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Inhibition of Methanogenesis from Acetate in Granular Sludge by Long-Chain Fatty Acids

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The effect of four saturated long-chain fatty acids (caprylic, capric, lauric, and myristic) and one unsaturated long-chain fatty acid (oleic) on the microbial formation of methane from acetate was investigated in batch anaerobic toxicity assays. The tests were carried out with granular sludge from an upflow anaerobic sludge bed reactor. In this sludge, *Methanothrix* spp. are the predominant acetoclastic methanogens. Lauric acid appeared to be the most versatile inhibitor: inhibition started at 1.6 mM, and at 4.3 mM the maximum specific acetoclastic methanogenic activity had been reduced to 50%. Caprylic acid appeared to be only slightly inhibitory. Oleic acid was almost as inhibitory as lauric acid. Although adsorption of the inhibitor on the cell wall might play an important role in the mechanism of inhibition, the inhibition was found to be correlated with concentration rather than with the amount per unit of biomass. In practical situations, as in anaerobic waste treatment processes, synergism can be expected to enhance the inhibition of methanogenesis. In the present research a background concentration of lauric acid below its MIC strongly enhanced the toxicity of capric acid and (to an even greater extent) myristic acid.

Anaerobic digestion is widely applied for the treatment of wastes and wastewaters. The increasing popularity of the process is mainly due to the fact that it couples the removal of organic compounds with the production of energy in the form of methane. In the anaerobic digestion of fatty-matter-containing waste and wastewater, by far the largest fraction of the organic compounds to be degraded is made up of the long-chain fatty acids that are esterified with glycerol to form neutral fats. Examples of waste streams containing considerable amounts of fatty matter are piggery wastes (16), sewage sludges (34), slaughterhouse wastewater (31), edible oil refinery wastewater (30), palm oil processing effluents (26), and wool scouring wastes (10).

Research by Hanaki et al. (14) showed that the anaerobic hydrolysis of neutral fats (viz., the breaking of the ester bonds) to glycerol and fatty acids proceeds easily but that the anaerobic digestion of the long-chain fatty acids is often hampered by an interruption of the balance between the metabolic activities that the anaerobic digestion process comprises, because these long-chain fatty acids are potential inhibitors of many of the bacteria involved in anaerobic digestion. In this respect it is essential to gain information concerning the effect of long-chain fatty acids on the methane-producing bacteria because they play an essential role in the mixed microbial population required for the effective degradation of complex organic material. If methanogenesis is inhibited, organic acids which are intermediary metabolites will accumulate in the digester environment, possibly resulting in a fatal pH drop and leading to a so-called "sour" digester in which the methanogenic bacteria cannot survive.

Long-chain fatty acids have been reported to be inhibitory at low concentrations for gram-positive microorganisms; gram-negative microorganisms are not affected by long-chain fatty acids (11, 22, 23, 27). Since methanogenic bacteria have a cell wall that resembles the cell wall of gram-positive bacteria (39), they can be expected to be susceptible to inhibition by long-chain fatty acids as well. In fact, long-chain fatty acids are not only widely applied as food preservatives (21); sometimes they are also added as supplements to the diets of ruminants to suppress the methanogenesis taking place in the rumen (2). This veterinary use of long-chain fatty acids has generated research concerning the inhibition by long-chain fatty acids of rumen populations, including methanogens. Most of this research was performed with poorly defined mixed rumen cultures or involved in vivo experiments in which the animals and not their rumen bacteria were monitored. Only a few in vitro experiments directly concerning methanogenesis in the rumen have been published (2, 4–6, 33). Related to this rumen-oriented research, some work has been published concerning pure-culture studies with methanogens (29). In the rumen methane is formed exclusively from hydrogen plus carbon dioxide (37), whereas in anaerobic digesters treating complex wastes, approximately 70% of the methane is produced via acetate (12). Inhibition of the acetoclastic methanogens by long-chain fatty acids might also affect the degradation of the long-chain fatty acids themselves, since it has been proved that in anaerobic digestion they are degraded via the acetate-yielding mechanism of β-oxidation (20, 35). To date, only two articles specifically concerning the effect of long-chain fatty acids on the production of methane from acetate have been published in the readily available international literature (13, 14). The present article is the first in which research concerning the effect of individual as well as mixed long-chain fatty acids on acetoclastic methanogenic populations is described.

MATERIALS AND METHODS

Reactors. The experiments were performed with perspex batch reactors with a working volume of 2.5 or 5 liters. The reactors were placed in a temperature-controlled room at 30 ± 1°C. The reactor contents were completely mixed every 30 min by means of stirring for 30 s at approximately 150 rpm.

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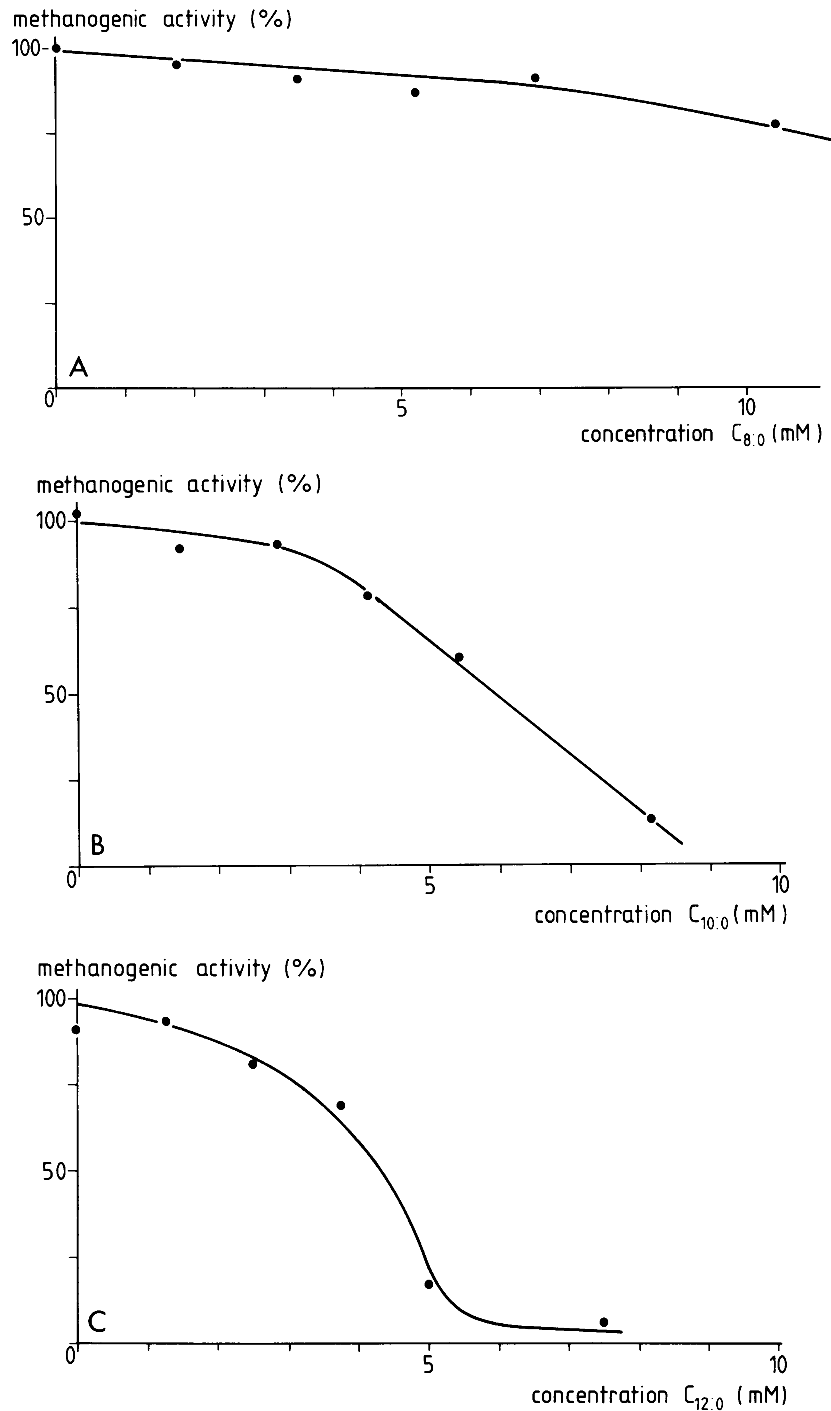

FIG. 1. Remaining methanogenic activity of acetate-fed granular sludge after exposure to caprylic acid (A), capric acid (B), lauric acid (C), myristic acid (D), and oleic acid (E).

Intermittent stirring was applied to avoid excessive erosion of the sludge granules. Methane production was determined by using a sodium hydroxide solution displacement system (36). The strength of the sodium hydroxide solution was such that all carbon dioxide was removed from the biogas.

Biomass. The methanogenic sludge used in the present experiments was obtained from the upflow anaerobic sludge bed reactor used to treat the wastewater of the potato processing factory of Aviko at Steenderen, The Netherlands. From the sludge obtained from the upflow anaerobic sludge bed reactor, the clay particles and the fine suspended sludge were removed by means of elutriation as described by Tramper et al. (32). The remaining sludge consisted of compact granules. A stock of granules was stored at 4°C. The predominant acetoclastic methanogens in the sludge granules were of the genus *Methanothrix*, of which at

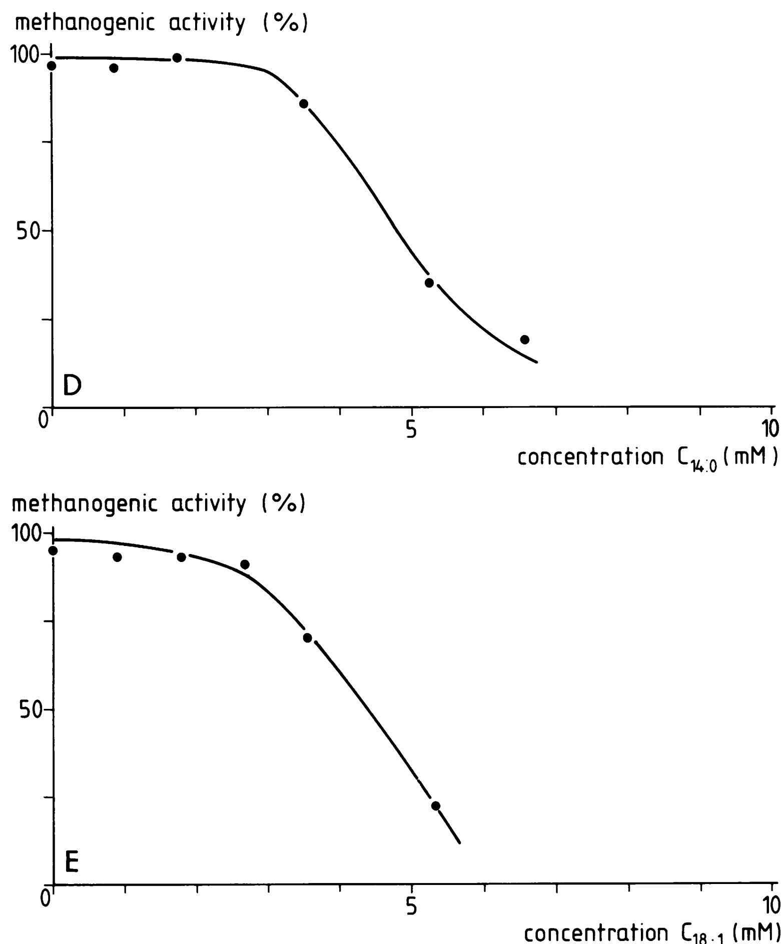

FIG. 1—*Continued*

present two species have been isolated and characterized (19, 28).

Basal medium. The basal medium used in all experiments contained (in milligrams per liter) NH_4Cl (174), $Na_2HPO_4 \cdot 2H_2O$ (37), and Na_2SO_4 (7). The medium was made up in Wageningen tap water, which contains approximately 35 mg of calcium ions per liter. One milliliter of a trace element solution was added per liter of medium as described by Zehnder et al. (38).

Chemicals. All chemicals were of analytical grade and were supplied by E. Merck AG, Darmstadt, Federal Republic of Germany, except for myristic acid, which was of technical quality and was supplied by BDH, London, England.

TABLE 1. Concentrations at which inhibition started and concentrations required for 50% inhibition in toxicity assays with acetoclastic methanogens exposed to single long-chain fatty acids at 30°C

Fatty acid	MIC (mM)	MIC_{50}[a] (mM)
$C_{8:0}$	6.75	>10
$C_{10:0}$	2.6	5.9
$C_{12:0}$	1.6	4.3
$C_{14:0}$	2.6	4.8
$C_{18:1}$	2.4	4.35

[a] MIC_{50}, MIC at which 50% of the methanogenic activity remained.

Long-chain fatty acids. The long-chain fatty acids used in the toxicity assays were as follows (common name or systematic name followed by abbreviation): caprylic or octanoic, $C_{8:0}$; capric or decanoic, $C_{10:0}$; lauric or dodecanoic, $C_{12:0}$; myristic or tetradecanoic, $C_{14:0}$; and oleic or *cis*-9-octadecanoic, $C_{18:1}$. In the abbreviation $C_{x:y}$, x and y indicate carbon chain length and number of double bonds, respectively.

Analysis. The gas chromatographic method used to determine the concentration of volatile fatty acids up to valeric acid has been described elsewhere (24). All other analyses were performed by standard methods (1).

Toxicity assay. For each long-chain fatty acid or combination of long-chain fatty acids, toxicity was assayed in a test run with six simultaneously operated 2.5-liter batch reactors, each testing a different concentration of the toxicant.

A test run started with the addition of a known amount of granular sludge (approximately 15 g of volatile solids) to reactors containing the basal medium and 3 g of acetate per liter. At this substrate concentration the rate of methane production is maximum and is not limited by substrate diffusional resistance in the granules. The pH was set at 7 ± 0.1 with sodium hydroxide.

On day 2 the acetate concentration was restored. The amount of acetate to be supplied was calculated from methane production: 2.41 g of acetate for each liter of methane that had been produced. If necessary, the pH was also

TABLE 2. MICs or toxicity threshold levels of long-chain fatty acids for various bacteria

Fatty acid	MIC (mM) for:					
	Methanothrix sp.[a]	*Bacillus megaterium*[b]	*Pneumococcus* sp.[c]	*Streptococcus* group A[c]	*Streptococcus* beta-hemolytic, non-group A[c]	*Staphylococcus aureus*[c]
$C_{8:0}$	6.75	2.0	>6.9	>6.9	>6.9	>6.9
$C_{10:0}$	2.6	1.0	1.45	1.45	2.9	2.9
$C_{12:0}$	1.6	0.15	0.062	0.124	0.249	2.49
$C_{14:0}$	2.6	0.15	0.218	0.547	2.18	4.37
$C_{18:1}$	2.4	0.05	>3.5	1.77	>3.5	>3.5

[a] Data are from this study.
[b] Data are from reference 9.
[c] Data are from reference 21.

restored by the addition of sodium hydroxide or hydrochloric acid.

On day 3 the acetate concentration and the pH were restored again. On this day methane production was measured at least every 30 min to determine the maximum specific acetoclastic methanogenic activity, which is the maximum rate of methane production from acetate per unit of volatile solids in the biomass. After the activity measurements had been completed (at the end of day 3), the long-chain fatty acid to be tested was added from an alkaline stock solution to five of the six reactors. From the known strength of the long-chain fatty acid solution the concentration in each reactor could be calculated. The pH in each reactor, which was increased by the addition of the alkaline long-chain fatty acid solution, was restored immediately by the addition of hydrochloric acid.

On day 4 the acetate concentration and the pH were restored again. Earlier work concerning anaerobic digestion of long-chain fatty acids (G. Heijnen, M.S. thesis, Wageningen Agricultural University, Wageningen, The Netherlands, 1982) has shown that granular sludge that is fed for the first time with long-chain fatty acids can degrade them only after a lag period of 1 or more days, so that methane production from the toxicants to be tested can be ruled out for the present experiments. After restoration of the acetate concentration and the pH the maximum specific acetoclastic methanogenic activity was determined as described for day 3.

The amount of inhibition of the conversion of acetate into methane was defined as the loss of maximum specific acetoclastic methanogenic activity in a reactor (at day 4), expressed as a percentage of the uninhibited maximum specific acetoclastic methanogenic activity in the same reactor (at day 3). In each test run one reactor was not supplied with a long-chain fatty acid to rule out the possibility of inhibition by causes other than the long-chain fatty acid addition.

Degradation test. The effect of lauric acid on its own anaerobic degradation was tested in 5-liter batch reactors. A known amount of granular sludge was put in a reactor after the basal medium and trace element solution that were already present had been made oxygen free by means of flushing with nitrogen gas. A known amount of lauric acid was added from a stock solution, immediately followed by 2.7 g of bicarbonate per g of lauric acid added, to provide some buffer capacity. During the experiment the reactor pH was kept at 7 ± 0.1 by the addition of sodium hydroxide or hydrochloric acid. After a lag period the methane production rate in each reactor became constant. From this methane production rate and the biomass content the specific methanogenic activity (liters of methane produced per gram of volatile solids in the biomass per hour) could be obtained. At least once a day a sample from the reactor contents was analyzed for volatile fatty acids.

RESULTS AND DISCUSSION

The maximum specific acetoclastic methanogenic activity that remained after the addition of the long-chain fatty acid to be tested can be expressed as a percentage of the uninhibited maximum specific acetoclastic methanogenic activity (Fig. 1A to E). From the 54 experiments performed in the present research project a mean uninhibited maximum specific acetoclastic methanogenic activity equivalent to a calculated specific acetate consumption rate of 35.21 mg/g of volatile solids per h (standard deviation, 6.68) was obtained. It is clear that there was an MIC or toxicity threshold level below which the maximum specific acetoclastic methanogenic activity was not affected by the presence of the long-chain fatty acid. At concentrations exceeding the toxicity threshold level the remaining maximum specific acetoclastic methanogenic activity decreased with increasing concentrations of long-chain fatty acid. The curves indicate that the susceptibility of the acetoclastic methanogens in the granular sludge varied with the type of long-chain fatty acid, a result which is also illustrated by the different toxicity threshold levels and concentrations needed for a reduction of the maximum specific acetoclastic methanogenic activity by 50% (Table 1). From these results it can be concluded that lauric acid was by far the most versatile inhibitor of the four types of saturated long-chain fatty acids that were tested. This conclusion is in accordance with results obtained with mixed rumen bacteria (2, 7, 9), pure cultures of *Lactobacillus* spp. (15, 23), and a variety of pure cultures of gram-positive bacteria as well as yeasts (22).

The MICs established in our experiments can be compared with data from toxicity tests with several gram-positive bacteria (Table 2). It appears that for each long-chain fatty acid the toxicity threshold level is variable among organisms. Given this fact, it may be concluded that our

TABLE 3. Rate of methane production from lauric acid at various concentrations and biomass loads in batch-fed reactors at 30°C

Lauric acid concn (mM)	Biomass load (mg/g of volatile solids)	Specific methanogenic activity (μl of CH_4/g of volatile solids per h)
2.50	95	388
3.75	153	397
5	31	261
10	111	24

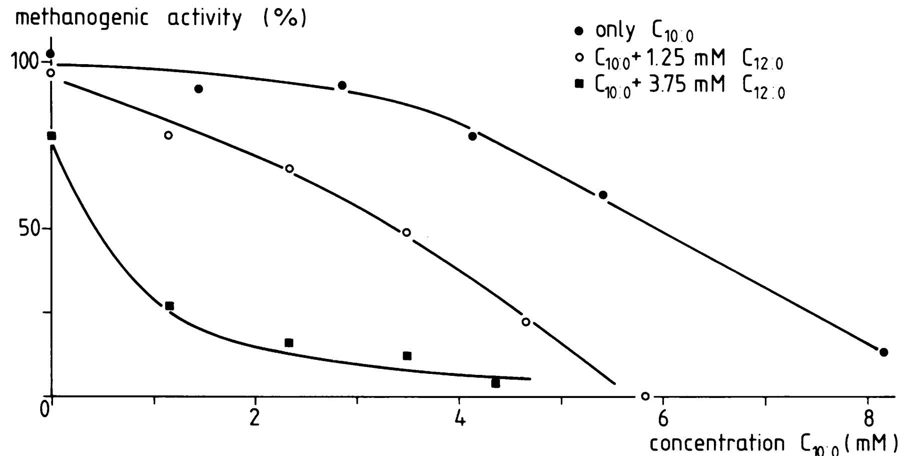

FIG. 2. Remaining methanogenic activity of acetate-fed granular sludge after exposure to capric acid with different background concentrations of lauric acid.

results were within the range of values reported in the literature. The toxicity threshold level for oleic acid that we established in our experiments is approximately 130 times higher than the toxicity threshold level for acetoclastic methanogenesis in a batch reactor reported by Hanaki et al. (13). Most probably this result is due to the fact that they totally excluded calcium and magnesium ions from their media to avoid precipitation of insoluble salts. We did not follow such a procedure to avoid any shortage of minerals which might influence the rate of methanogenesis. Moreover, all other reported research has been carried out in media containing at least some chemicals that form precipitates with long-chain fatty acids. For toxicity assays with pure cultures of hydrogenotrophic methanogens Prins et al. (29) reported 50% inhibition at 1.8 mM linolenic acid ($C_{18:3}$) and 3.2 mM linoleic acid ($C_{18:2}$). They did not test oleic acid. Since in general the toxicity of unsaturated long-chain fatty acids increases with the number of double bonds (6, 9, 23), the oleic acid concentration of 4.4 mM resulting in 50% inhibition in our experiments can be considered to be in accordance with the results of Prins et al. (29).

The mechanism of inhibition of bacterial metabolism caused by long-chain fatty acids is not completely revealed yet. Adsorption of long-chain fatty acids on cells might play an important role (8, 18). In that case the biomass load (viz., the amount of long-chain fatty acid added per unit of biomass) rather than the initial long-chain fatty acid concentration would be a realistic parameter to be used to decide whether inhibition can be expected from a certain dose of long-chain fatty acids. However, the results of our activity measurements concerning the anaerobic degradation of lauric acid (Table 3) indicated that the inhibitory action of lauric acid was related to the concentration rather than to the biomass load. In these experiments a buildup of volatile fatty acids indicated that at concentrations exceeding 3.74 mM methanogenesis was more inhibited than fermentation (viz., the actual lauric acid degradation).

Natural fats are always composed of a variety of long-chain fatty acids (17); therefore, the inhibitory effects of mixtures of long-chain fatty acids were also studied (Fig. 2 and 3). A background concentration of lauric acid of 1.25 mM, which itself is below the toxicity threshold level (Table

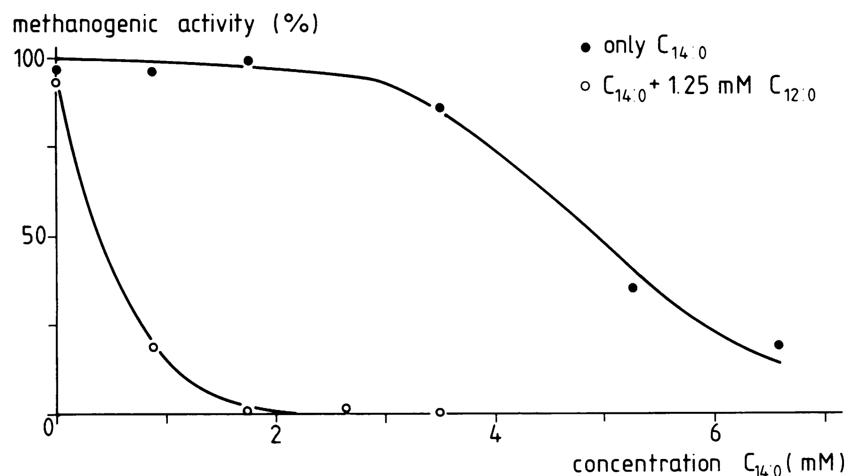

FIG. 3. Remaining methanogenic activity of acetate-fed granular sludge after exposure to myristic acid with or without a background concentration of 1.25 mM lauric acid.

1), appeared to enhance the inhibitory effect of capric acid (Fig. 2). This synergistic action of lauric acid was even more profound in the combination with myristic acid (Fig. 3). A background concentration of 1.25 mM lauric acid enhanced the toxicity of myristic acid to a greater extent than a background concentration of 2.5 mM lauric acid enhanced the toxicity of capric acid. The phenomenon of synergism in toxicity in anaerobic digestion has been extensively reviewed for the case of light-metal cation toxicity (25). For those toxicants the maximum synergistic action was also apparent at concentrations at which the synergist itself was not yet inhibitory. With respect to long-chain fatty acid toxicity it should be noted that antagonistic actions of various unsaturated fatty acids with at least 18 carbon atoms have been reported in the toxicity of saturated long-chain fatty acids with chains of 12 to 20 carbon atoms (3).

From our experiments it can be concluded that long-chain fatty acids are potential inhibitors of the microbial formation of methane from acetate. Degradation tests with lauric acid indicated that the inhibition was correlated with the concentration rather than with the biomass load. The concentrations at which the individual long-chain fatty acids appeared to become inhibitory for acetoclastic methanogenesis were in the same range as the concentrations reported for the inhibition of other gram-positive bacteria. In practice the methanogenic populations present in wastewater treatment facilities will mostly not be faced with a single long-chain fatty acid but with mixtures of long-chain fatty acids. In those cases extra care should be taken in the operation of the process, since the toxicity of a mixture of long-chain fatty acids can be enhanced significantly by synergism of the individual long-chain fatty acids.

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