdot 5H_2O$, 5; tap water, 1 liter.

TABLE 2. Results of the control experiments[a]

Medium	Effluent pH	Influent COD (mg/liter)	Effluent COD (mg/liter)	% COD removed	Biogas produced (liters/liter of reactor per day)	Composition of biogas			Specific yield of methane (ml of CH_4/g of COD removed)
						% CH_4	% CO_2	% H_2	
A	7.67 ± 0.05	5,052 ± 18	884 ± 75	82.3 ± 1.3	2.88 ± 0.21	90.1 ± 1.1	9.9 ± 1.1	0.009 ± 0.003	311 ± 24
AE	7.35 ± 0.06	4,921 ± 49	366 ± 44	92.6 ± 0.9	3.86 ± 0.08	83.3 ± 2.2	16.7 ± 2.1	0.028 ± 0.012	353 ± 12
F	7.03 ± 0.06[b]	5,255 ± 59	386 ± 47	92.7 ± 0.8	5.20 ± 0.17	60.1 ± 1.3	39.5 ± 1.3	0.132 ± 0.002	321 ± 10

[a] All values relate to steady-state conditions (n = 4). The loading rate was 10 g of COD per liter per day.
[b] The pH of the mixed liquor was controlled at 7.0 by an automatic pH control apparatus.

where α (absorption coefficient) is 1.83 at 35°C (19). The H_2S in solution is a weak acid and dissociates as follows:

$$H_2S \overset{k_1}{\rightleftharpoons} H^+ + HS^-$$

$$HS^- \overset{k_2}{\rightleftharpoons} H^+ + S^{2-}$$

At the neutral pH required for anaerobic treatment, only the first dissociation of H_2S is of importance (19). The equilibrium equation for the dissociation is:

$$[H^+]\,[HS^-]/[H_2S]_s = K_1$$

$$K_1 = 1.49 \times 10^{-7} \text{ at } 35°C \text{ (19)}$$

The concentration of free H_2S in solution was calculated from the concentration of total dissolved sulfide ($H_2S + HS^- + S^{2-}$), using the above K_1 value and pH of the mixed liquor in the reactor by the following equation (14):

$$f = (1 + K_1/10^{-pH})^{-1}$$

in which f is the free H_2S fraction of the total dissolved sulfide.

All results are expressed as means of values obtained during steady states.

RESULTS

Effect of sulfate on methane production. The results of the control experiments (without sulfate) are summarized in Table 2. By applying an organic loading rate of 10 g of COD per liter of reactor per day, the rate of biogas production from medium A (acetate), medium AE (acetate plus ethanol), and medium F (formate) were, respectively, 2.88, 3.86, and 5.20 liters/liter of reactor per day. The specific yields of methane were 311, 353, and 322 ml of CH_4 per g of COD removed for media A, AE, and F, respectively. It was noticed that the anaerobic digestion of medium F resulted in a much higher percentage of CO_2 and H_2 in biogas compared with media A and AE.

Table 3 summarizes the effects of sulfate on biogas production for media A and AE. When the level of sulfate added to the influent was increased up to 5 g of sulfate S per liter (or a ratio up to 100 g of sulfate S/100 g of removable COD), biogas production from both media A and AE was not severely inhibited. At the most, only about 12% inhibition occurred. However, at a concentration of 10 g of sulfate S per liter (a ratio of 200 g of sulfate S/100 g of removable COD), the rate of biogas production was strongly inhibited. The percent inhibition was about 88% for medium A and 24% for medium AE. In both reactors, microbial reduction of sulfate did not proceed very well either as evidenced from the low percentage of sulfate reduced, i.e., ca. 3% for medium A and 10% for medium AE (Table 4). To verify whether the decrease in biogas production was a result of the high sulfate-to-COD ratio or of salt toxicity, the influent was diluted to half strength, and the same ratio of 200 g of sulfate S per 100 g of removable COD together with the same loading rate of 10 g of COD per liter of reactor per day were maintained. It was noticed that the rate of biogas production returned to normal. This indicates that the reduction in the rate of biogas production at a sulfate concentration of 10 g of sulfate S per liter was probably not due to the high sulfate level or to the high sulfate-to-COD ratio, but rather to salt toxicity as a result of the addition of Na_2SO_4. At this sulfate concentration, the Na^+ concentration was about 15 g/liter. De Baere et al. (7), with a similar type of reactor, have shown that methanogenesis is inhibited 50% owing to the presence of 35 g of NaCl (= 14 g of Na^+) per liter.

The percentage of H_2S in the biogas and the levels of sulfide in the mixed liquor for media A and AE are given in Fig. 2 and 3, respectively. It was noticed that the percentage

TABLE 3. Effects of sulfate on biogas production (n = 5)

SO_4^{2-} S added		Medium A			Medium AE		
g/liter	g/100 g of COD	Biogas produced (liters/liter of reactor per day)	% CH_4 in biogas	% Inhibition[a]	Biogas produced (liters/liter of reactor per day)	% CH_4 in biogas	% Inhibition[a]
0.1	2	2.73 ± 0.15	88.2 ± 3.2	5	3.44 ± 0.17	84.1 ± 2.7	11
0.2	4	2.53 ± 0.14	87.1 ± 0.8	12	3.48 ± 0.11	82.7 ± 1.0	10
0.3	6	2.62 ± 0.04	86.4 ± 0.9	9	3.53 ± 0.10	83.5 ± 1.7	9
0.4	8	2.54 ± 0.04	91.8 ± 2.4	12	3.64 ± 0.07	81.9 ± 1.3	6
0.5	10	2.68 ± 0.16	87.9 ± 1.4	7	3.49 ± 0.25	82.7 ± 0.6	10
5.0	100	3.34 ± 0.54	85.5 ± 0.3	−16	3.57 ± 0.17	78.4 ± 2.0	8
10.0	200	0.35 ± 0.12	85.2 ± 0.03	88	2.94 ± 0.12	79.3 ± 4.8	24
5.0	200	2.64 ± 0.07	84.2 ± 3.1	8	3.30 ± 0.14	83.9 ± 0.5	13

[a] Calculated from data of the control experiments given in Table 2.

TABLE 4. Sulfur balance in the anaerobic digestion of synthetic media supplemented with sulfate (n = 5)

Medium	SO_4^{2-} S added		S before digestion (mg/liter)			S after digestion (mg/liter)				% S recovered	% SO_4^{2-} S reduced
	g/liter	g/100 g of COD	SO_4^{2-} S	S^{2-} S	Total	SO_4^{2-} S	S^{2-} S	H_2S S	Total		
A	0.1	2	76 ± 6	16 ± 4	92 ± 6	29 ± 11	51 ± 11	1 ± 0.3	81 ± 17	88	62
	0.2	4	184 ± 9	14 ± 1	198 ± 10	126 ± 10	47 ± 9	1 ± 0.2	174 ± 14	88	32
	0.3	6	260 ± 2	32 ± 13	292 ± 20	161 ± 5	89 ± 31	1 ± 0.2	251 ± 29	86	38
	0.4	8	335 ± 15	26 ± 6	361 ± 10	207 ± 43	70 ± 9	1 ± 0.1	278 ± 46	77	38
	0.5	10	480 ± 19	32 ± 8	512 ± 26	341 ± 27	95 ± 18	2 ± 0.5	438 ± 2	86	27
	5.0	100	4,783 ± 37	26 ± 6	4,809 ± 30	4,633 ± 42	63 ± 1	4 ± 0.8	4,700 ± 40	98	3
	10.0	200	8,598 ± 288	18 ± 4	8,616 ± 292	8,465 ± 96	54 ± 13	3 ± 0.6	8,522 ± 970	99	2
AE	0.1	2	71 ± 4	24 ± 5	93 ± 8	0	79 ± 1	14 ± 0.9	93 ± 2	100	100
	0.2	4	148 ± 6	35 ± 4	183 ± 11	2 ± 1	110 ± 9	17 ± 0.6	129 ± 7	71	99
	0.3	6	211 ± 10	57 ± 3	268 ± 12	7 ± 5	175 ± 7	23 ± 2.4	205 ± 7	76	97
	0.4	8	298 ± 9	66 ± 7	364 ± 10	109 ± 6	207 ± 29	20 ± 1.2	336 ± 27	92	63
	0.5	10	410 ± 16	84 ± 6	494 ± 18	172 ± 37	252 ± 7	23 ± 1.8	447 ± 42	90	58
	5.0	100	4,538 ± 166	68 ± 6	4,606 ± 159	4,093 ± 170	183 ± 36	32 ± 12	4,308 ± 154	94	10
	10.0	200	9,304 ± 198	33 ± 9	9,337 ± 188	8,761 ± 356	99 ± 30	39 ± 7	8,899 ± 315	95	6

of H_2S in the biogas was much higher in the presence of ethanol as sulfate levels were increased. Figure 3 reveals that the addition of 0.5 g of sulfate S per liter or 10 g of sulfate S per 100 g of removable COD gave the highest concentration of sulfide in the mixed liquor for both media.

The sulfur balance given in Table 4 shows that the percentage of sulfur recovered varied from 77 to 99% for medium A and 71 to 100% for medium AE. The percentage of the recovered sulfur was slightly higher at higher sulfate levels. The results in Table 4 show also that for medium A, sulfate was never exhausted as a substrate. On the other hand, sulfate was almost completely removed from medium AE up to a level of 0.3 g of sulfate S per liter (or 6 g of sulfate S per 100 g of removable COD).

Figure 4 gives the concentration of hydrogen in the biogas for various sulfate levels. Medium AE was found to sustain a much higher concentration of hydrogen compared with medium A. By increasing the level of sulfate added, the concentration of hydrogen decreased significantly up to 0.5 g of sulfate S per liter and then leveled off.

The results in Table 5 indicate that the addition of hydrogen gas to medium A enhanced the sulfate reduction remarkably. It was also noticed that by increasing the COD-ethanol fraction of medium AE, the percentage of sulfate reduced increased sharply (Table 6). Nevertheless, the MPB still acquired the major part of the electron flow as evidenced by the high gas production rates and the specific yields of methane.

Use of sodium formate as a substrate (medium F) yielded a low percentage of sulfate reduction (Table 7). Initially it was thought that the rather strong change of pH during growth on formate affected the sulfate-reducing bacteria (SRB). To verify this, the experiment was repeated at pH 7.0 with an automatic pH control apparatus. It was found that the percentage of sulfate reduction increased, but only very slightly.

Effects of sulfide addition. To determine the effects of sulfide on methane production and sulfate reduction, different levels of sulfide were added to media A and AE, in the absence and presence of sulfate. The results were expressed in relation to the concentration of free H_2S in the influent, since this is considered to be the form of sulfide which is toxic to the microorganisms (14). Figure 5 clearly indicates that methane production from medium A was inhibited by

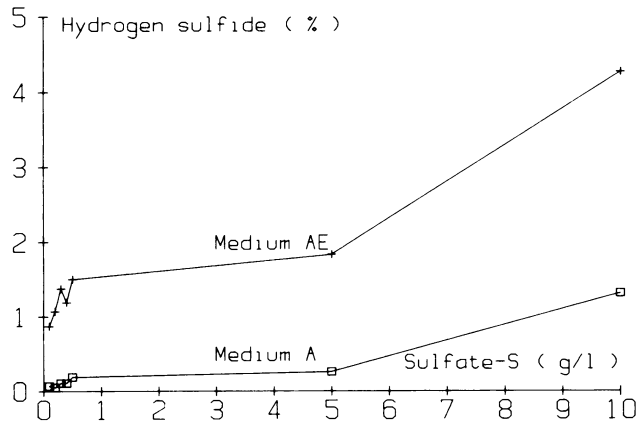

FIG. 2. Percentage of hydrogen sulfide in biogas from anaerobic digestion of synthetic media supplemented with different levels of sulfate.

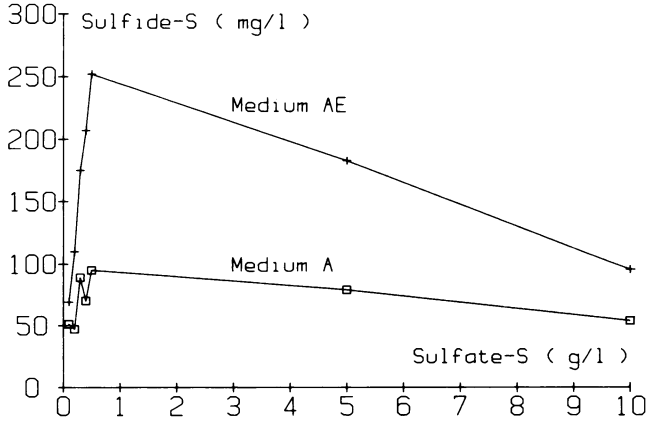

FIG. 3. Total sulfide concentrations in effluent from anaerobic digestion of synthetic media supplemented with different levels of sulfate.

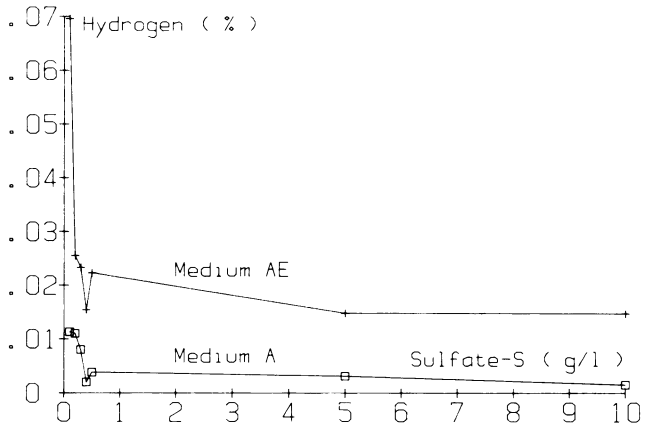

FIG. 4. Concentrations of hydrogen in biogas from anaerobic digestion of synthetic media supplemented with different levels of sulfate.

the free H_2S. The inhibitory effect increased with the increase in the level of free H_2S. Similar results were also obtained in the presence of sulfate (Fig. 6). The inhibitory effect was slightly more pronounced in medium A than in medium AE. These data also show that sulfate reduction decreased with the increase in the concentration of free H_2S in the influent. The decrease in the percentage of sulfate reduction was slightly less for medium A, possibly because acetate is not a good substrate for the SRB.

DISCUSSION

This study showed that sulfate levels up to 5,000 mg of sulfate S per liter (or 100 g of sulfate S per 100 g of removable COD) have no significant effect on methane production from synthetic media containing acetate or acetate together with ethanol digested in high-rate anaerobic reactors. At the most, only about 12% of the methane production was inhibited. Although it is generally reported in the literature that sulfate inhibits methane production, the present results are not the first to indicate the contrary. Zehnder et al. (32), for example, also found that both sulfate reduction and methanogenesis can occur simultaneously at relatively high sulfate concentration (320 mg of sulfate S per liter). Lettinga et al. (paper presented at the International Symposium on Advances in Anaerobic Digestion, Mexico, 1982) concluded that anaerobic digestion remains an effective treatment method for wastewaters containing sulfate concentrations as high as 1,700 mg of sulfate S per liter without any adverse effect on methane production. Szendrey (27) reported the highest sulfate level (6,000 mg of sulfate S per liter) so far which did not inhibit the methane production. His work concerned distillery slops digested in a downflow, fixed-bed reactor. The latter data correspond with the findings reported in this work.

With regard to sulfate reduction, the present results show that acetate alone is not a good substrate for the SRB as indicated by the lower percentage of H_2S in the biogas (Fig. 2) and the lower percentage of sulfate reduction (Table 4) compared with the medium containing acetate together with ethanol. In marine and brackish water sediments, however, acetate was reported to be the major electron donor for sulfate reduction (17, 28–30). *Desulfobacter postgatei* is one of the species of SRB which is highly specialized in utilizing only acetate as an organic substrate (28).

The high level of hydrogen in the biogas at low sulfate concentrations (Fig. 4) could be due to the following factors. At low sulfate levels, less hydrogen can be used by the SRB owing to the lack of an electron acceptor. In addition, the resulting low levels of sulfide might be inadequate for the MPB. Indeed, according to Ronnow and Gunnarsson (23), sulfide is consumed by MPB for two main purposes: primarily to produce sulfur compounds taking part in the energy production, and also to form sulfur-containing amino acids, proteins, etc., i.e., a general sulfur source required for growth.

Sulfate reduction was found to be strongly enhanced by the introduction of H_2 gas or hydrogen precursors such as ethanol. Similar observations have been reported by Smith and Klug (25), who found that H_2 stimulated the reduction of ^{32}sulfate S 2.5- to 2.8-fold. In their study on the effect of substrate on sulfate reduction (production of sulfide), Oremland and Polcin (21) reported that sulfide production increased by a factor of 2 to 4 when H_2 was added to acetate compared with when acetate alone was used as a substrate for the SRB. The results with sodium formate (medium F) in the present study indicate that the findings of Laanbroek et al. (16), who reported that certain species of SRB (*Desulfovibrio baculatus* H.L.21) from intertidal sediments are able to metabolize formate in the presence of sulfate, cannot be extrapolated directly to the digester systems. It is also interesting to note that formate, just as ethanol, gave rise to the formation of free H_2 gas in the range of 0.03 to 0.4% (Table 7). Yet formate did not stimulate the activity of the SRB. It therefore appears that in the case of ethanol, SRB possibly function as acetogens, using the electrons derived directly from ethanol to reduce sulfate.

The reducing equivalents (in terms of COD) potentially available as H_2 were calculated from the amount of H_2 added to medium A (Table 5) and also from the H_2 precursor (as transient H_2) in medium AE (Table 6), using the following equations:

TABLE 5. Effect of hydrogen gas on sulfate reduction and methane production from medium A (n = 4)

H_2 added (liters/day)	SO_4^{2-} S added		% SO_4^{2-} S reduced	Effluent S^{2-} S (mg/liter)	Effluent pH	Biogas produced (liters/liter of reactor per day)	Composition of biogas				% COD removed	Specific yield of methane (ml of CH_4/g of COD removed)
	g/liter	g/100 g of COD					% CH_4	% CO_2	% H_2S	% H_2		
0	0.25	5	38 ± 8	61 ± 7	7.8 ± 0.1	2.6 ± 0.1	87 ± 2	13 ± 2	0.08 ± 0.02	0.006 ± 0.001	96 ± 1	233 ± 13
3	0.25	5	79 ± 2	104 ± 12	8.0 ± 0.1	4.9 ± 0.2	51 ± 2	11 ± 5	7.10 ± 0.40	31 ± 4	96 ± 1	256 ± 4
0	0.50	10	27 ± 9	95 ± 18	7.8 ± 0.1	2.7 ± 0.2	88 ± 1	12 ± 2	0.18 ± 0.04	0.004 ± 0.001	93 ± 3	241 ± 8
3	0.50	10	74 ± 1	143 ± 10	7.8 ± 0.1	4.1 ± 0.2	57 ± 9	9 ± 4	7.20 ± 1.20	27 ± 7	98 ± 1	234 ± 51

TABLE 6. Effect of a hydrogen precursor (ethanol) on sulfate reduction and methane production (n = 4)

COD composition		SO_4^{2-} S added		% SO_4^{2-} S reduced[a]	Biogas produced (liters/liter of reactor per day)	Composition of biogas				Specific yield of methane (ml of CH_4/g of COD removed)
% Acetate	% Ethanol	g/liter	g/100 g of COD			% CH_4	% CO_2	% H_2S	% H_2	
100	0	0.5	10	31 ± 8	2.7 ± 0.4	88 ± 1	12 ± 1	0.20 ± 0.01	0.0040 ± 0.0014	245 ± 19
50	50	0.5	10	58 ± 13	3.2 ± 0.4	82 ± 1	16 ± 0.5	1.51 ± 0.16	0.0223 ± 0.0045	297 ± 9
25	75	0.5	10	86 ± 6	3.5 ± 0.1	82 ± 1	16 ± 1	2.20 ± 0.23	0.0184 ± 0.0031	331 ± 33
0	100	0.5	10	99 ± 2	3.8 ± 0.2	81 ± 1	16 ± 1	2.76 ± 0.07	0.0150 ± 0.0028	338 ± 14

[a] In all cases the amount of sulfate added = 0.5 g of sulfate S per liter (or 10 g of sulfate S per 100 g of removable COD).

TABLE 7. Anaerobic digestion of medium F (formate) supplemented with different levels of sulfate (n = 4)

Expt[a]	SO_4^{2-} S added		% SO_4^{2-} S reduced	Effluent S^{2-} S (mg/liter)	Effluent pH	Biogas produced (liters/liter of reactor per day)	Composition of biogas				% COD removed	Specific yield of methane (ml of CH_4/g of COD removed)
	g/liter	g/100 g of COD					% CH_4	% CO_2	% H_2S	% H_2		
1	0	0	0	0	8.9 ± 0.1	3.3 ± 0.2	78 ± 6	22 ± 6	0	0.40 ± 0.05	88 ± 1	310 ± 19
	0.5	10	13 ± 6	33 ± 19	8.9 ± 0.1	3.7 ± 0.1	68 ± 9	32 ± 9	0.13 ± 0.06	0.04 ± 0.02	89 ± 3	284 ± 43
	1.0	20	32 ± 3	52 ± 14	8.9 ± 0.1	3.6 ± 0.3	71 ± 3	29 ± 3	0.09 ± 0.03	0.07 ± 0.01	92 ± 1	273 ± 24
	2.0	40	24 ± 5	124 ± 22	8.8 ± 0.1	3.3 ± 0.0	69 ± 1	31 ± 1	0.29 ± 0.06	0.09 ± 0.01	92 ± 0	229 ± 3
2	0	0	0	0	7.03 ± 0.06	5.2 ± 0.2	60 ± 1	40 ± 1	0	0.13 ± 0.002	93 ± 1	321 ± 10
	0.5	10	47 ± 7	66 ± 8	7.00 ± 0.11	5.1 ± 0.4	63 ± 7	36 ± 7	0.43 ± 0.06	0.06 ± 0.003	92 ± 0.4	329 ± 11
	1.0	20	32 ± 6	68 ± 8	7.00 ± 0.00	4.9 ± 0.2	66 ± 5	34 ± 4	0.36 ± 0.07	0.05 ± 0.006	93 ± 1	325 ± 4
	2.0	40	31 ± 0.4	74 ± 1	7.00 ± 0.00	4.6 ± 0.4	69 ± 0.1	31 ± 0.1	0.39 ± 0.03	0.03 ± 0.004	94 ± 0.3	311 ± 30

[a] Experiment 1, pH of the mixed liquor was not controlled; experiment 2, pH of the mixed liquor was controlled at 7.0.

As free H_2: $2H_2 + O_2 \rightarrow 2H_2O$

1 mol of $H_2 \equiv \frac{1}{2}$ mol of COD $\equiv$ 16 g of COD

As transient H_2: $C_2H_5OH + H_2O \rightarrow CH_3COO^- + H^+ + 2H_2$

1 mol of $C_2H_5OH \equiv$ 2 mol of $H_2 \equiv$ 1 mol of COD $\equiv$ 32 g of COD

In the presence of H_2, sulfate reduction is given by the following equation:

$$SO_4^{2-} + 4H_2 + H^+ \rightarrow HS^- + 4H_2O$$

1 mol of HS^- produced $\equiv$ 4 mol of H_2 consumed

FIG. 5. Effect of free H_2S on methane production from anaerobic digestion of medium A.

The reducing equivalents potentially available as H_2 were calculated and expressed as the percentage of the total reducing equivalents present in the system (Table 8). In addition, the amounts of H_2 used for sulfate reduction were also calculated assuming that all sulfate reduced was due to H_2. Only values relating to the experiments in which sulfate was not limiting were included. The results in Table 8 illustrate the true potential of SRB, i.e., scavenging H_2. The interesting aspect is that under the given reactor conditions, the SRB, even for their preferred substrate H_2, apparently endure stiff competition from the hydrogenotrophic MPB.

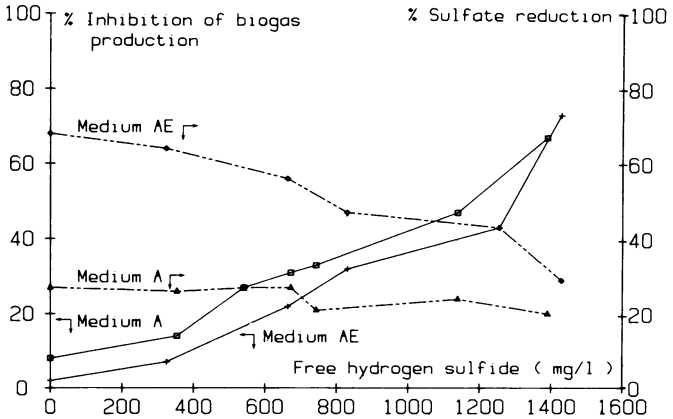

FIG. 6. Effect of free H_2S on methane production and sulfate reduction of synthetic media supplemented with 0.5 g of SO_4^{2-} S per liter. Symbols: □, ▲, medium A; +, ◆, medium AE; full line, percent inhibition of biogas production; dotted line, percent sulfate reduction.

TABLE 8. H_2 flux used by SRB for the reduction of sulfate (n = 4)

SO_4^{2-} S added		H_2 available (mM)[a]	% of total reducing equivalents available as H_2	Sulfide formed (mM)	H_2 equivalent of sulfide formed (mM)	% of the available H_2 used for sulfate reduction[b]
g/liter	g/100 g of COD					
0.5[c]	10	0	0	3.5 ± 0.9	13.8 ± 3.9	0
5.0[c]	100	0	0	4.7 ± 0.2	18.8 ± 0.9	0
10.0[c]	200	0	0	4.7 ± 0.7	18.7 ± 2.9	0
0.5[d]	10	93.8	23	12.3 ± 0.6	49.2 ± 2.5	38–52
0.5[e]	10	52.0	16	8.3 ± 0.7	33.4 ± 2.8	38–64
5.0[e]	100	52.0	16	12.2 ± 3.0	48.6 ± 12.0	57–93
10.0[e]	200	52.0	16	5.0 ± 0.3	19.8 ± 1.4	2–38
0.5[e]	10	78.0	25	13.5 ± 2.6	54.0 ± 10.6	52–69

[a] Calculated from the amount of H_2 added to the medium in the form of H_2 gas or ethanol.
[b] The lower values are calculated on the basis that the same amount of sulfate is reduced by acetate-utilizing SRB as in footnote *a*; the higher values on the basis that all sulfate reduction is due to H_2 or transient H_2.
[c] Acetate as the only substrate.
[d] Acetate plus H_2 as substrate.
[e] Acetate plus ethanol as substrate.

From the results in Table 8, it appears that probably on the average only 50% of the available H_2 is captured by the SRB.

For the acetate-ethanol medium (medium AE), the ratio between both substrates was chosen in such a way that about 16 to 25% of the total reducing equivalents were available as H_2 (Table 8). In digesters treating complex wastes such as sewage sludge, about 30% of the substrate flow proceeds via the H_2 intermediates (12). Hence, the data observed for medium AE relate to practical situations in which normally the major part of the methanogenesis is determined by acetate metabolism.

In contrast to sulfate, the sulfide ions and more particularly the free H_2S in the liquid phase were found to influence strongly the acetoclastic MPB (Fig. 5). Kroiss and Wabnegg (14) reported a 50% inhibition at about 50 mg of free H_2S per liter. Our results in Fig. 5 and 6 indicate that the acetoclastic MPB and also the hydrogenotrophic MPB were only significantly inhibited at much higher free H_2S concentrations (more than 1,000 mg of free H_2S per liter). Adaptation of the MPB to free H_2S, particularly in reactors with fixed biomass as in our case, could be the reason why the inhibition only occurred at very high levels of free H_2S. Butlin et al. (2) had also observed an adaptation of MPB to relatively high levels of H_2S (ca. 100 mg of free H_2S per liter). Our results in Fig. 6 indicate that the SRB themselves were also affected by the increase in the level of free H_2S in the reactors. However, it still has to be proven whether the inhibition of MPB and SRB by free H_2S is due to the toxicity of free H_2S to these organisms or merely due to the unavailability of the essential metallic elements for the MPB and SRB owing to their precipitation as insoluble sulfides.

From the practical point of view, the results obtained in the present study show that the anaerobic treatment of sulfate-rich wastewaters by a high-rate anaerobic reactor poses no problem in relation to the inhibitory effects of sulfate on methanogenesis. Under any of the conditions tested, sulfate reduction was found to be able to outcompete or inhibit the methanogenesis. The reactor employed was of the fixed-film type. The retention of cells on the polyurethane matrix is of major importance and is studied in more detail elsewhere (11). Only at very high sulfate concentrations (more than 10 g of sulfate S per liter) was the digestion affected, not so much by sulfate or sulfide produced but by the salt concentration. The presence of H_2S in the biogas resulting from the microbial reduction of sulfate, however, can be technically problematic with regard to the use of the biogas. If the wastewater is subjected to two-phase anaerobic digestion, the first phase should be directed to produce a maximum amount of acetate and a minimum amount of hydrogen precursors. As shown in the Results, acetate alone gave very low levels of H_2S in the biogas as a result of a very low sulfate reduction. The presence of hydrogen precursors such as ethanol, however, yielded a much higher percentage of H_2S in the biogas. Segers et al. (24) have shown that by controlling the pH of the acid fermentation system, different product patterns can be obtained. Ethanol, for example, was a dominant product at pH 4.0 to 4.5, while acetate was dominant between pH 6.0 and 7.0. Thus, for sulfate-rich wastewaters, further work to direct the fermentation to a maximum formation of acetate in the acidification phase of the two-phase anaerobic digestion is therefore warranted.

ACKNOWLEDGMENTS

This work was made possible through financial support from the Algemeen Bestuur voor Ontwikkelingssamenwerking (ABOS) of Belgium.

We also wish to thank C. Graveel for her diligent typing of the manuscript.

LITERATURE CITED

1. **American Public Health Association.** 1971. Standard methods for the examination of water and wastewater, 13th ed. American Public Health Association, New York.
2. **Butlin, K. R., S. R. Selwyn, and D. S. Wakerley.** 1956. Sulfide production from sulfate-enriched sewage sludges. J. Appl. Bacteriol. **19**:3–15.
3. **Callander, I. J., and J. P. Barford.** 1983. Precipitation, chelation and the availability of metals as nutrients in anaerobic digestion. II. Application. Biotechnol. Bioeng. **XXV**:1959–1972.
4. **Capestany, G. J., J. McDaniels, and G. L. Opgrande.** 1977. The influence of sulfate on biological treatment of phenolbenzaldehyde waste. J. Water Pollut. Control Fed. **49**:256–261.
5. **Cappenberg, T. E.** 1974. Interrelation between sulfate-reducing bacteria and methane-producing bacteria in bottom deposits of a freshwater lake. I. Field observation. Antonie van Leeuwenhoek J. Microbiol. Serol. **40**:285–295.
6. **Cottenie, A., M. Verloo, L. Kiekens, G. Velghe, and R. Camerlync.** 1982. Chemical analysis of plants and soils. Laboratory of Analytical and Agrochemistry, State University of Ghent, Ghent, Belgium.

7. **De Baere, L. A., M. De Vocht, P. Van Assche, and W. Verstraete.** 1984. Influence of high NaCl and NH_4Cl salt levels in methanogenic associations. Water Res. **18**:543–548.
8. **De Vocht, M., P. Van Meenen, P. Van Assche, and W. Verstraete.** 1983. Influence of sedimentation and adhesion on selection of methanogenic associations. Process Biochem. **18**:31–34.
9. **Eggers, E., J. Ebberhorst, and F. Van Hernen.** 1981. Verwijdering van zwavelwaterstof uit gistingsgas. H_2O **8**:173–182.
10. **Huysman, P., P. Van Meenen, P. Van Assche, and W. Verstraete.** 1983. Factors affecting the colonisation of non-porous and porous packing materials in model upflow methane reactors. Biotechnol. Lett. **5**:643–648.
11. **Isa, Z., S. Grusenmeyer, and W. Verstraete.** 1986. Sulfate reduction relative to methane production in high-rate anaerobic digestion: microbiological aspects. Appl. Environ. Microbiol. **51**:580–587.
12. **Kaspar, H. F., and K. Wuhrmann.** 1978. Kinetic parameters and relative turnovers of some important catabolic reactions in digesting sludge. Appl. Environ. Microbiol. **36**:1–7.
13. **Khan, A. W., and T. M. Trottier.** 1978. Effect of sulfur-containing compounds on degradation of cellulose to methane by a mixed culture obtained from sewage sludge. Appl. Environ. Microbiol. **35**:1027–1034.
14. **Kroiss, H., and F. P. Wabnegg.** 1983. Sulfide toxicity with anaerobic wastewater treatment, p. 72–85. *In* W. J. van der Brink (ed.), Proceedings of the European Symposium on Anaerobic Wastewater Treatment (AWWT). AWWT Symposium Secretariat, TNO Corporate Communication Department, The Hague, Netherlands.
15. **Kugelman, I. J., and K. K. Chin.** 1971. Toxicity, synergism and antagonism in anaerobic treatment processes. Adv. Chem. Ser. **105**:55–90.
16. **Laanbroek, H. J., H. J. Geerlings, A. A. C. M. Peijnenburg, and J. Siesling.** 1984. Competition for L-lactate between *Desulfovibrio*, *Veillonella*, and *Acetobacterium* species isolated from anaerobic intertidal sediments. Microb. Ecol. **9**:341–354.
17. **Laanbroek, H. J., and N. Pfennig.** 1981. Oxidation of short chain fatty acids by sulfate reducing bacteria in freshwater and in marine sediments. Arch. Microbiol. **128**:330–335.
18. **Lawrence, A. W., and P. McCarty.** 1965. The role of sulfide in preventing heavy metals toxicity in anaerobic treatment. J. Water Pollut. Control Fed. **37**:392–409.
19. **Lawrence, A. W., P. L. McCarty and F. J. A. Guerin.** 1966. The effects of sulfide on anaerobic treatment. Air Water Pollut. Int. J. **10**:207–221.
20. **Lovley, D. R., and M. J. Klug.** 1983. Sulfate reducers can outcompete methanogens at freshwater sulfate concentrations. Appl. Environ. Microbiol. **45**:187–192.
21. **Oremland, R., and S. Polcin.** 1982. Methanogenesis and sulfate reduction: competitive and non-competitive substrates in estuarine sediments. Appl. Environ. Microbiol. **44**:1270–1276.
22. **Oremland, R. S., and B. F. Taylor.** 1978. Sulfate reduction and methanogenesis in marine sediments. Geochim. Cosmochim. Acta **42**:209–214.
23. **Ronnow, P. H., and A. H. Gunnarsson.** 1981. Sulfide-dependent methane production and growth of a thermophilic methanogenic bacterium. Appl. Environ. Microbiol. **42**:580–584.
24. **Segers, L., L. Verstrynge, and W. Verstraete.** 1981. Product patterns of non-axenic sucrose fermentation as a function of pH. Biotechnol. Lett. **3**:635–640.
25. **Smith, R. L., and M. J. Klug.** 1981. Electron donors utilized by sulfate-reducing bacteria in eutrophic lake sediments. Appl. Environ. Microbiol. **42**:116–121.
26. **Speece, R. E., and G. F. Parkin.** 1983. The response of methane bacteria to toxicity, p. 23–35. *In* R. L. Wentworth, D. A. Stafford, B. I. Wheatley, W. E. Edelmann, G. Lettinga, Y. Minoda, P. Mulas del Pozo, E. J. Nyns, F. G. Pohland, J. F. Rees, L. van den Berg, W. Verstraete, and R. F. Ward (ed.), Proceedings of the 3rd International Symposium on Anaerobic Digestion. Evans and Faulkner, Inc., Watertown, Mass.
27. **Szendrey, L. M.** 1983. Start-up and operation of the Bacardi cooperation anaerobic filter, p. 365–377. *In* R. L. Wentworth, D. A. Stafford, B. I. Wheatley, W. E. Edelmann, G. Lettinga, Y. Minoda, P. Mulas del Pozo, E. J. Nyns, F. G. Pohland, J. F. Rees, L. van den Berg, W. Verstraete, and R. F. Ward (ed.), Proceedings of the 3rd International Symposium on Anaerobic Digestion. Evans and Faulkner, Inc., Watertown, Mass.
28. **Thauer, R. K.** 1982. Dissimilatory sulfate reduction with acetate as electron donor. Philos. Trans. R. Soc. London B Biol. Sci. **298**:467–471.
29. **Widdel, F., and N. Pfennig.** 1977. A new anaerobic sporing, acetate oxidizing, sulfate reducing bacterium, *Desulfotomaculum acetoxidans*. Arch. Microbiol. **112**:119–122.
30. **Widdel, F., and N. Pfennig.** 1981. Studies on dissimilatory sulfate reducing bacteria that decompose fatty acids. I. Isolation of new sulfate-reducing bacteria enriched with acetate from saline environments. Description of *Desulfobacter postgatei* gen. nov., sp. nov. Arch. Microbiol. **129**:395–400.
31. **Winfrey, M. R., and J. G. Zeikus.** 1977. Effect of sulfate on carbon and electron flow during microbial methanogenesis in freshwater sediments. Appl. Environ. Microbiol. **33**:275–281.
32. **Zehnder, A. J. B., K. Ingvorsen, and T. Marti.** 1982. Microbiology of methane bacteria, p. 45–68. *In* D. E. Hughes, D. A. Stafford, B. I. Wheatley, W. Baarder, G. Lettinga, E. J. Nyns, W. Verstraete, and R. L. Wentworth (ed.), Anaerobic digestion—1981. Elsevier/North-Holland Biomedical Press, Amsterdam.