TOXICITY IN ANAEROBIC DIGESTION

with emphasis on the effect of ammonia, sulfide and long-chain fatty acids on methanogenesis

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BIBLIOGRAPHIC ABSTRACT

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The dissertation concerns the problem of toxicity in anaerobic digestion, which to a large extent is the problem of inhibition of methanogenic conversions by chemical compounds. The dissertation begins with an extensive literature review in which the microbiology of anaerobic digestion and the chemical and physical requirements of the bacteria involved are discussed. A literature review concerning the general aspects of toxicity in anaerobic digestion is also included. The general aspects that are discussed include: synergism and antagonism, concentration dependency, influence of environmental factors (e.g. temperature and pH) on the effective concentration of toxicants, acclimatization of methanogenic populations to the presence of toxicants in the wastewater to be treated. The impact of modern reactor design on the ability of anaerobic wastewater treatment systems to deal with toxicant containing wastewater is also discussed, as is the fact that biofilms increase the toxicant tolerance of methanogenic populations.

Many of the general aspects of toxicity in anaerobic digestion that are discussed in the literature review are illustrated in the chapters concerning research projects. In the chapter concerning long-chain fatty acids toxicity the phenomena of synergism and lowering the effective toxicant concentration by means of formation of insoluble molecules are shown. The influence of pH on toxicity is shown in the chapter on sulfide toxicity and the chapter on ammonia inhibited growth rate. The latter chapter also deals with the influence of temperature. The chapter on ammonia inhibition of methanogenic activity illustrates the concentration dependency and the acclimatization potential of methanogenic biomass. The extreme tolerance of methanogenic populations for toxicants is illustrated in the chapter on amminia inhibition of methanogenic inhibition and in the chapter on manure digestion.

keywords: anaerobic digestion; anaerobic waste water treatment; methane; biogas; toxicity; inhibition; long-chain fatty acids; sulfide; ammonia; acclimatization; adaptation; synergism; antagonism.

INTRODUCTIONARY ACCOUNT

Historical background

My interest in anaerobic digestion arose in 1980, when as a student I was member of the "Projektgroep Veenkoloniaal Afvalwater", a group of seven students that studied the socio-economic and environmental problems caused by the potato-starch industry in the north-eastern part of The Netherlands.[1] Most problems were caused by the enormous pollution potential: without treatment the water pollution caused by the potato-starch industry would amount to 12 million population equivalents, which is one third of the total wastewater production in The Netherlands. The "Projektgroep Veenkoloniaal Afvalwater" proposed some technical solutions for the water pollution of the potato-starch industry. Anaerobic digestion was the heart of these proposed solutions.

Since the wastewater of the potato-starch industry contains a lot of protein, ammonia inhibition could be an argument against the use of anaerobic wastewater treatment processes. Therefore, the first research I ever did concerning the subject of the present thesis (which is toxicity in anaerobic digestion) was within the framework of the "Projektgroep Veenkoloniaal Afvalwater" and was aimed at finding out to what extent ammonia toxicity could limit the use of anaerobic wastewater treatment at the potato-starch industry. Because the wastewater of the potato-starch industry is rather cold, I also did a lot of research concerning anaerobic digestion at psychrophilic conditions.[2]

The practical work concerning ammonia toxicity in anaerobic digestion was used to write the three articles compiled in chapter 4 of this thesis. The data processing and actual writing of the articles was done as part of my work during my occupation as a temporarily employed additional researcher at the anaerobic digestion group of Lettinga at the Department of Water Pollution Control of the Wageningen Agricultural University. Chapter 5 of this thesis also stems from my period as additional researcher. The scientific interest I had in toxicity problems in anaerobic digestion was further stimulated by the work I could do for a research project concerning the anaerobic treatment of wastewater from the edible oil industry.[3] The toxicity problems encountered with anaerobic treatment of these wastewaters concern sulfide and long-chain fatty acids. My research resulted in the articles compiled in the chapters 2 and 3.

The work described in chapter 6 was done as part of a feasibility study concerning anaerobic digestion of highly concentrated ('thick') animal waste slurries.

A lot of data processing, literature search and writing of articles has been done as part of my work as member of the permanent staff of the Department of Water Pollution Control of the Wageningen Agricultural University, with *Valorization and treatment of organic wastes* as my principal field of work. Anaerobic digestion is one of the many methods to make value of organic wastes, but because of the low water content in organic wastes toxicity problems are abundant.

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[1] Projektgroep Veenkoloniaal Afvalwater (april 1981) Geen kater van schoon water, uitgave Vakgroep Waterzuivering, verkrijgbaar in het Centraal Magazijn van de Landbouwuniversiteit onder bestelnummer 06451107

[2] Koster, I.W. & G. Lettinga (1985) Application of the upflow anaerobic sludge bed (UASB) process for treatment of complex wastewaters at low temperatures, Biotechnol. Bioeng., 27: 1411-1417

[3] Rinzema, A. (1988) Anaerobic treatment of wastewater with high concentrations of lipids or sulfate, Ph. D. thesis, Wageningen Agricultural University

Scope and organization of this thesis

Except for chapter 1 the chapters of this thesis are the result of laboratory research concerning some details of toxicity in anaerobic digestion. The choice of toxicants to be studied was limited to ammonia, sulfide and long-chain fatty acids. This choice was to a great extent influenced by fund-raising opportunities. One chapter in this thesis concerns toxicity in anaerobic manure digestion; the other chapters concern research of a more general nature. Except in the manure digestion study, in all studies methanogenic populations in granular form and dominated with *Methanothrix sp.* were used. The reason for the use of granular sludge is that UASB reactors (which generally contain granular sludge) are by far the most widely applied reactors in anaerobic wastewater treatment systems.

This thesis contains as chapter 1 a comprehensive literature study concerning the microbial, chemical and technological aspects of toxicity in anaerobic digeston.
This chapter serves as an introduction to anaerobic digestion and the problems that can be met when the waste or wastewater to be digested contains potential toxicants that may disrupt the digestion process by inhibiting the formation of methane.

The toxicity of long-chain fatty acids is dealt with in chapter 2. The results in this chapter illustrate the phenomenon of synergism. Non-inhibitory concentrations of lauric acid enormously enhanced the toxicity of capric acid. Chapter 2 also provides an illustration of the fact that the formation of insoluble salts can lower the concentration of toxicants to non-inhibitory levels.

Chapter 3 concerns the toxicity of sulfide. It is an illustration of the pH influence on toxicity. It also illustrates the potential of biofilms to diminish the effect of toxicants, in this case by providing a biofilm pH which differs from the pH in the bulk environment.

In chapter 4 a study concerning ammonia toxicity over the concentration range from non-inhibitory up to extremely high levels is presented. The potential of methanogenic populations to adapt to the presence of toxicants is dealt with in detail, and special attention is paid to a quantitative comparison of the specific activity of methanogenic biomass before and after adaptation.

Chapter 5 contains one of the few reports on the effect of toxicants (in this case: ammonia) on the growth rate of methanogenic biomass. Also this chapter illustrates the effect of temperature and pH on ammonia inhibition.

Chapter 6, in which research concerning anaerobic manure digestion at extreme ammonia concentrations is presented, is included in this thesis to give an example of a practical situation in which anaerobic digestion is desirable, but hampered by the presence of high concentrations of a toxicant.

Acknowledgements

Most of the practical work concerning the research described in the chapters 2, 3, 5 and 6 has been done by students and analysts, who are mentioned either in the list of authors or in the acknowledgements of the respective articles. I thank all of them for their co-operation.

CONTENTS OF THE THESIS

CHAPTER 1

MICROBIAL, CHEMICAL AND TECHNOLOGICAL ASPECTS OF TOXICITY IN ANAEROBIC DIGESTION

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MICROBIAL AND BIOCHEMICAL ASPECTS OF ANAEROBIC DIGESTION

Introduction

In anaerobic digestion organic matter is transformed into methane via a series of successive bacteriological processes. Bacterial methane production is common in nature and it even occurs in extreme ecosystems such as glacier ice (Berner et al., 1975), acid tundra peat (Svensson, 1983), thermophilic (55-80 °C) agricultural waste digesters (Varel, 1983), thermophilic waste water treatment systems (Wiegant, 1986), sedimentary rocks from oil fields (Belyaev & Ivanov, 1983), hypersaline sediment of the Dead Sea (Dosoretz & Marchaim, 1988) and possibly also in submarine hydrothermal vents at pressures of about 250 bar and temperatures exceeding 300 °C (Baross et al., 1982). The subject of this thesis is methanogenesis at mesophilic temperatures in waste or waste water treating digesters, stressed only by the occurrence of potential toxicants such as ammonia, sulphide or long-chain fatty acids [1]).

Anaerobic digestion is a general term for the process of biological degradation of organic material with the exclusion of oxygen which can take place in solid waste treatment, manure digestion, sludge digestion, waste water treatment, etc.

The first time that anaerobic digestion was reported to be a useful method for waste treatment was in 1881 when the French engineer Louis Mouras reported about an anaerobic waste water treatment system more or less similar to the septic tank which is still in use today (McCarty, 1982). Since that time the applications of anaerobic digestion have grown steadily, as has the knowledge of the microbiology, chemistry and technology involved. In recent years, interest in anaerobic digestion has grown considerably, because anaerobic digestion applied to waste and waste water treatment requires much less energy than the conventional aerobic treatment methods. Moreover, the methane product of anaerobic digestion can serve as a useful fuel. A critical review of the microbial, chemical and technological aspects of the anaerobic digestion of organic material is presented elsewhere (Koster, 1988). In this chapter a comprehensive introduction of the conversion processes in anaerobic digestion will be presented, followed by a general introduction to the phenomenon of toxicity in anaerobic digestion. Since methanogens are by far the most sensitive of the bacteria involved in anaerobic digestion, emphasis will be on inhibition of methanogenesis.

Methanogenic bacteria are part of a unique genealogical group (Woese, 1981; Balch et al., 1979). Apart from the eukaryotes (cells which have well-formed nuclei, e.g., fungi) and the prokaryotes (cells which do not have nuclei, the common bacteria) there is the primary group of the Archaebacteria, which includes three very different kinds of bacteria: extreme halophiles, thermoacidophiles, and methanogens. Archaebacteria differ from prokaryotes at the molecular level, especially in ribosomal RNA sequences. Methanogenic bacteria also differ from other bacteria in the composition of the cell wall (Kandler & König, 1978). The genealogical differences between methanogens and common bacteria makes that several compounds which are severe toxicants for common bacteria do not affect methanogens. Cell-wall active antibiotics may serve as example. Because muramic acid is not present in the cell walls of methanogenic bacteria, they are resistant to cell-wall active antibiotics such as penicillin, vancomycin, D-cycloserine and cephalosporin (Sahm, 1983). On the other hand, the specificity of methanogens also makes that there are inhibitory compounds which are very specific for methanogens, even at high concentrations (Sparling & Daniels, 1987).

Comparing natural ecosystems with digesters

Much information concerning methane-producing bacterial populations can be derived from natural anaerobic ecosystems such as sediments or the rumen. The rumen especially

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[1] It should be noted that in this text the term "digesters" is used to describe any reactor in which anaerobic digestion occurs, including the manure- or sewage-sludge treating reactors for which the term is usually reserved.

has been the subject of extensive microbial research (Hobson & Wallace, 1982[a]; Hobson & Wallace, 1982[b]). From the rumen a novel high-rate process for the digestion of solid cellulosic waste has been derived (Gijzen, 1987). It should be realized, however, that these natural ecosystems in many cases are not in all aspects comparable with anaerobic waste water treatment systems or sludge (or manure) digesters. Marine sediments are characterized by a sulfate pool which cannot be exhausted (Zehnder, 1978). Under conditions of sulfate sufficiency, sulfate-reducing bacteria will win the competition for hydrogen and acetate from the methanogenic bacteria (Winfrey & Zeikus, 1977; Laanbroek & Veldkamp, 1982).

The main difference between the rumen and a manure digester or an anaerobic waste water treatment system is on the basis of the turnover time (Hobson, 1973). The rumen turnover time is about 1 day, whereas a manure digester turnover time is always longer, generally about 30 days. This implies that many bacteria whose growth rates are such that they are washed out of the rumen are able to occupy a habitat in a manure digester which in fact is fed with material that has passed the rumen.

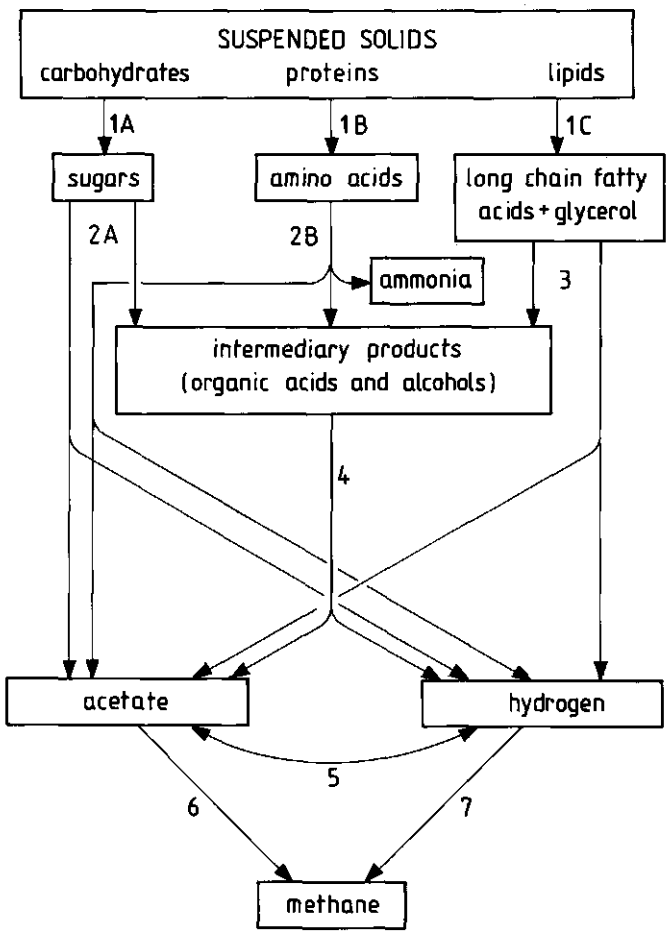

Fig. 1 *Substrate flows in anaerobic digestion of complex organic material.*
1: hydrolysis, 2: fermentation, 3: oxidation of long-chain fatty acids, 4: intermediary acetogenesis, 5: nonmethanogenic conversion of methanogenic substrates, 6/7: methanogenesis

Substrate flows in anaerobic digestion

The effective conversion of complex organic material into methane depends on the combined activity of a miscellaneous microbial population consisting of various genera of obligate and facultative anaerobic bacteria. The activities of the mixed population present in an anaerobic digester can be summarized in seven distinct processes (Figure 1):

1. Hydrolysis or liquefaction of suspended solids
2. Fermentation of amino acids and sugars
3. Anaerobic oxidation of long-chain fatty acids
4. Anaerobic oxidation of intermediary products, mainly volatile fatty acids such as propionic acid, butyric acid, etc.
5. Nonmethanogenic conversions of acetate and hydrogen
6. Acetoclastic or acetotrophic methanogenesis
7. Hydrogenotropic methanogenesis

These processes can be arranged into four distinct metabolic stages (McInerney et al., 1980):

1. In hydrolysis or liquefaction, complex, nonsoluble organic compounds are solubilized by enzymes excreted by hydrolytic bacteria. Since these enzymes work outside the bacteria, they are called exoenzymes. In fact, hydrolysis is the conversion of polymers into monomers.
2. In acidogenesis, soluble organic compounds, including the products of the hydrolysis, are converted into organic acids such as acetic acid, propionic acid and butyric acid.
3. In acetogenesis or intermediary acidogenesis, the products of the acidogenesis are converted into acetic acid, hydrogen, and carbon dioxide.
4. In methanogenesis, methane is produced from acetic acid or from hydrogen plus carbon dioxide. Methane can also be formed directly from some other substrates, from which formic acid and methanol are the most important.

A diagram of the four metabolic stages that can be distinguished in anaerobic digestion is shown in Figure 2. In well-balanced anaerobic digestion, all products of a previous metabolic stage are converted into the next one, so that the overall result is a nearly complete conversion of the biodegradable organic material in the influent into end products such as methane, carbon dioxide, hydrogen sulfide, ammonia, etc. without significant build-up of intermediairy products.

In the next paragraphs the four metabolic stages of anaerobic digestion will be discussed in more detail. However, the discussion is limited to the aspects that influence inhibition or can themselves be influenced by inhibition of certain processes in anaerobic digestion. A much more detailed description can be found elsewhere (Koster, 1988).

Hydrolysis of Suspended Solids

Hydrolysis of complex organic compounds is a slow process, which depends on the action of extracellular enzymes such as cellulases, amylases, proteases and lipases. The rate of hydrolysis is to a great extent influenced by pH and detention time (Ghosh & Klass, 1978; Verstraete et al., 1981; Gujer & Zehnder, 1983).

The optimum pH for hydrolysis is different for various substrates. For easily degradable carbohydrates, hydrolysis and acidogenesis proceed at maximum rates in the pH range 5.5 to 6.5 (Zoetemeyer et al., 1982; Zoetemeyer, 1982). The optimum pH for hydrolysis of proteins is at pH 7 or even higher (Breure & van Andel, 1984; Breure, & van Andel, 1983). The optimum pH for hydrolysis of lipids has never been assessed. In an acid-phase sludge digestion operated at pH 5.15, lipids are not degraded at all (Eastman & Ferguson, 1981). O'Rourke (1968) found that in a conventional (one-phase) sludge digestion operated in the pH range 6.7 to 7.4 lipid hydrolysis is not the rate-limiting step in the anaerobic digestion of lipids. The fact that he recovered most of the lipid COD as fatty acids indicates that the anaerobic oxidation of these fatty acids was the rate limiting step. The

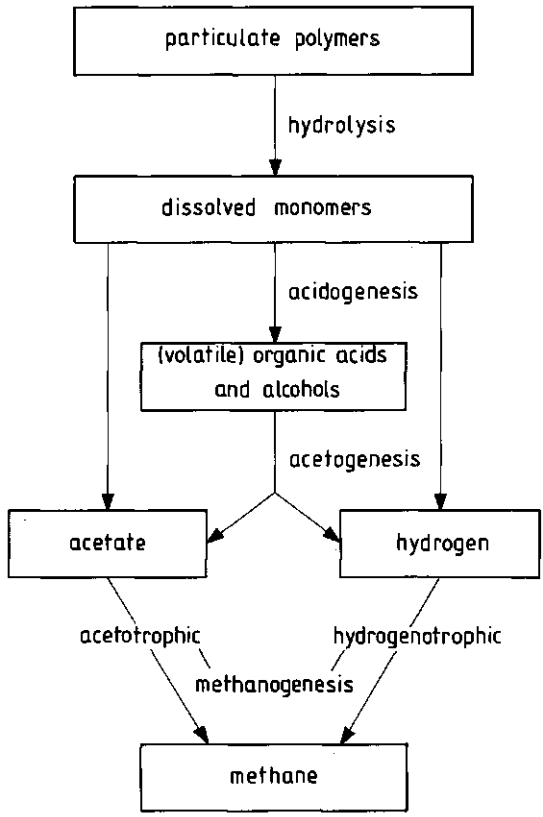

Fig. 2 *Metabolic stages and products in anaerobic digestion of complex organic material*

optimum pH for the hydrolysis of solid cannery vegetable wastes has been reported to be pH 6.5 (Roy et al., 1983). For hydrolysis of a mixture of garbage and sewage sludge the pH optimum is at pH 5.8 (Verstraete et al., 1981).

In sludge digestion at 35 °C hydrolysis becomes the rate-limiting step at hydraulic retention times exceeding 15 days (Pfeffer, 1968). In a separate acid-producing reactor fed with sewage sludges the hydrolysis is always the rate-limiting step (Eastman & Ferguson, 1981; Ghosh, 1981). Hydrolysis also is the rate-limiting step in the anaerobic degradation of proteins (Nagase & Matsuo, 1982). Lipids are hydrolyzed very slowly, with the result that hydrolysis might be the rate-limiting step in the anaerobic degradation of wastes containing considerable amounts of lipids (Henze & Harremoës, 1983). Below 20 °C, the anaerobic degradation of lipids in primary sewage sludge digestion is nil, even though methanogenesis continues at a reduced rate (O'Rourke, 1968). The slow rate of hydrolysis appeared to be the limiting factor to the application of one-stage anaerobic treatment of slaughterhouse waste water, especially at low temperatures (approximately 20 °C) (Sayed, 1982; Sayed et al., 1984). That particular (swine) slaughterhouse waste water contained 40 to 50% suspended solids, 5% of the total solids as grease.

Acidogenesis

Fermentation of amino acids and sugars

Fermentations can be defined as those biological processes that occur in the dark and that do not involve respiratory chains with oxygen or nitrate as electron acceptors. The absence of respiratory chains causes a low ATP yield for the fermentative reactions. Con-

sequently, the amount of biomass attained per mole of substrate is much smaller than with aerobes and, in addition to cell material, large amounts of fermentation end products are formed (Gottschalk, 1979).

Most bacteria present in anaerobic digesters are obligate anaerobes. However, a small fraction of the fermentative population is also able to use oxygen. In general, approximately 1% of the nonmethanogenic population in a digester consists of facultative anaerobic bacteria (Toerien & Hattingh, 1969).

The products of the fermentative population vary depending on environmental conditions applied. In an acid producing reactor of a two-phase digester the product pattern from the fermentative bacteria is influenced by the pH. At pH values below pH 6, the main product of the fermentation of glucose is butyric acid, but with increasing pH the product pattern changes, first to lactic acid and subsequently to acetic acid, formic acid, and ethanol (Zoetemeyer et al., 1982). Hydrolysis and subsequent fermentation of gelatin in an acid-producing reactor of a two-phase digester at pH values above pH 6 result in the following products: acetic acid, propionic acid, valeric acid, and minor amounts of other volatile fatty acids. However, at pH values below pH 6, the relative amount of acetic acid decreases and the relative amount of propionic acid increases (Breure & van Andel, 1984).

Removal of hydrogen by hydrogenotrophic bacteria (e.g., methanogenesis, sulfate reduction, or denitrification) can significantly influence the kinds of products formed by fermentative bacteria (Wolin & Miller, 1982). When hydrogen is continuously consumed by hydrogenotrophic bacteria, the fermentative bacteria are able to produce further-oxidized products than they would be capable of at increased hydrogen levels, which supplies more energy per unit of substrate to the bacteria (Wolin, 1976; Wolin, 1979; Mah, 1983).

In fermentation, the oxidation of substrate is coupled with the reduction of metabolic intermediates (Wolfe, 1983). Some fermentative bacteria are able to form H_2 from reduced pyridine nucleotides, but the accumulation of H_2 inhibits this reaction. Other major mechanisms for formation of H_2 include those from formate and pyruvate. These routes are not susceptible to inhibition by H_2. The importance of the H_2-inhibited and -uninhibited routes in the fermentation of glucose can be illustrated by the fermentation pathway of *Ruminococcus albus* (Wolin, 1979). In pure culture, this bacterium ferments glucose to ethanol, acetic acid, hydrogen, and carbon dioxide. However, in coculture with a hydrogenotrophic bacterium, it ferments glucose to acetic acid, hydrogen, and carbon dioxide; ethanol is not a product. The effect of instant removal of hydrogen by methanogenic bacteria on the products formed by other bacteria was first demonstrated during the studies on the nonmethanogenic bacterium isolated from *M. omelianskii*. In pure culture, this so-called S-organism ferments pyruvate to ethanol, acetic acid, carbon dioxide, and only a trace of hydrogen. In coculture with a hydrogenotrophic methanogen, acetic acid, carbon dioxide, and methane (but not ethanol) are formed. When cocultured with a methanogenic bacterium that keeps the hydrogen partial pressure low, the S-organism uses the electrons generated during pyruvate catabolism for H_2 production rather than for the production of ethanol (Reddy et al., 1972). Inhibition of methane formation from H_2 will cause an elevation of the hydrogen partial pressure. This will trigger a chance in the fermentation product pattern.

An important end product of the fermentation of amino acids is ammonium. Ammonium is the source of nitrogen for methanogenic bacteria (Bryant et al., 1971; Kenealy et al., 1982). On the other hand, at concentrations exceeding about 700 mg nitrogen per liter, ammonium inhibits methanogenesis (Koster & Lettinga, 1983; Koster & Lettinga, 1984).

In anaerobic digestion, alkalinity is mainly provided by ammonium and bicarbonate ions. A reduction in the ammonium content of a digester leads to a decrease in alkalinity and perhaps pH, thus illustrating that, apart from being essential as a nutrient, ammonium is also essential for the buffering capacity (Kotzé et al., 1969). The contribution of ammonium ions to the buffering capacity in an anaerobic digester will only be of considerable importance if the ammonium concentration is of magnitude of a few grams N per liter, such as in some industrial waste waters, manure, and concentrated sewage sludge.

Anaerobic oxidation of long-chain fatty acids

Experiments with ^{14}C-labeled, long-chain fatty acids revealed that the anaerobic degradation of long-chain fatty acids occurs by ß-oxidation (Jeris & McCarty, 1965; Weng & Jeris, 1976). Unsaturated fatty acids are hydrogenated before being degraded by ß-oxidation. The overall reaction for ß oxidation of even-carbon-numbered fatty acids, including the formation of hydrogen (which is the main sink for electrons) is as follows:

$$CH_3(CH_2)_nCOO^- + nH_2O \rightarrow (\tfrac{1}{2}n + 1)CH_3COO^- + \tfrac{1}{2}nH^+ + nH_2$$

In this equation n is an even number. Anaerobic ß-oxidation of odd-carbon-numbered fatty acids results in the same products plus propionate. Anaerobic ß-oxidation of long-chain fatty acids is thermodynamically unfavourable unless the hydrogen partial pressure is maintained at a very low level (Hanaki et al., 1981). For this reason, pure cultures of long-chain fatty acids degrading bacteria cannot exist. The dependence of a low hydrogen partial pressure makes that long-chain fatty acids degradation can be inhibited indirectly via inhibition of hydrogen consuming organisms, such as methanogens.

Table 1: Some acetogenic reactions

$CH_3CHOHCOO^- + 2\ H_2O \rightarrow CH_3COO^- + HCO_3^- + H^+ + 2\ H_2$	$\Delta G_0' =$	-4.2 kJ/mol
$CH_3CH_2OH + H_2O \rightarrow CHCOO^- + H^+ + 2\ H_2$	$\Delta G_0' =$	+9.6 kJ/mol
$CH_3CH_2CH_2COO^- + 2\ H_2O \rightarrow 2\ CH_3COO^- + H^+ + 2\ H_2$	$\Delta G_0' =$	+48.1 kJ/mol
$CH_3CH_2COO^- + 3\ H_2O \rightarrow CH_3COO^- + HCO_3^- + H^+ + 3\ H_2$	$\Delta G_0' =$	+76.1 kJ/mol

Values of free energy changes from Thauer et al. (1977)

Acetogenesis

The products of the acidogenic bacteria are converted into substrates for the methanogenic bacteria by the acetogenic bacteria, which produce acetic acid, hydrogen and carbon dioxide. Some acetogenic reactions are shown in Table 1, together with the associated values for the standard free-energy change ($\Delta G_0'$) (Thauer et al., 1977). The standard conditions for the calculation of free-energy changes in biochemical reactions are the following: temperature of 25°C, pH 7.0, pressure of 1 atm, activity of all solutes is 1 mol/kg, and water is a pure liquid. At these standard conditions the acetogenic conversion of ethanol, butyrate and propionate is not possible, because these reactions do not yield energy (i.e., the free-energy change is positive). The actual free-energy change for the reaction aA + bB → cC + dD may be calculated from the standard free-energy change by means of the following equation (Chang, 1977):

$$\Delta G' = \Delta G_0' + R.T.\ \ln \frac{[A]^a.[B]^b}{[C]^c.[D]^d} \qquad (1)$$

in which R is the molar gas constant (8.31 $J.mol^{-1}.T^{-1}$) and T is the absolute temperature. A very low hydrogen partial pressure is necessary to make the acetogenic conversion of ethanol, butyrate and propionate possible. This is illustrated in Figure 3.

The high substrate affinity of the hydrogen-consuming microorganisms in methanogenic ecosystems makes it possible to maintain sufficiently low hydrogen concentrations. The Michaelis-Menten half-saturation constant (K_m) for hydrogen has been reported to be 5.8 μM for rumen fluid, 6.0 μM for the contents of a sewage sludge digester, and 7.1 μM for sediment taken from the pelagic zone of a hypereutrophic lake (Robinson & Tiedje, 1982). These values are equivalent with a hydrogen partial pressure of 6.89×10^{-3}, 7.09×10^{-3}, and 7.82×10^{-3} atm, respectively (Schumpe et al., 1982; Wilhelm et al., 1977).

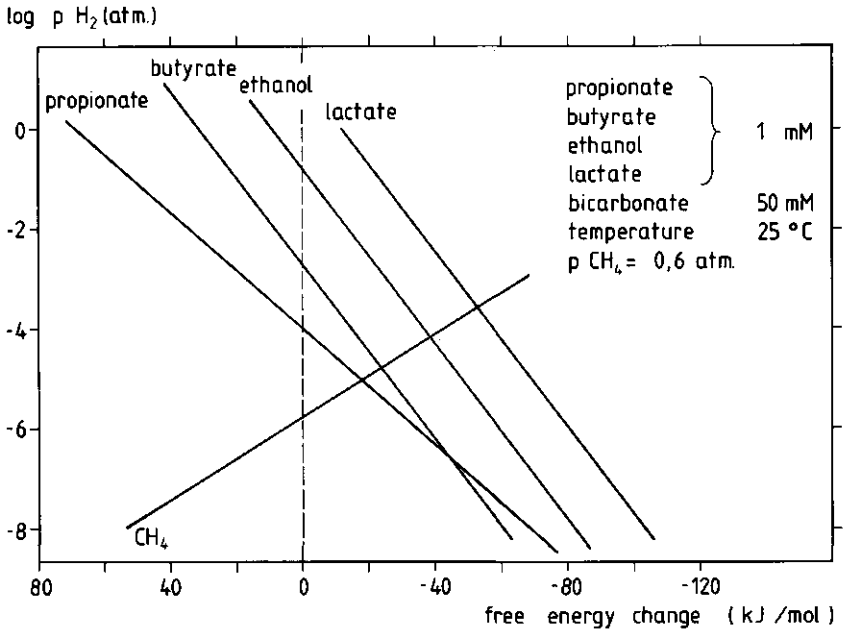

Fig. 3 *Effect of the partial pressure of hydrogen (pH_2) on the free energy change ($\Delta G'$) for the acetogenic conversion of lactate, ethanol, propionate and butyrate, as well as for the formation of methane from hydrogen and carbon dioxide.*

In a well-balanced methane fermentation, the hydrogen partial pressure does not exceed 10^{-4} atm (Zehnder, 1978), and is in most cases approximately 10^{-6} atm (Zehnder et al., 1982). Such a low hydrogen partial pressure can only be maintained if all hydrogen produced by acidogenic and acetogenic bacteria is instantly and very effectively removed by hydrogen-consuming bacteria. It can be calculated that, in a well-balanced methane fermentation, a hydrogen molecule will be consumed within 0.5 sec after being produced, which means a maximum diffusion path of <0.1 mm (Gujer & Zehnder, 1983). This illustrates that the symbiotic relationship between hydrogen-producing and hydrogen-consuming bacteria is not only biochemical, but spatial as well. This symbiotic relationship makes it impossible to obtain pure cultures of acetogenic bacteria. It is obvious that acetogenic conversion can easily be disturbed by a hydrogen build-up caused by inhibition of hydrogenotrophic methanogens.

Table 2: Methanogenic reactions

$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$	$\Delta G_0' = -28.2$	kJ/mol CH_4
$4\ CH_3OH \rightarrow 3\ CH_4 + CO_2 + 2\ H_2O$	" $= -102.5$	" "
$4\ HCOO^- + 2\ H^+ \rightarrow CH_4 + CO_2 + 2\ HCO_3^-$	" $= -126.8$	" "
$4\ H_2 + CO_2 \rightarrow CH_4 + 2\ H_2O$	" $= -139.2$	" "
$4\ CO + 2\ H_2O \rightarrow CH_4 + 3\ CO_2$	" $= -185.1$	" "
$4\ CH_3NH_2 + 2\ H_2O + 4\ H^+ \rightarrow 3\ CH_4 + CO_2 + 4\ NH_4^+$	" $= -101.6$	" "
$2\ (CH_3)_2NH + 2\ H_2O + 2\ H^+ \rightarrow 3\ CH_4 + CO_2 + 2\ NH_4^+$	" $= -86.3$	" "
$4\ (CH_3)_3N + 6\ H_2O + 4\ H^+ \rightarrow 9\ CH_4 + 3\ CO_2 + 4\ NH_4^+$	" $= -80.2$	" "

Values of free energy changes from Zehnder et al. (1982)

Methanogenesis

In methanogenic environments supplied with a complex organic substrate, approximately 70% of the methane is produced by acetate cleavage (Jeris & McCarty, 1965; Smith & Mah, 1966; Mah et al., 1977; Boone, 1982). Approximately 30% of the methane is produced by carbon dioxide reduction with hydrogen. Some methanogenic bacteria are able to utilize other substrates, such as formic acid, methanol, or methylamines, but except for the case of some methanolic waste waters, these substrates play only a minor role in anaerobic waste water treatment. The methanogenic reactions and free energy changes are shown in Table 2. *Methanosarcina barkeri* by far is metabolically the most versatile of all methanogenic bacteria isolated in pure culture so far. It has been shown to form methane by all the reactions compiled in Table 2, except for the reaction with formate (Shapiro & Wolfe, 1980).

The relatively slow growth rate of acetotrophic methanogenic bacteria (which grow 5 to 10 times slower than hydrogenotrophic methanogenic bacteria) is a consequence of the fact that energetically acetic acid is a poor substrate.

The acetotrophic methanogenic bacteria can be divided into two groups: (1) the *Methanosarcina* group, characterized by a maximum specific growth rate (μ_m) of 0.3 day^{-1} and a substrate saturation constant (K_s) of 200 mg/l and (2) the *Methanothrix* group, characterized by a maximum specific growth rate of 0.1 day^{-1} and a substrate saturation constant of 30 mg/l (Gujer & Zehnder, 1983). The facultative acetotrophic methanogenic bacterium *Methanococcus mazei*, which was reported in 1980 (Mah, 1980), is now believed to belong to the genus *Methanosarcina*. Because of a high substrate affinity (i.e. small K_s) *Methanothrix* sp. will outcompete other acetotrophic methanogenic bacteria at detention times above approximately 15 days (at 35°C). For this reason, in most digesters *Methanothrix* sp. will be the predominant acetate-cleaving organism.

In general, of the total bacterial population involved with methane production from a complex substrate, the methanogenic bacteria are the most sensitive with respect to pH, temperature, toxic compounds, etc. A stable anaerobic digestion process can only be accomplished if the methanogenic bacteria function well, because they are needed to remove acids formed in earlier metabolic stages and they play an important role in maintaining a low hydrogen concentration. Therefore, monitoring of the concentrations of methanogenic substrates is a very suitable way of controlling the performance of a methane-producing system. An increase in the hydrogen partial pressure is an indication of an instantaneous upset of the process, whereas the augmentation of acetic acid is an early warning for a possible forthcoming pH drop (Zehnder & Koch, 1983). Since hydrogen is rather difficult to analyze in the extremely low concentration range present in anaerobic digestion processes, the propionic acid concentration is often used as an indirect parameter for the activity of the hydrogenotrophic population (Kennedy & van den Berg, 1982).

CHEMICAL AND PHYSICAL REQUIREMENTS OF METHANE FERMENTATIONS

Acidity and buffering capacity

A crucial factor in the operation of anaerobic digestion processes is the acid tolerance of the fermentative bacteria. Apart from methanogenesis in acid bogs, which might be explained by the existence of neutral microenvironments (Zehnder et al., 1982) and direct methanogenesis from methanol (Lettinga et al., 1979), which might be related to the fact that methanol is unionisable, the methanogenic activity severely drops at pH values below pH 6. Methanogenesis is optimal in the pH range 6.7 to 7.4 (Zehnder, 1978, Clark & Speece, 1971). Many fermentative bacteria are still active at pH values below pH 6 (Russel & Dombrowski, 1980). In the fermentation of green crops as silage, bacterial activity only stops at pH 4 (McDonald, 1981; McDonald, 1982; Wieringa & de Haan, 1961). The same happens in the acidification of other organic material, e.g., waste tomatoes (Koster, 1984).

Under balanced digestion conditions, the biochemical reactions tend to automatically maintain the pH in the proper range. The acidogenic reactions alone would result in a reduction of the pH caused by the production of organic acids, but this effect is counter-

acted by the degradation of these acids and the concomitant reformation of the bicarbonate buffer during the methanogenic reactions. However, if an imbalance of the digestion process occurs, for example, by a sudden change in temperature, the introduction of a toxic compound, or an overloading with degradable organic material, the acid-producing bacteria will outpace the acid-consuming bacteria, therefore causing a build-up of organic acids in the system. If a balanced digestion is not restored, the buffering capacity of the system is exhausted, followed by a decrease in pH. Since a decreasing pH hinders methanogenesis (= acid consumption) more than it hinders acidogenesis, a "sour" digester will be the result. Once the digester has become sour, the time needed to restore a methanogenic bacterial population after the environmental stress has been removed will be very long, since the viable methanogenic population left in the digester will be small and methanogenic bacteria are very slow growing. In fact, the best way to achieve a balanced anaerobic digestion again will be, in many cases, to start with a new seed (Sahm, 1984).

The fact that methanogenesis is an acid consuming process implies that acidic waste waters can be subjected to anaerobic digestion without addition of neutralizing chemicals, provided the influent flow rate is such that the acid input rate does not exceed the acid consumption rate. It has been shown that even a waste stream with a pH in the range 3.2-4.2 can be treated anaerobically at loading rates as high as 22 kg COD/(m^3.dag) without any need for chemical neutralization (Koster, 1986[a]).

In anaerobic digesters where methanogenic bacteria are the main hydrogen-consuming organisms, even a relatively small pH drop (which may be caused by a toxic compound inhibiting methanogenesis) may be the start of a severe upset of the digestion process, because it influences the hydrogen metabolism by inhibiting the hydrogenotrophic methanogenic bacteria, thus leading to a build-up of hydrogen in the digester environment. Hydrogen build-up is a very rapid process, and therefore is an almost immediate warning for digester instability (Heyes & Hall, 1981; Mosey, 1983). Since the product pattern of the fermentative bacteria is influenced by the pH and to an even greater extent by the hydrogen concentration in the digester environment, a pH drop followed by a build-up of hydrogen will lead to a change in the composition of the fermentation products which are the substrates for the acetogenic and methanogenic bacteria. This substrate change might cause a decrease in the conversion rate of volatile acids, thus accelerating the pH drop which initially was very small.

The pH can also indirectly influence the rate of anaerobic digestion processes. For many compounds with a potential toxicity for bacteria active in anaerobic digestion, the molecular form is much more toxic than the ionized form. This has been shown to be the

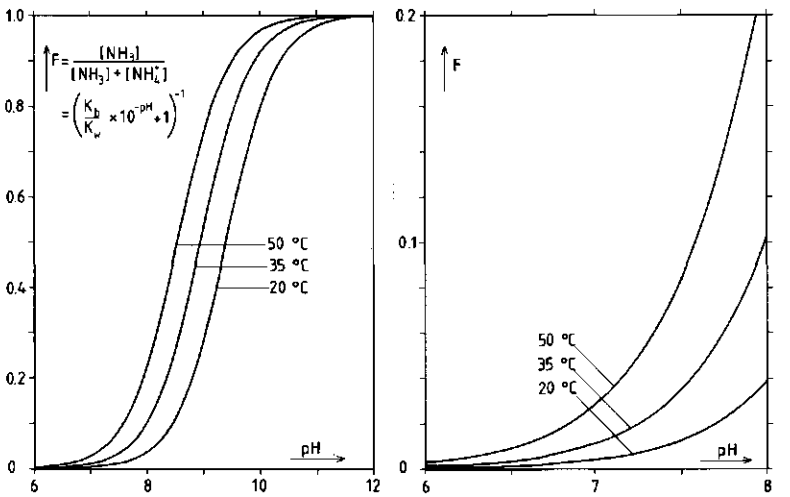

Fig 4 *The effect of pH on the dissociation of ammonium at various temperatures.*

case for acetic acid (Andrews, 1969; Andrews & Graeff, 1971; Duarte & Anderson, 1982),ammonia (De Baere et al., 1984; McCarty & McKinney, 1961) and hydrogen sulfide (Kroiss & Plahl-Wabnegg, 1983). The effect of pH on the dissociation equilibrium of ammonium is shown in Figure 4. The effect of pH on the dissociation of hydrogen sulfide is shown in figure 1 of chapter 3 of this thesis.

The precipitation of toxic heavy-metal ions such as carbonate salts is also influenced by the pH. In situations where heavy-metal toxicity occurs, it might be advisable to operate the digester in the pH range 7.4 to 8.0 instead of operating in the pH optimal for uninhibited methanogenesis (Yang et al., 1979).

Temperature

Since the early research efforts concerning the effect of temperature on the anaerobic digestion of sewage slude, which were performed in the 1930s (Rudolfs & Heukelekian, 1930; Fair & Moore, 1932; Fair & Moore, 1934; Fair & Moore, 1937; Imhoff, 1936; Imhoff, 1939), it is clear that in anaerobic digestion at least two different temperature ranges, corresponding with two different groups of bacteria, exist. Mesophilic methanogenic bacteria grow at temperatures up to 40 °C, with an optimum at about 35 °C. Thermophilic methanogenic bacteria grow at temperatures over 50 °C, with an optimum between 55 and 75 °C, depending on the particular species. It is generally assumed that in anaerobic digesters operated at psychrophilic temperatures (i.e., temperatures below 20 °C) the methanogenic population is basically similar to the one in digesters operated at mesophilic conditions, but recently evidence for the existence of psychrophilic methanogenic bacteria has been presented (Svensson, 1984).

Undoubtedly, thermophilic anaerobic digestion will become a very attractive alternative to mesophilic anaerobic digestion in the near future. At thermophilic temperatures higher loading rates can be achieved, higher methane yields can be obtained from complex wastes, and the killing of pathogens is much more effective (Wiegant & Lettinga, 1981). In various cases waste water temperatures are in the thermophilic range or thermophilic temperatures can be applied by using waste heat available from the production process generating the waste water. Important drawbacks of thermophilic anaerobic digestion are the high costs for heating in case waste heat is not available and the fact thermophilic anaerobic digesting presumably is more sensitive (compared with mesophilic anaerobic digestion) to toxicants. Thermophilic digestion has been reported to be more sensitive to high ammonium concentrations than mesophilic digestion, which presumably is caused by an increase in the fraction of free ammonia (NH_3) with temperature (van Velsen et al., 1979; van Velsen & Lettinga, 1981; Wiegant & Lettinga, 1982). The effect of temperature on the dissociation equilibrium of ammonium is shown in Figure 5.

Nutrient requirements

One of the major advantages of anaerobic digestion over aerobic waste water treatment is the low growth yield of the bacteria involved, resulting in relatively small quantities of excess sludge. The low cell yield during anaerobic digestion also implies a relatively small uptake of nutrients from the waste water to be treated.

Since in waste water treatment the substrate for the biomass in the treatment process is usually expressed in terms of COD, the nutrient requirement of the biomass present in a digester is often expressed as a ratio to the COD concentration in the waste or waste water to be treated.

It is not possible to give a general value for the appropriate COD to nutrient ratio, because it is subjective and depends on the required COD removal, the detention time, and the allowable nutrient leakage in the effluent (Lohani & De Dios, 1984). Apart from these considerations, the COD to nutrients ratio required for a stable anaerobic digestion process depends mainly on the yield coefficient of the biomass, that is, the amount of biomass produced per amount of digested organic material.

Based on an extensive literature review, Henze and Harremoës (1983) concluded that the yield coefficient decreases with a decreasing organic loading rate, resulting in a required COD to nitrogen ratio of 1000:7 or more at organic loading rates lower than 0.5 kg

COD per kilogram VSS per day, whereas at organic loading rates of 1.0 kg COD per kilogram VSS per day or higher a COD to nitrogen ratio of 400:7 would be necessary. This relationship between the yield coefficient and organic loading rate is deduced from results obtained in experiments with a variety of different waste waters, so a considerable effect of waste water composition on the yield coefficient should have been accounted for. Nevertheless, the resulting relationship between the COD to nitrogen ratio and organic loading rate might be useful in certain cases. Lettinga et al., (1981) account for the different yield coefficients of methanogenic plus acetogenic bacteria (assumed biomass yield on a waste water containing mainly volatile fatty acids: 0.05) and acidogenic bacteria (assumed biomass yield on a waste water containing mainly carbohydrates: 0.15). They advise a COD to nitrogen to phosphorus ratio of 1000:5:1 if volatile fatty acids serve as the main substrate, as is the case when the waste water is already acidified in the sewer system, and a COD to nitrogen to phosphorus ratio of 350:5:1 if complex, not-yet-acidified material serves as the substrate.

However, there are indications that a simple nutrients to COD ratio is only sufficient if it takes into account that for the nutrients the concentration itself (instead of the total dosage) can be the rate-limiting factor. Unfortunately the microbial kinetic constants (especially K_S) are unknown for almost all inorganic nutrients including trace elements (Speece, 1987[a]). In a recent study concerning the anaerobic digestion of sucrose containing influents in UASB-reactors it was concluded that a COD:N:P ratio of 500:5:1 (w/w) was only sufficient if the ammonia-nitrogen concentration was at least 100 mg N/l (Mendez et al., 1989).

Apart from the macro-nutrients N, P and S, the environment of a methanogenic bacterial population should contain trace elements, mainly metal ions. On the basis of a number of case histories and a lot of experiments with acetate to methane conversion studies in their own laboratory Speece et al. (1986) argued that the need of available trace metals is grossly under-estimated. They claim that in about half of the cases in which trace metal supplementation has stimulated the rate of anaerobic digestion, "common wisdom" would have indicated that no such definciency should have existed because the feed stock was rich in these total trace metals.

The addition of nutrients and trace metals to enhance anaerobic digestion has been reported several times. It has been found that in the methanogenic conversion of methanol solutions as well as in the anaerobic treatment of waste water containing methanol and higher alcohols one or more trace metals are of eminent importance with respect to the stability of the conversion process (Lettinga et al., 1979 & 1981). Trace metal additions are further discussed in this chapter's section on toxicity, especially the paragraph concerning the concentration dependent effect of compounds on methanogens. Addition of phosphate has been reported for a UASB-reactor treating liquid sugar factory waste water in which methanogenesis dropped to an intolerable low level before phosphate was applied (Lettinga et al., 1980). Phosphate addition also appeared to be necessary in the anaerobic treatment of rendering wastes (Lettinga et al., 1983).

It is very difficult, if not impossible, to give exact figures for the amount of nutrients and trace elements that should be added to secure a stable digestion process, even if the theoretically required amount has been determined and kinetic constants are taken into account, because it is the *available* amount of nutrients and trace elements that counts. The precipitation of metals with carbonate or sulfide can cause serious metal deficiences. Precipitation and chelation of metal ions play a very important role in anaerobic digestion, determining the availability of metals as nutrients (Callander & Barford, 1983[a]; Callander & Barford, 1983[b]). Phosphorus deficiency can occur in the presence of excessive amounts of iron, aluminium, and calcium ions that cause precipitation of insoluble phosphate salts (Pfeffer & White, 1964).

TOXICITY: EFFECT OF INHIBITORY CHEMICALS ON METHANE FERMENTATIONS

Introduction

To maintain a stable anaerobic digestion process, there should be a balance between the acid production rate (as a result of hydrolysis and acidogenesis) and the acid consumption rate (as a result of acetogenesis and methanogenesis). There is little if any information available concerning the direct inhibition of acetogenic bacteria by chemicals. The indirect inhibition of acetogenesis via a build-up of hydrogen as a result of inhibition of hydrogenotrophic methanogenesis is well-understood. It is described in detail in an earlier section of this chapter.

Since the susceptibility of methanogenic bacteria to toxic compounds in the environment is much greater than that of the acidogenic bacteria, the presence of toxic (= inhibitory) compounds in the waste or waste water that is being digested can easily disrupt the balance between the acid production rate and the acid consumption rate, therefore being the start of a fatal upset of the digestion process. The introduction of a toxic compound in a digester will lead to inhibition of methanogenesis. Even if the toxicant affects acidogenesis, methanogenesis will be slowed down to a much greater extent than acidogenesis, resulting in a build-up of volatile fatty acids in the reactor environment. If the digester loading rate is not adjusted to the lower acid consumption capacity of the methanogenic biomass, the ultimate consequence will be a pH-drop below the physiological tolerance level of methanogenic bacteria, so that recovery of the digester is only possible by means of completely starting-up again from the point of addition of fresh, viable methanogenic seed sludge.

Since it would be rare to find an industrial waste water completely devoid of all potential toxicants, there is a commonly held belief that anaerobic digestion is not appropriate for treatment of most industrial waste waters. However, this misbelief is based mostly on results obtained in the early days of scientific interest in anaerobic digestion as a waste water treatment process. At that time, experiments concerning toxicity problems in anaerobic digestion were performed for periods too short to allow any adaptation of the bacteria to occur, or they were performed with systems characterized by a relatively short sludge retention time such as in the anaerobic contact process. The modern high-rate anaerobic waste water treatment systems are all characterized by a very high retention time of the biomass, which is present as biofilms (Lettinga et al., 1984). This increases the potential to tolerate toxic compounds in the waste water to be treated. At present also many mechanisms such as adaptation and antagonism that increase the tolerance of the anaerobic digestion process are known and more or less understood. Moreover, it should be realized that in many cases the actual *effective* concentration of a potential toxicant in the digester environment is much less than the concentration in the waste water to be treated.

Because methanogens are the most vulnerable to toxicity and they play a key-role in anaerobic digestion, the next sections will concentrate on the effect of toxicants on methanogenic bacteria.

Concentration dependent effect of chemicals on methanogens

For compounds that are normally present in anaerobic environments, three concentration ranges, each characterized by a different effect of the particular compound on the methanogenic bacteria, can be distinguished (McCarty, 1964). Initially, an increasing concentration will result in an increasing bacterial activity. This concentration range is followed by a range in which the concentration is optimal (an increasing concentration neither stimulates nor inhibits the bacterial activity). Increasing the concentration further than the optimal range will result in inhibition of the bacterial activity. Depending on the nature of the compound, this inhibition starts at concentrations of several tens to several hundreds of mg/l. In general, this inhibition increases as the concentration increases. This type of dose-effect relation is valid for trace elements (e.g. nickel, sodium), nutrients (e.g. ammonium) and substrates (e.g. acetate).

Sodium has been reported as an essential component of media in which methanogenic bacteria are cultivated, because a transmembrane Na^+ gradient is involved in the biochemi-

cal process of methanogenesis (Peinemann et al., 1988) and because sodium plays a role in controlling the internal pH of methanogens (Schoenheit & Beimhorn, 1985). Apparent K_S values for Na^+ ranging from 9-25 mg/l have been found with pure cultures of hydrogenotrophic methanogens (Perski et al., 1982). For a hydrogenotrophic methanogen strongly resembling *Methanobacterium formicicum* optimal growth was found at 350 mg Na^+/l (Patel & Roth, 1977), a value which is in accordance with the findings of Kugelman and Chin (1971) who in their famous comprehensive study concerning toxicity in anaerobic digestion suggested 230 mg Na^+/l as the optimum sodium concentration in waste treatment processes. Inhibition of methanogenesis has been reported at concentrations that are 20-100 times higher (Kugelman & McCarty, 1965; Lettinga & Vinken, 1981; De Baere et al., 1984; Dolfing & Bloemen, 1985; Rinzema et al., 1988).

The high nickel content of methanogenic bacteria (Scherer et al., 1983) reflects the fact that they belong to the unique Archaebacteria and not to the common prokaryotes. Nickel is generally not essential for growth of bacteria, but it is essential for methanogenic bacteria because they contain nickel tetrapyrroles (e.g., factor F_{430}) which are involved as catalysts in methane formation (Thauer, 1982; Diekert et al., 1981).

A specific nickel uptake and transport system has been identified in the acetoclastic methanogen *Methanothrix concilii* (Baudet et al., 1988).

In the presence of nickel, the specific acetate utilization rate of an acetate-enriched methanogenic culture (in which *Methanosarcina* was the predominant organism) was two to five times higher than in the absence of nickel. This stimulatory effect of nickel was enhanced by concomitant addition of iron and cobalt (Speece et al., 1983). Growth of a pure culture of *M. barkeri* on methanol was found to be dependent on cobalt and molybdenum, whereas nickel and selenium had a stimulatory effect on growth on methanol (Scherer & Sahm, 1981). In the presence of 0.117 mg Ni^{2+}/l the methane production by a pure culture strain of *Methanothrix soehngenii* was very much stimulated (Fathepure, 1987). In the same study nickel was found to be inhibitory at 0.294 mg/l.

Apart from these reports on enriched or pure cultures, there are also some reports concerning nickel addition in anaerobic digestion of complex substrates by a mixed population. The addition of nickel together with iron and cobalt greatly enhanced the performance of an anaerobic fixed film expanded bed reactor treating a powdered, whey-based solution. In this study, the methanogenic bacteria were more affected by nutrient limitation (i.e. shortage of iron cobalt and nickel) than were the nonmethanogenic bacteria (Kelly & Switzenbaum, 1983). The conversion of acetic acid to methane and carbon dioxide by the mixed microbial population from an anaerobic fixed film digester treating bean-blanching waste water was stimulated by the addition of nickel and cobalt, especially if these elements were added in combination. Molybdenum addition was only slightly stimulatory when added in combination with nickel and cobalt (Murray & van den Berg, 1981). During treatment of a petrochemical waste water in a fixed film reactor the reactor efficiency markedly increased after addition of trace metals, including nickel (Nel et al., 1985). The concentrations applied were in the order of magnitude of 10^{-2}-10^{-3} mg/l. Inhibition of methanogenesis during sludge digestion has been reported for nickel concentrations exceeding 10 mg/l (Hayes & Theis, 1978). Acetate fed methanogenic enrichment cultures were reported to be inhibited by nickel at concentrations of 100-200 mg/l (Yang et al., 1979; Parkin et al., 1981).

It has been established that ammonia is the nitrogen source for methanogenic bacteria (Bryant et al., 1971; Kenealy et al., 1972). McCarty (1964) reported stimulation of methanogenic activity in the ammonia-nitrogen concentration range 50-200 mg/l. The optimum concentration of ammonia-nitrogen for methanogenesis by granular sludge from UASB-reactors treating sugar factory waste water has been reported to be in the range 130-390 mg/l (Dolfing & Bloemen, 1985). For a similar sludge methanogenesis was found to be inhibited by ammonia-nitrogen concentrations exceeding approximately 700 mg/l (Koster & Lettinga, 1984). In general, ammonia has been identified as potential inhibitor of methanogenesis which plays a key-role in the process stability of all kinds of anaerobic digestion of animal wastes (Kroeker et al., 1979).

Acetate is an important substrate for methanogenic bacteria, but at higher concentrations it also acts as inhibitor of methanogenesis (Anderson & Duarte, 1980; Duarte & Anderson, 1982; Andrews, 1969; Andrews & Graef, 1971; Belay et al., 1986). Probably hydro-

genotrophic methanogenic bacteria are less susceptible to acetate than acetoclastic methanogens. *Methanobacterium formicicum*, which is the main hydrogen consuming methanogen in manure digesters, was found to tolerate 10 g/l acetate without any sign of inhibition (Hobson & Shaw, 1976).

Effective concentration of potential inhibitors

In many cases, the extent of the inhibitory effect of a compound is influenced by the pH. Examples of compounds showing a pH-related toxicity are ammonia (McCarty & McKinney, 1961; De Baere et al., 1984; Koster & Koomen, 1988), acetate (Andrews, 1969; Andrews & Graef, 1971; Duarte & Anderson, 1982; Attal et al., 1988), and sulfide (Kroiss & Plahl-Wabnegg, 1983; Lawrence et al., 1966; Koster et al., 1986; Rinzema & Lettinga, 1988).

The unionized forms of these compounds are supposed to be the toxic agents, whereas the ionized forms have much less effect. It has been shown that the pH-controlled operation and a suitable choice of temperature can prevent the inhibitory effect of ammonia in manure digestion (Braun et al., 1981)

Another mechanism that decreases the effective concentration of potential toxicants is the formation of insoluble precipitates. Precipitation of metal sulfides is probably the best example, since sulfide is the end product of the anaerobic conversion of sulfur-containing organic material. The precipitation of metal sulfides can be used to prevent sulfide toxicity, but the mechanism is better known in relation to toxicity of heavy metals (Lawrence & McCarty, 1965). The precipitation of metal carbonates also plays a role in the prevention of heavy metal toxicity (Mosey & Hughes, 1975; Mosey, 1976). More than 70% of the heavy metal removal occurring in a completely mixed anaerobic filter treating landfill leachate was reported to be due to precipitation as carbonates (DeWalle et al., 1979). Precipitation of metal carbonates needs a relatively high pH to be important. For the formation of cadmium carbonate, a pH above pH 7.2 is necessary; for the formation of zinc carbonate the pH should be even higher than pH 7.7. In cases of potential heavy-metal toxicity, it is advised to add sulfate (which will be reduced to sulfide) to the waste water and operate the digester at a somewhat higher pH than normal (Hayes & Theis, 1978; Yang et al., 1979). Addition of sodium sulfide in combination with hydrated lime has successfully been used to cure "sour" sludge digesters suffering from inhibited methanogenesis initiated by heavy metals (Regan & Peters, 1970).

A somewhat different method of lowering the concentration of inhibitory heavy metals is binding them to Al-silicates. In laboratory scale sludge digesters addition of synthetic Al-silicates (zeolite A) decreased the toxicity of copper, cadmium, zinc and mercury (Roland & Smid, 1978).

Cyanide toxicity can also be reduced by means of precipitation. A coking operation waste water containing 5 mg/l of cyanide was successfully treated by an immobilized-cell anaerobic system when iron was added to precipitate the cyanide (Speece, 1983). Complexation of cyanide is an even better method to reduce its toxicity (Lettinga, personal communication).

Another example of precipitation reducing toxicity is the interaction between calcium ions and long-chain fatty acids (Hanaki et al., 1981; Koster, 1987). Metal cations also form precipitates with long-chain fatty acids and can therefore also be applied to reduce long-chain fatty acids inhibition of methanogenesis (Galbraith & Miller, 1973). Experiments with lauric acid showed that interaction between the methanogens and the inhibitor is very rapid, and that the precipitates should be formed within minutes to prevent inhibition (Koster, 1987).

With many organic inhibitory compounds adsorption plays an important role in reducing the concentration in the environment of the methanogens (Johnson & Young, 1983). Therefore addition of activated carbon to digesters can reduce or completely eliminate inhibitory effects of organic compounds known to be toxic to methanogenic biomass (Ng et al., 1988). Activated carbon additon to digesters treating waste water from coal conversion processes (which are rich in aromatic inhibitory compounds) increases digestibility (Harper et al., 1983; Suidan et al., 1987). In the Anaerobic Expanded Bed Granular Activated Carbon reactor the activated carbon granules provide an excellent surface for microbial attachment, but also its adsorptive properties provide a buffer capacity for absorbing shock loads of

inhibitory compounds as present in coal and petroleum distillation waste waters (Suidan et al., 1988).

The effective concentration of volatile inhibitory components in a digester can be lower than in the influent as a result of stripping from the digester fluid with the biogas. This phenomenon plays an important role in the anaerobic treatment of sulfate-rich waste waters, from which a considerable part of the potentially inhibitory sulfide is removed via the biogas (Kroiss & Plahl-Wabnegg, 1983; Rinzema & Lettinga, 1988). Vinyl chloride was found to be inhibitory at concentrations exceeding 5-10 mg/l in batch toxicity tests, whereas in semicontinuous assays even the highest concentration of 64 mg/l did not result in adverse digester performance. This could be explaned by volatilization of the vinyl chloride during the semi-continuous digestion (Stuckey et al., 1980).

Antagonism and synergism

Antagonism is the reduction of the toxic effect of one compound by the presence of another. Syngerism is an increase in the apparent toxicity of one compound caused by the presence of a second compound. As with toxicity, antagonism and synergism are concentration dependent. Antagonism and synergism have been studied in detail using acetate consuming enrichment cultures exposed to various cations (Kugelman & McCarty, 1965[a]; Kugelman & McCarty, 1965[b]; Kugelman & Chin, 1971). Table 3 gives an overview of antagonistic and synergistic relationships in cation toxicity adapted from the work of Kugelman and Chin (1971).

Table 3: Antagonism and synergism in cation toxicity in anaerobic digestion

primary toxicant	antagonist	synergist
Na^+	K^+	NH_4^+, Ca^{2+}, Mg^{2+}
K^+	Na^+, Ca^{2+}, Mg^{2+}, NH_4^+	
Ca^{2+}	Na^+, K^+	NH_4^+, Mg^{2+}
Mg^{2+}	Na^+, K^+	NH_4^+, Ca^{2+}
NH_4^+	Na^+	K^+, Ca^{2+}, Mg^{2+}

They reported that peak antagonism is achieved at 0.01 M with monovalent cations as antagonists and at 0.005 M when divalent cations serve as antagonists. They also found that the concentration of the synergist at which the synergistic effect begins is below the concentration at which the synergistic cation will produce inhibition when present alone.

Results of experiments with pure cultures of methanogenic bacteria confirm the antagonistic role of sodium in ammonia toxicity. Sprott and Patel (1986) found that sodium ions countered ammonia toxicity with *Methanosarcina barkeri*, *Methanobacterium bryantii* and *Methanothrix concilii*. They applied concentrations which were several times higher than those recommended by Kugelman and Chin (1971) for optimum antagonistic action. Antagonism of ammonia toxicity by sodium has also been found with *Methanobacterium thermoautotrophicum* (Schönheit & Beimhorn, 1985). In contrast with the findings of Kugelman and Chin (1971), Sprott and Patel (1986) also reported antagonsim of magnesium and calcium for ammonia inhibition of some species.

The biochemical background of synergism and antagonism in cation toxicity for methanogenic bacteria is beginning to be revealed. One possible mechanism of ammonia toxicity is passive diffusion of NH_3 into the cytoplasm, where it disrupts the internal cell pH by taking up protons to establish the NH_3/NH_4^+ equilibrium. In order to try to maintain the internal pH, the cells will activate systems which actively import protons. Many methanogenic species can achieve this by exchanging potassium from the cytoplasm with protons from the envrionment, a so-called K^+/H^+-antiporter (Sprott et al., 1984 and 1985). This might explain the synergistic action of potassium with ammonia toxicity. A higher

potassium concentration in the environment increases the concentration gradient against which the H^+/K^+-antiporter has to operate.

Some methanogenic bacteria have a Na^+/H^+-antiporter which is involved in the energy fluxes during methanogenesis. This antiporter is competatively inhibited by ammonia (Schönheit & Beimhorn, 1985), so an increasing environmental sodium concentration will depress the inhibitory action of ammonia.

Synergism and antagonism have mainly been researched for cation toxicity, but some reports concerning other inhibitory compounds are known. In pure cultures of thermophilic methanogenic bacteria the toxicity of copper and cadmium decreased when trace amounts of nickel were added in the medium (Ahring & Westermann, 1985). Since nickel is an essential trace element for methanogens, it is not clear whether this nickel addition really suppressed the biochemical action of coppper and cadmium, or that the addition of nickel stimulated the general well-being of the methanogens to such an extent that the inhibitory effect of the metals became masked by the generally improved activity.

Another report about synergism in toxicity for methanogenic bacteria concerns the inhibitory action of long chain fatty acids. A background concentration of lauric acid of 1.25 mM (which is below the minimum inhibitory concentration of 1.6 mM) was found to enhance the toxicity of capric acid moderately, whereas at the same background concentration the toxicity of myristic acid was enormously enhanced (Koster & Cramer, 1987).

Acclimatization of methanogenic populations to toxicants

One of the most important aspects of toxicity in anaerobic digestion is the capacity of the methane producing bacterial population to get acclimatized to the presence of potential toxicants in the waste or waste water to be digested. Acclimatization takes place during application of gradually increasing concentrations of toxicants (McCarty, 1964; Melbinger & Donnellon, 1971; Guthrie et al., 1984; De Baere et al., 1984), but also after a period of apparent death (i.e. zero gas production caused by the toxic compound) recovery of the bacterial activity can occur (Koster & Lettinga, 1983; van Velsen, 1979; Parkin & Speece, 1982; Koster, 1986; Parkin et al., 1983; Healy & Young, 1979; Yang et al., 1980). Acclimatization can be the result of adaptation of the methanogenic bacteria. Such adaptation implies that the cells are able to change their biochemical behaviour in such a way that they can deal with envrionmental stress that before their adaptation caused complete inhibition of activity and/or growth. A probably more common way of acclimatization is the introduction in the inhibited methanogenic population of the capability to degrade the toxic component. Both types of acclimatization will be discussed in more detail in the next paragraphs.

Adaption of methanogenic bacteria

Pure culture studies have supplied definite proof of the hypothesis that methanogenic bacteria can alter themselves in order to become more or less resistant to toxic compounds. This may be called "true" adaptation. In cultures of *Methanobacterium* strain FR-2 that were exposed to bacitracin spontaneous mutants were found to occur at the frequency of 10^{-7}. These mutants tolerated bacitracin at concentrations 8-16 times that of the wild type organism (Harris & Pinn, 1985). Pure cultures of *Methanobacterium autotrophicum* rapidly acquired resistance towards fluoro-uracil, which probably inhibits nucleic acid synthesis (Grinbergs et al., 1988). Dellinger and Ferry (1984) found that pure cultures of *Methanobacterium formicicum* showed inhibition of growth when exposed to monensin, but that eventually the cultures recovered from this inhibition. Before or during the recovery, nor during growth after the recovery monensin was inactivated. Hence the recovery must have been due to biochemical changes of the organism.

"True" adaptation has also been reported in studies concerning more or less undefined mixed methanogenic cultures. Such a recovery of methane production after an apparent death phase during which "true" adaptation occurred is shown in figure 5, which is based on results of a batch experiment in which diluted potato juice (approximately 2.3 g nitrogen per liter) was digested by unadapted granular methanogenic sludge. This sludge has a toxicity threshold level for ammonia-nitrogen of approximately 1700 mg/l (Koster & Lettinga, 1984). In the initial stage of the digestion, methane production was possible because a

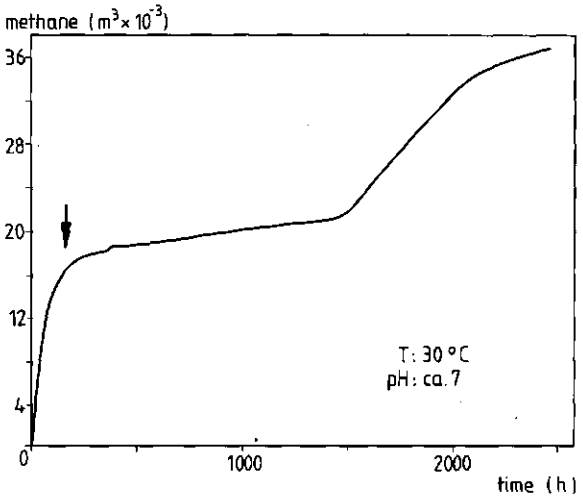

Fig. 5 *Cumulative methane production as a function of time in the anaerobic digestion of twice-diluted potato juice. Ammonia concentrations at the start, prior to the adaptationon period (arrow) and at the end were 620, 2000 and 2300 mg N/l respectively.*

large fraction of the nitrogen was still bound in proteins and amino acids, but as soon as these had been broken down and the nitrogen had been converted into ammonia, the methane production stopped. Kinetic analysis of the methane production after the lag phase indicated that growth of ammonia-resistant mutants or other (during non-inhibitory cultivation non-dominant) species was very unlikely (Koster, 1986), so that probably "true" adaptation had occurred during the lag phase. Similar observations were reported by Parkin et al. (1983), who concluded that the recovery of methanogenesis after a period of zero gas production resulting from exposure to toxicants was too fast to be accounted for by bacterial (re)growth alone.

Other evidence of "true" adaptation in mixed methanogenic cultures was provided by Yang and Speece (1986). Their radio tagged chloroform studies indicated that recovery of methane production in acetate fed enrichment cultures occurred due to adaptation of the methanogens to the presence of chloroform in their environment, and not from the disappearance of chloroform by microbial degradation. Other experiments with radio-active cyanide have led to similar conclusions concerning the adaptation of methanogens to cyanide toxicity (Speece & Parkin, 1983).

Microbial degradation of the toxicant

If a certain compound which is a potential inhibitor of methanogenesis can be degraded in an anaerobic environment, it should in principle be possible to treat waste streams containing that compound anaerobically without inhibition problems, just by providing the right conditions for microbial break-down of the inhibitor in the digester. This principle is similar to the approach of treating an acidic waste water with an extremely high organic acids concentration in a one stage UASB reactor without pretreatment or chemical neutralization, just by means of adjusting the influent flow to the acids consumption rate (Koster, 1986[a]).

A lot of petrochemicals that are classified as toxicants can in fact be degraded anaerobically, so petrochemical waste waters need not be excluded from anaerobic digestion based biological treatment systems if these systems have a cell residence time that is long enough to allow the proper petrochemical degrading bacteria to colonize the digester biomass (Speece, 1987[b]). A recent overview of the state-of-the-art in anaerobic degradation of petrochemicals and other halogenated molecules as well as other aromatic compounds is presented by Hollinger et al. (1988). A review of the role of phenolic compounds (many of

which are degradable methanogenic toxicants) in anaerobic digestion is presented by Field and Lettinga (1988). Since organic toxicants are the subject of the Ph.D. thesis of J. Field (planned for 1989) this will not be dealt with in detail here. Instead some examples will be presented.

Several toxic compounds in paper making waste water are anaerobically degradable so that acclimatization of methanogenic populations can occur (Benjamin et al., 1984). Examples of the practical implications of this finding are the biodegradation of chlorophenols and chlorocatechols which enables anaerobic digestion of Kraft-bleaching effluents (Salkinoja-Salonen, 1983) and the biodegradation of pentachlorophenol in methane producing reactors, thus enabling anaerobic treatment of waste waters containing 5 mg/l of pentachlorophenol while 0,2 mg/l is the toxicity level for methanogenic bacteria (Guthrie et al., 1984). Unfortunately, not in all types of industry related to paper making the organic toxicants in the waste water can be degraded in the digester. For example tannin compounds were found to be responsible for the majority of methanogenic toxicity in waste water from debarking and bark pressing operations in the forest industry, while these tannins were also found <u>not</u> to belong to the readily degradable substrates in these waste waters during anaerobic digestion (Field et al., 1988). Moreover, in batch toxicity assays the methanogenic toxicity of gallotannic acid (a model tannin) was found to be persistent despite the rapid anaerobic degradation (Field & Lettinga, 1987). Many toxicants in forest industry waste water which cannot be degraded during anaerobic digestion may be eliminated with a variety of detoxification methods (either biological, physical or chemical) prior to the digester (Welander et al., 1988).

Acrylic acid has been reported as a versatile inhibitor of methanogenesis, with a 50% inhibition level of 0.86 g/l (Chou et al., 1978). However, in methane producing biofilm reactors an acrylic acid degrading microflora could be obtained so that waste water containing acrylic acid and its methyl-, ethyl- and butylesters could be treated successfully. In the same reactors even an influent containing acrylic acid as the only substrate at 10-12 g/l could be treated without recirculation at loading rates up to 2.5 kg/(m^3.day). The effluent acrylic acid concentration was zero and the COD treatment efficiency was above 98 % (Dohányos et al, 1988).

Long-chain fatty acids are also examples of anaerobically degradable compounds that are versatile inhibitors of methanogenic conversions taking place in anaerobic waste water treatment processes (Hanaki et al., 1981; Koster & Cramer, 1987). Long chain fatty acids containing waste waters can be treated satisfactorily in continuously operated digesters (McCarty, 1964), although shock loads should be avoided and good mixing should be provided (Rinzema, 1988).

Formaldehyde is an organic toxicant which is present in the waste water of many petrochemical industries, where it is often accompanied by phenol. Although formaldehyde and phenol both are used as desinfectants, at lower concentrations they are biodegradable, so that degradation of these compounds can eliminate their toxicity in anaerobic digestion (Speece, 1985). That it may be very difficult to achieve anaerobic digestion of formaldehyde containing waste water was shown by De Bekker et al. (1983) who concluded from research with waste water containing 20 g/l acetate and 3-5 g/l formaldehyde that "both in batch and (continuous) upflow experiments it appears to be impossible to treat formaldehyde containing waste water successfully in an anaerobic system, for bacterial die-off surpasses bacterial growth". Nevertheless, in later years reports appeared claiming that waste waters containing phenol and formaldehyde can be treated in UASB reactors. One waste water with 800 mg/l phenol and 1200 mg/l formaldehyde could be treated at a loading rate of 8-9 kg COD/(m^3.day) with more than 95% removal of the toxicants (Koevoets et al., 1986). Another waste water containing 450 mg/l phenol and 900 mg/l formaldehyde could be treated in lab-scale UASB reactors after an adaptation period of only 6 weeks. Phenol and formaldehyde were removed with an efficiency of 95 and 99% respectively. The success of this research has led to the construction of full scale UASB reactors treating phenol and formaldehyde containing waste waters at several factories (Borghans & van Driel, 1988).

In all of the above-mentioned examples the organic toxicants could be degraded completely. However, it should be noted that often a slight change of the molecule is enough to detoxify it. For example, inhibition of methanogenic mixed cultures by 4-nitro-

phenol, 2-nitrophenol and nitrobenzene was found to be reversible as a result of biological reduction of the nitro group to the less toxic amino group.

Acclimatization of methanogenic populations to the presence of toxicants in the waste water by means of biodegradation is not limited to organic compounds. Nitrite and nitrate have been identified as direct (viz. not only via competition for electrons from the substrate) inhibitors of methanogenic bacteria (Balderston & Payne, 1976). Slug additions of 50 mg/l of nitrate have been reported as harmful to methanogenic bacteria (Kugelman & Chin, 1971). Nitrogen oxides can be eliminated by denitrifying bacteria, which can easily be achieved in digesters producing methane. In fact denitrification has an electro-chemical advantage over methanogenesis so that denitrification cannot be prevented in methane producing digesters (Zehnder, 1978). Dairy-factory waste water containing nitrate up to 8.9 g/l has been reported to be treated anaerobically in a 2-step UASB system in which the first reactor acts mainly as a denitrification reactor (Zaal, 1983).

Sulfite is used in starch production as a bactericide and bleacher. Sulfate reducing bacteria have been reported to colonize the sludge in a single stage UASB reactor treating maize-starch waste water so that reduction of the sulphite to sulfide prevents inhibition of the methanogens present in the digester (Maaskant & Hobma, 1981). The potato starch industry uses a 2-phase anaerobic waste water treatment system. In the first reactor simultaneous acidogenesis and sulphite reduction takes place, so that the highly toxic sulphite does not enter the methane producing reactor (Wijbenga et al., 1983). A 2-step anaerobic digestion process in which the frist reactor acts as a sulphite remover by means of biological reduction of sulphite to sulphide which is to be stripped out of the water has been reported for anaerobic treatment of waste water from a sodium based sulphite paper mill (Särner, 1989).

A highly specific biological detoxification incorporated in an anaerobic digestion process can be applied to hydrogen peroxide containing waste water from chemi-thermomechanical pulping operations. In the first reactor of a 3-step process, the hydrogen peroxide is eliminated by the enzyme catalase which is present in some of the bacteria grown in the second (acidogenic) reactor. Before the waste water flows into the third (methanogenic) reactor the biomass is separated and directed to the first reactor where the catalase destroys the hydrogen peroxide (Gunnarsson & Welander, 1986; Welander et al., 1988).

Technological aspects of toxicity in anaerobic digestion

Reactor design and operation

In the anaerobic treatment of waste water containing potentially toxic components the biomass retention time is a crucial parameter for design as well as operational purposes. This case holds for chronic as well as transient exposure to toxicants (Parkin & speece, 1982). The biomass retention time should be large enough to allow the methanogenic biomass to go through a phase of diminished activity and growth without being washed out of the system in the meantime. Such periods of diminished activity and growth occur during the acclimatization period in case of chronic exposure to toxicants and during the period of exposure and the recovery period thereafter in case of transient exposure. The importance of the biomass retention time in the process of dealing with methanogenic toxicity is illustrated by the findings of Yang and Speece (1985) that the system with the longest biomass retention time could recover from the highest slug dose of cyanide. In fact at a biomass retention time of 50 days the highest dose of cyanide from which recovery was possible was 10 mg/l, whereas at a biomass retention time of 12.5 days this was only 0.5 mg/l. Also in situations of chronic presence of toxicants in the waste water a relatively long biomass retention time is required to make up for the increased generation time (= decreased growth rate) of the methanogenic bacteria (Parkin & Speece, 1982; Speece & Parkin, 1983; Parkin & Speece, 1983). In general, anaerobic toxicity assays are based on the measurement of methanogenic activity. Although activity and growth rate normally are coupled, stress such as the presence of toxic chemicals can cause uncoupling of growth and activity. For a pure culture study with *Methanosarcina barkeri* it has been reported that sulfide caused a negative net growth rate (viz. a dying population) while the methanogenic activity of the culture was not lost (Mountfort & Rodney, 1979). There are only two reports concerning the effect of toxicants (in both cases: ammonia) on the growth rate of

methanogenic bacteria. In both cases the growth rate decreased with increasing toxicant concentrations (Sprott & Patel, 1986; Koster & Koomen, 1988).

To take full advantage of the acclimatization potential of methanogenic bacterial populations to undegradable soluble toxicants digesters allowing very high biomass concentrations are required. In case of chronic toxicity, methanogenic bacteria can become adapted (see earlier paragraphs of this chapter), but generally the specific methanogenic activity will be much less than at uninhibitory conditions. For example, at 5 g/l of ammonia-N the maximum specific methanogenic activity of well-adapted granular sludge was found to be only 22% of the value assessed at 1 g/l of ammonia-N (Koster & Lettinga, 1988). With such a decreased specific biomass activity, economical operation of a waste water treatment system is only possible if the digester contains a very dense sludge.

Another important feature in the design of reactors bound for operation with toxicant containing waste water is the fluid behaviour. If the digester contents are well mixed, the concentration to which the biomass is exposed may in many cases be less than the concentration in the waste water itself. In case of transient toxicity (viz. a slug dose of toxicant appears in the waste water) a short hydraulic retention time is beneficial. It has been established that in general the recovery from exposure to toxicants is shorter with a shorter exposure time (Speece & Parkin, 1983). Methanogenic cultures recovered from a 100 mg/l cyanide dosage during 1 hour within 4 days, whereas a 24 hours exposure to a similar dosage required a nearly 11 days recovery period (Yang et al., 1980). Moreover, after the 1 hour exposure the methanogenic activity dropped to approximately 45% of its uninhibited level, whereas the 24 hours exposure caused several days of zero methanogenic activity. Parkin & Speece (1983) concluded that for maximum process efficiency, stability and flexibility in case of either chronic or transient toxicity a quasi plug-flow fluid behaviour is required.

The requirements of high biomass retention potential and short hydraulic retention times (preferably combined with quasi plug-flow fluid characteristics) can be met in many modern, high-rate anaerobic reactor designs. The upflow fixed bed anaerobic filter developed by Young & McCarty (1967 & 1969) was the first of a series of digester designs in which flocculation and probably even granulation of sludge and bacterial adhesion to heavy support materials allowed very high liquid loading rates without risking the washout of biomass.

The modern high-rate anaerobic digesters are available in a wide variety such as anaerobic expanded bed reactors, anaerobic fluidized bed reactors, anaerobic filters with random packing material or structured adhesion support material (operated either in upflow or downflow mode), or upflow anaerobic sludge bed reactors. Several review articles comparing the several types of high-rate digesters with high biomass retention potential have been published (van den Berg & Kennedy, 1983; Callander & Barford, 1983; Switzenbaum, 1983[a] & 1983[b]; Henze & Harremoës, 1983). Probably the most often applied system is the upflow anaerobic sludge Bed (UASB) reactor, which is characterized by the absence of biomass carrier material (Lettinga et al., 1980) and in which the biomass mostly grows in granules (Hulshoff Pol et al., 1983). In a review of the UASB reactor and its many applications it is claimed that by July 1984 51 plants had been installed and commissioned (Lettinga et al., 1984).

The biomass retention potential of the modern high-rate reactors, which can be typified as "fixed film" reactors, allows for biomass retention times of over 100 days. For the anaerobic attached film expanded bed reactor it has been claimed that biomass retention times in excess of a year would not be impossible (Jewell & Morris, 1981).

Due to the static packing material the fluid flow in anaerobic filters tends to be plug-wise. In order to increase the mixing towards the more optimal quasi plug-flow behaviour effluent recycle is applied (Okkes, 1983). Lithium tracer studies showed that the flow pattern in a large (liquid volume = 5600 m^3) downflow submerged anaerobic filter comprising a recirculation loop was close to complete mixing (Racault, 1989). Anaerobic fluidized bed reactors may be considered as a completely mixed reactor when the effluent to feed recycle ratio exceeds 2 (as it usually does) (Shieh & Mulcahy, 1983). Radioactive tracer studies with a hybrid UASB reactor (floating filter material instead of gas-liquid-solids separator) indicated that with and without effluent recycle the fluid flow pattern was of the perfectly mixed type (Samson & Guiot, 1985). The fluid flow pattern in UASB

reactors maybe the best approximation of a quasi plug-flow that can be obtained with high-rate digesters, since UASB reactors are not equipped with mixing devices and effluent recycle is strongly being dissuaded (Lettinga et al., 1983[a]). The fluid flow in UASB reactors can be described as a small series of interconnected perfectly mixed reactors, generally one ideal mixer for the compact granular sludge bed near the bottom of the reactor, also one ideal mixer for the flocculant sludge blanket above the granular sludge bed, and if the sludge height is several meters the transition area between sludge bed and blanket can also be regarded as an ideal mixer (Heertjes & van der Meer, 1978; van der Meer, 1979; Heertjes et al., 1982; Bolle et al., 1983).

Biofilms

A biofilm is either a dense layering of micro-organisms attached to an inert surface, or a dense aggregation of micro-organisms held together by electrostatic forces or synthetic or natural polymers. A significant advantage of biofilms over biomass in suspension is that reactions can take place within their depths, even though the bulk liquid composition may not be favourable for this. The presence of biofilms has been acknowledged as one of the reasons for the operating stability of the modern high-rate anaerobic digesters, since biofilms provide micro-environments with an extremely low hydrogen partial pressure allowing propionate degradation even when the composition of the reactor gas indicates that in the bulk environment propionate degradation would be thermodynamically unfavourable (McCarty & Smith, 1986). The presence of biofilms may also enhance process stability of anaerobic digestion during exposure to toxicants and increase the tolerance to toxicants as will be discussed in the following alineas.

Biofilms maybe increase tolerance to toxicants in a way that the film composed of dead and living bacteria together with extracellular polymers formed by the bacteria and organic and inorganic precipitates can act as a more or less impenetrable barrier for some toxicants. This theory has been suggested as explanation for some phenomena in anaerobic treatment of toxic forest industry waste water, but it has not yet been proved satisfactorily (Welander et al, 1988).

In case of degradable toxicants, biofilms may cause a mass transfer resistance in the boundary layer of liquid near the biofilm and cause limitations for inward diffusion of the toxicant, which together with the toxicant degradation potential in the biofilm can result in toxicant concentrations near the end of the biofilm which are below the level at which inhibition occurs, even when in the bulk liquid the toxicant concentration exceeds the maximum toxicity tolerance level. This phenomenon has been proved to occur during exposure of granular methanogenic sludge from an UASB-reactor to capric acid (Rinzema, 1988 [chap. 2]).

Another important feature of biofilms is that they generally contain a back-up reservoir of biomass which is hardly used during normal operation, but becomes activated when the presence of toxicants has inhibited the normally active biomass. Using some general data about anaerobic biofilms it can be concluded that in more or less completely mixed biofilm reactors of which the effluent requirements are in the same range as the substrate saturation constant (K_m), the penetration of the substrate will be only several tenths of mm (Henze & Harremoës, 1983). However, a survey of the characteristics of the granules from five full-scale UASB-reactors showed that the diameter could be as high as 3 mm (Hulshoff Pol et al., 1986). The fact that with UASB granular sludge a positive relationship between the apparent substrate saturation constant for acetate and granule size has been found also indicates that a significant portion of the methanogenic bacteria inside the granules is metabolically active, evenso they seem to be extremely substrate limited (Dolfing, 1985). It is clear that without any difficulties these bacteria will increase their activity when more substrate comes available as a result of inhibited activity in the outer layers of the biofilm. The potential of the reservoir of "stand-by biomass" in granular sludge to mask the effect of toxicants has been demonstrated in research in which at lower substrate concentrations (viz. with incomplete substrate penetration at uninhibitory conditions) the effect of increasing sodium concentrations on the over-all sludge activity was much less than at high substrate concentrations (Rinzema et al., 1988).

The environmental conditions in a biofilm may differ significantly from the bulk liquid environment. Probably the most important environmental factor that influences toxicity is

the pH. Biofilm denitrification models have indicated that due to resistance to out diffusion of bicarbonate and carbonate in the biofilm the pH will be higher than in the surrounding liquid. This effect will be strongest in conditions of low alkalinity (Arvin & Harremoës, 1989). These model predictions have been verified by pH measurements at the rear end of denitrifying biofilms (Arvin & Kristensen, 1979 & 1982). Since acetoclastic methanogenesis also is a bicarbonate generating process, also in methanogenic biofilms pH gradients similar to that in denitrifying biofilms should be expected. Indirect proof of the occurrence of a gradient towards a higher pH in the centre of granular sludge has been obtained in research concerning anaerobic digestion at sub-optimal pH (ten Brummeler et al., 1985) and in research concerning sulfide toxicity (Koster et al., 1986). The recent development of micro-electrodes has enabled direct measurement of the pH inside granular methanogenic sludge (de Beer, 1988; de Beer & van den Heuvel, 1988). Depending on the nature of the granule (especially the amount of calcium carbonate precipitate in the texture) and the substrate concentration outside the granule, the pH-rise towards the centre of the granule can amount to approximately 1 pH unit (de Beer, personal communication). The increased internal pH has been found to increase to tolerance of methanogenic granular sludge for sulfide enormously; at sulfide concentrations at which suspended sludge reportedly fails the granular sludge was hardly affected (Koster et al., 1986).

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CHAPTER 2

LONG-CHAIN FATTY ACIDS INHIBITION OF ACETOCLASTIC METHANOGENESIS IN GRANULAR SLUDGE

GENERAL INTRODUCTION

Inhibition of methanogenesis caused by long-chain fatty acids can occur in anaerobic treatment of lipids containing wastewaters such as the wastewater from slaughterhouses, meat processing industries, rendering operations, bone processing industries, palm oil mills, edible oil refineries, dairy-factories and margarine factories. The long-chain fatty acids are present in animal and vegetable oils and fats as glycerol esters, which during anaerobic digestion are hydrolysed as a result of the activity of exo-enzymes. The research reported in this chapter has been carried out within the framework of a project-study concerning the feasibility of anaerobic treatment (preferably with UASB-reactors or hybrids thereof) of wastewaters from edible oil refining processes. These wastewaters may contain lipids, but since they originate from processes including chemical ester splitting ('soaping') they certainly contain free long-chain fatty acids.

Long-chain fatty acids are well-known inhibitors of microbial activity. They are used as organic preservatives in foods, and they are often included in the diet of ruminants in order to inhibit methanogens which compete with the animal for the intermediairy products of the digestion. As a logic result of their use as feed additive a lot of research has been done concerning the effect of long-chain fatty acids on methanogenic populations, but unfortunately all this work concerns hydrogenotrophic methanogens. (Due to the short retention time the slower growing acetotrophic methanogens can hardly survive in rumen systems). Since in anaerobic wastewater treatment generally at least 70 % of the methane production originates from acetate it was decided that research concerning long-chain fatty acids inhibition of acetotrophic methanogenesis should be carried out.

When the research started, only one study about the subject was available in the international literature [Hanaki et al., Biotechnol. Bioeng., 23(1981) 1591-1610]. In that study dispersed biomass was used. In the present research granular biomass was used, since granular sludge UASB-reactors were the primary option for treatment of the edible oil refinery wastewaters in the project-study. Long-chain fatty acids may show a different physical reaction with dispersed biomass than with granular biomass. The biofilm may protect the inner biomass, while the outer biomass is inactivated by adsorbed long-chain fatty acids. This was an additional reason to use granular sludge in the present research.

Synergism and antagonism are well-known features in light metal cation toxicity for methanogens. Very little is known about synergism and antagonism in long-chain fatty acids toxicity; only one article concerning lactic acid bacteria could be found. The article "Inhibition of methanogenesis from acetate in granular sludge by long-chain fatty acids" included in this chapter is the first in which this phenomenom is described in detail for acetotrophic methanogenic biomass.

As pointed out in chapter 1 of this thesis, the available concentration rather than the total amount present of an inhibitory substance determines the actual extent of inhibition that will occur. The available concentration of long-chain fatty acids can be lowered enormously by adding precipitating chemicals. In the second article included in this chapter (entitled "Abatement of long-chain fatty acid inhibition of methanogenesis by calcium addition") the counteracting effect of calcium chloride on lauric acid inhibition of acetoclastic methanogenesis in granular sludge similar to that used in the initial toxicity assays is described in relation with the time lapse between toxicant dosing and calcium dosing.

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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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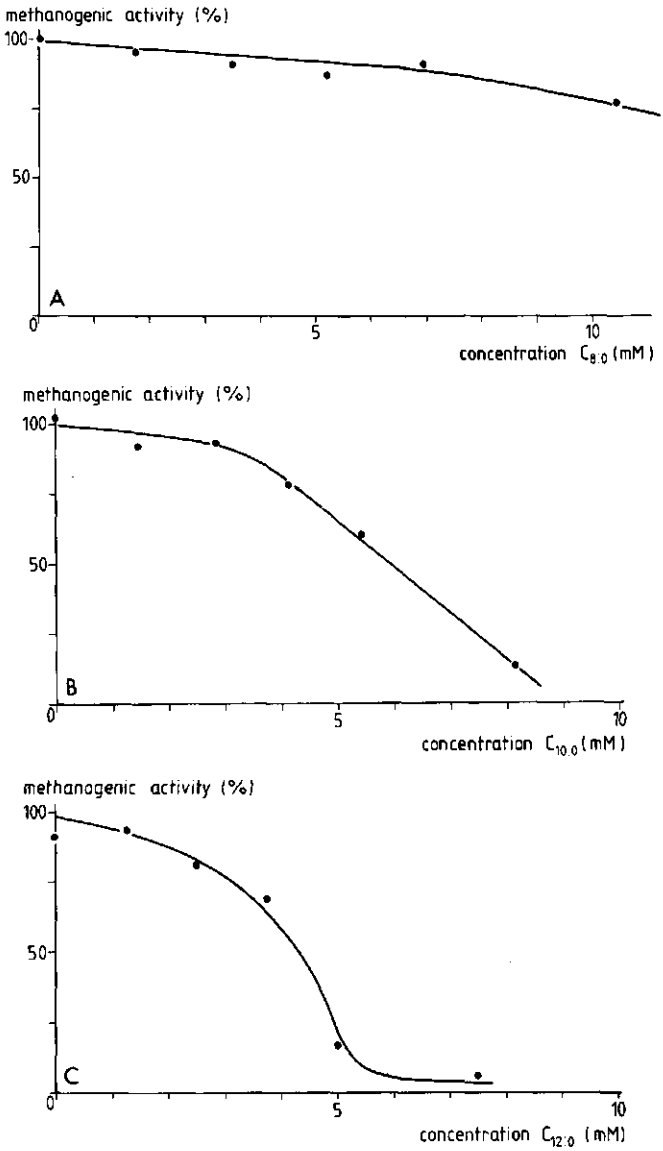

FIG. 1. Remaining methanogenic activity of acetate-fed granular sludge after exposure to caprylic acid (A), capric acid (B), lauric acid (C), myristic acid (D), and oleic acid (E).

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

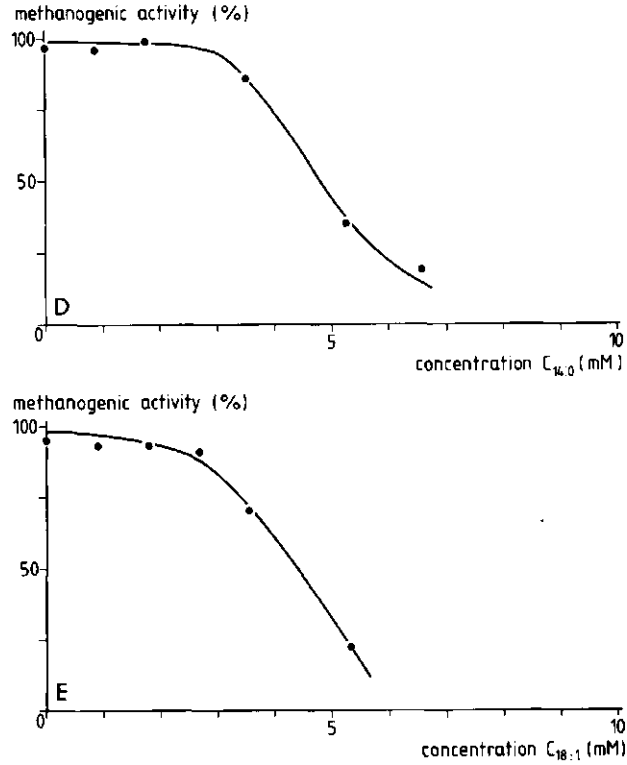

FIG. 1—*Continued*

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

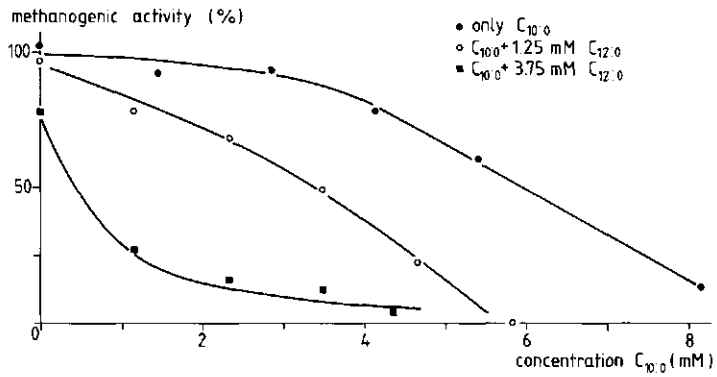

FIG. 2. Remaining methanogenic activity of acetate-fed granular sludge after exposure to capric acid with different background concentrations of lauric acid.

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

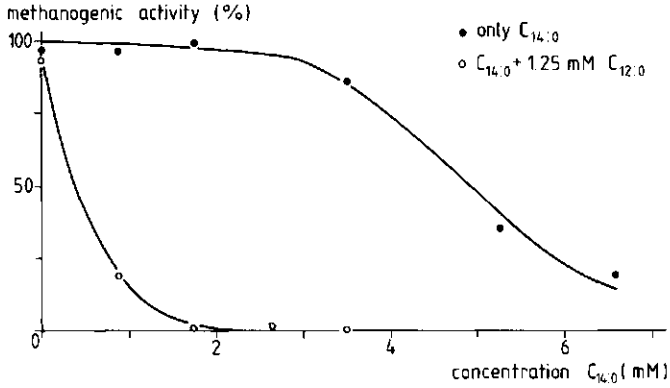

FIG. 3. Remaining methanogenic activity of acetate-fed granular sludge after exposure to myristic acid with or without a background concentration of 1.25 mM lauric acid.

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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Abatement of Long-chain Fatty Acid Inhibition of Methanogenesis by Calcium Addition

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ABSTRACT

Long-chain fatty acids are versatile inhibitors for methanogenic bacteria. Research concerning lauric acid toxicity proved that addition of calcium chloride can reverse lauric acid inhibition of methanogenesis, provided the addition is applied within several minutes after the inhibitory lauric acid concentration has entered the methanogenic environment. The antagonistic effect of calcium chloride is caused by precipitation of the inhibitory long-chain fatty acids as calcium salts.

It has been proved that lauric acid has a strong affinity for methanogenic sludge. The rapid reaction of lauric acid with methanogenic sludge causes the need for almost immediate addition of calcium chloride to prevent inhibition of methanogenesis. Of the methanogenic potential of granular sludge 50% was lost after only 7·5 min of exposure. Addition of calcium chloride after an exposure period of 6 h had no effect at all.

INTRODUCTION

Long-chain fatty acids have been proved to be inhibitory for methanogenic bacteria (Hanaki *et al.*, 1981; Koster & Cramer, 1987). This makes the anaerobic treatment of wastewaters containing long-chain fatty acids relatively troublesome, because methanogenic bacteria play a crucial role in anaerobic wastewater treatment. Long-chain fatty acids are abundant in wastewaters containing natural fats, such as the effluents from dairies, slaughterhouses, palm-oil mills and edible-oil refineries.

Anaerobic treatment operations applied to wastewaters in which long-chain acids can occasionally be present in amounts that are inhibitory for methanogenic bacteria should be provided with a control mechanism to prevent toxic concentrations of the acids from entering the methane-producing reactor. Since the fatty acids can be degraded anaerobically, it should not be necessary to entirely prevent them entering the anaerobic reactors, but the concentration present in the wastewater should be kept below the maximum allowable. Addition of calcium ions to wastewaters in which the concentration of long-chain fatty acids is too high might be an attractive way of preventing the acids from upsetting an anaerobic treatment system by inhibiting the methanogenic bacteria, because calcium salts of long-chain fatty acids are relatively insoluble.

The aim of the research described here was to find out how much time would be available, after the start of the exposure of a methanogenic population present in granular sludge to toxic concentrations of long-chain fatty acids, in which to add calcium ions in order to prevent a fatal inhibition of the methanogenesis. Lauric acid was chosen as the model long-chain acid because it is the strongest potential inhibitor for methanogens of the acids that can be present in any wastewater (Koster & Cramer, 1987).

METHODS

Since approximately 70% of the methane produced during anaerobic wastewater treatment originates from acetate, the effect of calcium addition on lauric acid toxicity was investigated with an acetate-fed methanogenic sludge. The sludge consisted of granules taken from the Upflow Anaerobic Sludge Bed (UASB) reactor treating the wastewater of the potato processing factory of Aviko at Steenderen, The Netherlands.

The effect of 7·5 mM lauric acid on the maximum specific acetoclastic activity of granular methanogenic sludge after various exposure times was assessed using the anaerobic toxicity assay described by Koster & Cramer (1987). In a series of completely mixed batch reactors the uninhibited (in the absence of lauric acid) maximum specific acetoclastic methanogenic activity was assessed by monitoring the methane production from an excess amount of acetate by a known amount of biomass (24 g VSS per reactor). This was followed by a test run with the lauric acid present in the reactors. Lauric acid (7·5 mM) was introduced at the start of the test run, and calcium chloride was also introduced. In one reactor the calcium chloride was added simultaneously with the lauric acid; in the other reactors the calcium chloride was added after a period of exposure of the sludge to the acid. The amount of calcium ions added was equimolar to the amount of lauric acid.

Two experiments related the effect of the exposure time on methanogenic activity to the behaviour of lauric acid in the presence of methanogenic sludge. In 1 litre Erlenmeyer flasks approximately 7·7 g VSS per litre of digested sewage sludge or granular sludge (as used in the activity tests) were supplied with 23·2 mM and 50 mM sodium laurate, respectively, under a nitrogen atmosphere. Both flasks were put on an orbital shaker. At given intervals samples were taken and immediately centrifuged at 13 000 *g* for 15 min and the concentration of lauric acid in the supernatant determined. In these experiments the initial concentration of sodium laurate was higher than in the activity tests, because a relatively high concentration was required for the gas chromatographic analysis. All experiments were performed at 30 ± 1°C and pH 7.

Lauric acid concentrations were determined using a GLC equipped with a 2 m glass column packed with Supelco-port (100–120 mesh) coated with 10% Fluorad 431. Nitrogen saturated with formic acid was used as carrier gas at a flow rate of 50 ml min^{-1}. The column temperature was 190°C, the temperature of the injection port was 220°C and the temperature of the flame ionization detector was 240°C. Prior to injection the samples were diluted four times with methanol.

All other analyses were according to *Standard Methods* (American Public Health Organization, 1975).

RESULTS AND DISCUSSION

The mean uninhibited maximum specific acetoclastic methanogenic activity was 0·58 mmol CH_4.(g VSS.h)$^{-1}$.

The relationship between the maximum specific methanogenic activity remaining after exposure of the sludge to lauric acid and the time elapsed between the addition of sodium laurate and the addition of calcium chloride is shown in Fig. 1. Before the addition of calcium chloride the main salt of lauric acid present was sodium laurate. After the addition of calcium chloride there was enough calcium present to completely convert the sodium laurate into calcium laurate. The solubility of sodium laurate is 197 mM at 30°C, whereas the solubility of calcium laurate is only 0·042 mM at 26·7°C (Stephen & Stephen, 1963). Hence, it is safe to assume that the addition of calcium chloride in an amount equimolar to the amount of sodium laurate already present caused precipitation of all the laurate. Therefore, the period between addition of sodium laurate and the addition of calcium chloride can be interpreted as the period during which the sludge was exposed to the laurate.

In Fig. 1 the point corresponding with exposure time zero shows the effect

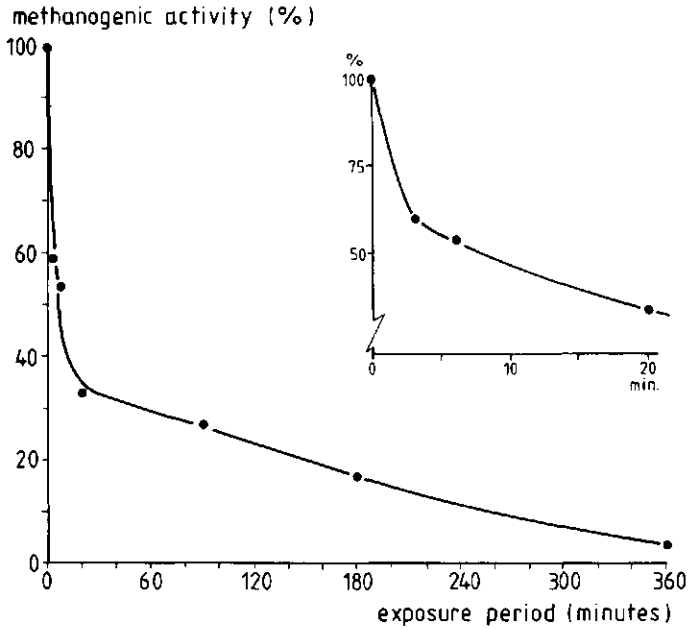

Fig. 1. The remaining maximum specific acetoclastic methanogenic activity of granular sludge after exposure to 7·5 mM sodium laurate at pH 7 (expressed as a percentage of the uninhibited maximum specific acetoclastic methanogenic activity) as a function of the exposure time. The exposure time was determined by the time of calcium chloride addition (see text). Inset, time 0–20 min on full graph.

of 7·5 mM calcium laurate on the methanogenic activity. In this case there is no inhibition. Earlier work showed that 7·5 mM sodium laurate caused 94% inhibition of the methanogenesis from acetate (Koster & Cramer, 1987). This difference between the inhibitory potential of the sodium salt or the calcium salt of a particular long-chain fatty acid agrees with results obtained in research concerning the effect of the presence of long-chain fatty acids in the feed of ruminants on the functioning of the rumen system. In these studies calcium was found to be a strong antagonist to toxicity of long-chain fatty acids for a wide range of rumen bacteria (El Hag & Miller, 1972; Galbraith *et al.*, 1971; Galbraith & Miller, 1973). In an early review of the effect of unsaturated long-chain fatty acids on Gram-positive bacteria, calcium was also mentioned as a compound that can be used to reverse the inhibitory action of long-chain fatty acids (Kodicek, 1949).

From the results shown in Fig. 1 it may be concluded that addition of calcium chloride in order to prevent inhibition of methanogenesis from acetate by lauric acid should be made as soon as possible after the appearance of an inhibitory laurate concentration in the environment of the acetoclastic methanogens. There is no exposure period without any

inhibitory effect. After 3 min of exposure to 7·5 mM of sodium laurate 40% of the activity was lost; after 20 min only 33% of the original activity remained. After 6 h, 4% of the uninhibited activity remained. This is similar to the remaining activity if there is no addition of calcium chloride (Koster & Cramer, 1987), so it can be concluded that after an exposure time of 6 h calcium addition does not produce any immediate restoration of methanogenic activity. In research with a mixture of long-chain fatty acids in which the effect of calcium chloride addition after exposure times exceeding 4 h was investigated, Hanaki *et al.* (1981) found that in all cases the methane production rate was lower than the rate after an exposure time of 5 min. However, addition of calcium chloride after an exposure time of 8 h still resulted in a decrease for 1–2 days of the approximately 30 day lag-period required for recovery of the gas production. Long-term effects were not within the range of the present work.

The instant inhibitory action of lauric acid means that for application in practice of calcium chloride addition to prevent lauric acid toxicity, either an on-line lauric acid analyzer or a device that can instantly detect any upset of the methane production rate in the reactor should be available. A fast toximeter for protection of anaerobic wastewater-treatment systems has been developed recently (Heijnen *et al.*, 1986). If such devices are not available, continuous liming could be applied as a safeguard. The methanogenic population present in an anaerobic wastewater treatment system can be protected to a certain extent against toxicants with a high physico-chemical affinity for organic material such as cell walls by operating the system as a two-phase process. Toxicants with a strong affinity for cell walls will react with the cells in the first, acidogenic, reactor and therefore should not reach the methanogenic population in the second reactor. Because of the high growth rate of its biomass the first reactor is much easier to restart than the second.

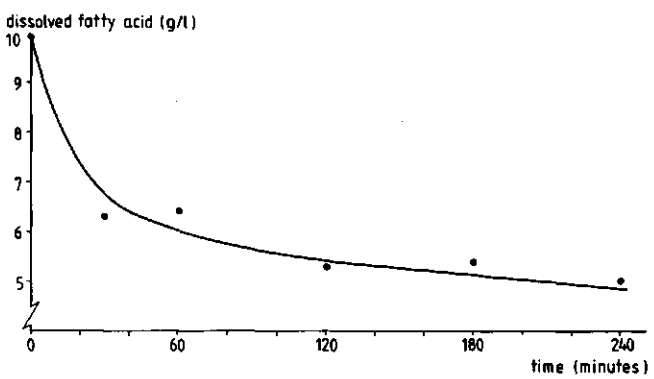

Fig. 2. The concentration of dissolved lauric acid in the presence of 7·7 g VS litre^{-1} of granular methanogenic sludge at 30°C and pH = 7 as a function of contact time.

The necessity of an almost immediate addition of calcium chloride to save the methanogenic potential of the sludge if it becomes exposed to lauric acid indicates that the acid interacts very rapidly with the sludge. This was confirmed by the results of the experiments in which the concentration of dissolved lauric acid in the presence of granular sludge (Fig. 2) or dispersed sludge (Fig. 3) was monitored for several hours. Within half an hour a considerable amount of lauric acid had disappeared from solution. Probably this cannot be attributed totally to adsorption. Research concerning

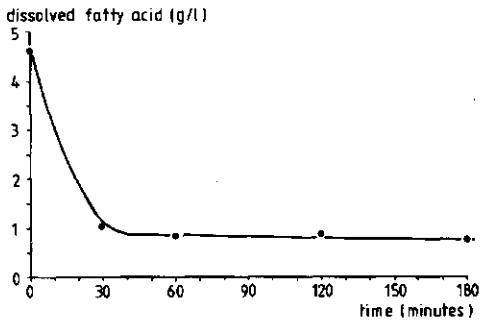

Fig. 3. The concentration of dissolved lauric acid in the presence of 7·7 g VS litre^{-1} of digested sewage sludge at 30°C and pH = 7 as a function of contact time.

adsorption of capric acid onto granular sludge showed that such adsorption processes are rather slow (Keurentjes & Rinzema, 1986). It might be that the rapid disappearance from solution of lauric acid in the presence of methanogenic sludge is caused by precipitation with calcium- and other metal-ions from the cell contents. This loss of vital ions from the cells could account for the loss of their methanogenic activity.

Although the present research dealt only with lauric acid, the general picture of how to deal with a sudden inhibitory concentration of lauric acid that can be derived from its results is most probably also valid for cases with other long-chain fatty acids. Only the allowable exposure time will be different.

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CHAPTER 3

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SULFIDE INHIBITION OF THE METHANOGENIC ACTIVITY OF GRANULAR SLUDGE AT VARIOUS pH-LEVELS

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Abstract—The effect of sulfide on the formation of methane from acetate in granular sludge originating from a UASB reactor has been determined using a new batch anaerobic toxicity assay. The assay is based on measurement of the methane concentration in the closed head space of a serum bottle, thus allowing operation at constant pH and without loss of sulfide via off-gases.

Sulfide toxicity appeared to be correlated with the free hydrogen sulfide concentration in the pH range 6.4–7.2. However this correlation did not hold at pH = 7.8–8.0. Free hydrogen sulfide concentrations leading to 50% inhibition were 250 mgS l^{-1} in the pH range 6.4–7.2 and 90 mgS l^{-1} at pH = 7.8–8.0.

The high tolerance for sulfide toxicity exhibited by the granular sludge can probably be attributed to the existence of a pH gradient in the sludge granules leading to an increased internal pH.

Key words—sulfide, toxicity, inhibition, methanogenesis, anaerobic digestion, Methanothrix, granular sludge, anaerobic toxicity assay

INTRODUCTION

In dark anaerobic environments hydrogen sulfide is the metabolic end-product of the degradation of sulfur containing compounds, either organic or inorganic (Zinder and Brock, 1978; Dunnette *et al.*, 1985). The sulfur content of methanogenic bacteria is unusually high when compared to aerobic microorganisms (Speece, 1983). Sulfide is the major sulfur source of methanogenic bacteria (Zehnder and Wuhrmann, 1977; Zeikus, 1977; Mountfort and Asher, 1979; Scherer and Sahm, 1981; De Zeeuw, 1984). At concentrations exceeding the ppm range in which sulfide stimulates methanogenesis it has an inhibitory effect. When considering that sulfide-inhibition of methanogenesis is one of the major factors that have prevented the commercial application of the anaerobic digestion process to the wastewaters of several major sectors of international industries, sulfide might be ranked as one of the most important inhibitors (Anderson *et al.*, 1982).

Sulfide toxicity is very much dependent on pH, because unionized hydrogen sulfide is able to pass through the cell membrane (Schlegel, 1981; Speece, 1983). The effect of pH on the unionized fraction, of the total amount of sulfide present, is shown in Fig. 1. Below pH = 9 all ionized sulfide will be present as bisulfide (Mosey and Jago, 1977).

Inhibition of methanogenesis by sulfide has been reported several times (Bannink and Muller, 1951; Rudolfs and Amberg, 1952; Aulenbach and Heukelekian, 1955; Butlin *et al.*, 1956, Lawrence *et al.*, 1964; Yang *et al.*, 1979; Kroiss and Plahl-Wabnegg, 1983; Parkin *et al.*, 1983) but lack of precise pH control and variations of sulfide concentration (caused by removal of hydrogen sulfide with the biogas leaving the test reactor) during most of these experiments has prevented a clear interpretation of sulfide toxicity data so far.

In the present article we describe an anaerobic toxicity assay which enables accurate measurement of the specific methanogenic activity (viz. methane production rate per unit biomass) at a constant pH and a constant sulfide concentration. In this assay acetate is used as substrate, because in the anaerobic di-

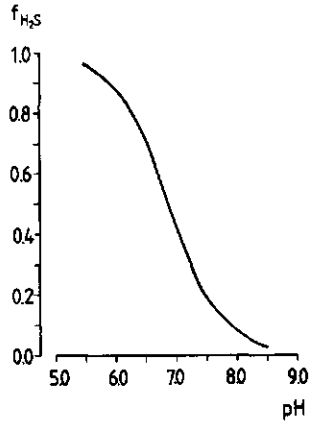

Fig. 1. The fraction fH_2S of the total dissolved sulfide present as free hydrogen sulfide as a function of pH. Calculated using data from Lawrence *et al.* (1964).

gestion of organic material approx. 70% of the methane is produced via acetate (Zehnder, 1978). Usually, industrial wastewaters emenable to sulfide toxicity during anaerobic treatment will contain a lot of sulfate. Although the use of acetate for sulfate reduction is thermodynamically favored over cleavage of acetate to produce methane (Archer, 1983), in anaerobic wastewater treatment processes applied to sulfate-rich wastewaters almost all acetate is used for methanogenesis (Hoeks *et al.*, 1983, 1984; Mulder, 1984; Rinzema and Lettinga, 1986). As source of active methanogenic biomass to be exposed to sulfide we used granular sludge from an Upflow Anaerobic Sludge Bed (UASB) reactor because to date the UASB reactor is by far the most widely applied high-rate anaerobic wastewater treatment system (Van den Berg, 1984; Lettinga *et al.*, 1984). At present granular sludge to be used as seed material for full scale digesters can be obtained on the free market.

MATERIALS AND METHODS

Biomass

The methanogenic mixed culture used in our experiments originated from the UASB reactor of the Aviko potato processing factory at Steenderen, The Netherlands. From this sludge a clean fraction of granules with a diameter in the range 1–2.5 mm was obtained by means of elutriation. The elutriation procedure has been described elsewhere (Tramper *et al.*, 1984). These granules were stored in an acetate-fed batch reactor at 30°C. Apart from the sulfide, the environment in this reactor was similar to the environment in the anaerobic toxicity assay. The predominant acetoclastic methanogenic bacterium in Aviko granular sludge strongly resembles *Methanothrix*, a genus which has such a strong affinity for acetate that it will outcompete all other known acetoclastic methanogens at conditions prevailing in anaerobic high rate wastewater treatment systems (Gujer and Zehnder, 1983). At present two different species have been isolated and characterized (Huser *et al.*, 1982; Patel, 1984).

Chemicals

The basal medium used in the anaerobic toxicity assay contained (mg l^{-1}): $NaHCO_3$ (1000), NH_4Cl (40), KCl (25), $MgCl_2 \cdot 6H_2O$ (11), KH_2PO_4 (9), Na_2SO_4 (9), yeast extract (50). The medium was made up in Wageningen tap water, which contains approx. 35 mg l^{-1} calcium ions. Per litre of medium one ml of a trace element solution according to Zehnder *et al.* (1980) was added. All chemicals were of analytical grade and were supplied by Merck, Darmstadt, F.R.G., except for the gases, which were supplied by Hoekloos, Schiedam, The Netherlands. The yeast extract was supplied by Gist-brocades, Delft, The Netherlands.

Analyses

Volatile Suspended Solids (VSS) were determined according to *Standard Methods* (APHA, 1975). Sulfide was determined photometrically (Trüper and Schlegel, 1964). Acetic acid was determined with a gaschromatograph equipped with a 2 m × 4 mm (i.d.) glass column packed with Supelcoport (100–120 mesh) coated with 10% Fluorad FC 431. The temperature of the column, the injection port and the flame ionization detector were 130, 220 and 240°C respectively. Nitrogen saturated with formic acid was used as carrier gas at a flow rate of 50 ml min^{-1}. Methane was determined with a gaschromatograph equipped with mol sieve 5A (mesh 60–80). The temperature of the column, the injection port and the flame ionization detector were 120, 200 and 250°C respectively. Nitrogen was used as a carrier gas at a flow rate of 20 ml min^{-1}.

The pH was determined with a pH-electrode which was put in the liquid immediately after opening the reaction bottle, in order to avoid a pH rise before measurement due to release of carbon dioxide from the liquid.

Anaerobic toxicity assay

At each pH-range to be tested at least two test runs of six activity measurements were performed. Each test run consisted of two activity measurements at "blank" conditions and four activity measurements at different sulfide concentrations. Activity tests were performed in 1.16 l. glass serum bottles sealed with a 4 mm rubber septum kept in place by a screw-cap. Each serum bottle contained 500 ml of the basal medium and substrate plus a known amount of granular sludge in the range of 0.04–0.20 g VSS per bottle, depending on the expected inhibition.

Taking into account the pH change to be induced by the subsequent flushing operation, before closing a serum bottle the pH was corrected to the desired level by adding sodium hydroxide from a concentrated stock solution. At the start of each experiment the liquid was flushed with nitrogen if the pH had to be near 8 or with a 70% nitrogen/30% carbon dioxide mixture for all other pH's. Flushing was continued until the redox indicator (resazurin) present in the trace element solution changed from light pink to colourless. The serum bottles were placed in a reciprocating shaker situated in a temperature controlled room kept at 30 ± 1°C. The shaker was operated 1 min each 5 min at a frequency of 150 strokes per min.

Except for two test runs at pH = 7.0–7.2 with substrate concentrations of 500 and 2000 mg l^{-1} acetate all activity measurements were performed at 1000 mg l^{-1} acetate. After one day of incubation the pH and the acetate concentration were measured and corrected if necessary. At this time in four out of six serum bottles sulfide was added from a 1 M Na_2S stock solution. After flushing of the gas phase the bottles were incubated one more day. The actual measurement of the specific methanogenic activity was performed on the third day after incubation had been started. The concentration of acetic acid and the pH were measured and corrected if necessary. Sulfide was also measured so that after having calculated the amount of sulfide present in the gas phase Na_2S could be added to make up for the loss of sulfide during subsequent flushing of the gas phase. During a period of 2–3 h, starting one h after flushing of the gas phase, every 20 min two 25 μl samples of the head space of each serum bottle were taken with a gas tight syringe and instantly injected in the gas chromatograph to be analysed for methane. From the slope (S) of the resulting progress line of relative methane concentration and the accessory amount of VSS the specific methanogenic activity expressed as kg COD turnover per unit VSS per day can be calculated using the following equation:

$$A = \frac{S}{100} \cdot V \cdot 24 \cdot f \cdot \frac{1}{\text{VSS}}$$

where

A = specific methanogenic activity (kg CH_4-COD kg^{-1} VSS d^{-1})
S = slope of progress line (% h^{-1})
V = volume of head space (m^3)
f = conversion factor for m^3 CH_4 to kg COD
VSS = Volatile Suspended Solids (kg).

At 30°C the value of f is 2.57 kg COD m^{-3}.

RESULTS

In order to check whether or not an acetate concentration of 1000 mg l^{-1} was high enough to avoid substrate diffusion induced rate limitation,

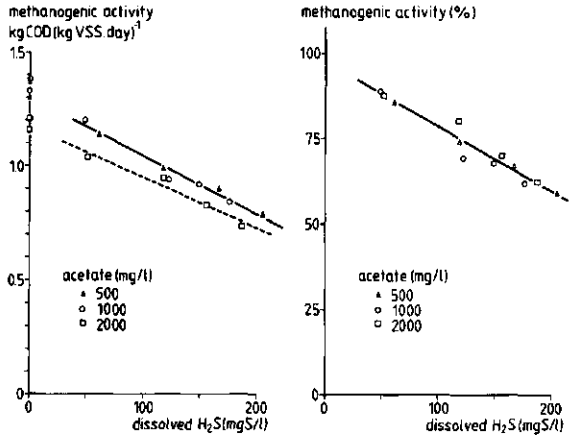

Fig. 2.(a) (left) and 2(b) (right). The specific methanogenic activity of granular sludge as a function of the free hydrogen sulfide concentration in the liquid at pH ≈ 7.0–7.2, determined at various acetate concentrations. The dotted line in Fig. 2(a) refers to measurements at 2000 mg l^{-1} acetate, the continuous line refers to measurements at the other two concentrations. In Fig. 2(b) the remaining activity is given as a percentage of the mean uninhibited activity at the concomitant acetate concentration.

three test runs at pH = 7.0–7.2 with 500, 1000 and 2000 mg l^{-1} acetate respectively were performed. The result of this series of experiments is shown in Fig. 2. In Fig. 2(a) the absolute values of the specific methanogenic activities are given, whereas in Fig. 2(b) the remaining specific methanogenic activity is given as a percentage of the average value of the two blanks at a similar acetate concentration.

In Table 1 the specific methanogenic activities in the experiments without exposure to sulfide in the various pH-ranges tested are tabulated. Because of the apparent (very slight) pH dependency of the uninhibited specific methanogenic activity the effect of sulfide at a particular pH-range should be related to the specific methanogenic activity at that particular pH-range. Therefore we will give the results of our anaerobic toxicity assay as the proportionally remaining specific activity, which means the specific methanogenic activity at a certain sulfide concentration expressed as the fraction of the uninhibited specific methanogenic activity in the same pH-range. This reference specific methanogenic activity is the average value of the results of all blank experiments performed at a certain pH-range.

In Figs 3 and 4 the proportionally remaining specific methanogenic activity at three different pH-ranges is shown as a function of the total sulfide concentration and the free hydrogen sulfide concentration respectively.

Table 1. Uninhibited maximum specific methanogenic activity (kg COD kg^{-1} VSS d^{-1}) of acetate-fed granular sludge at three different pH ranges

pH	Maximum specific methanogenic activity	SD	No. of tests
6.4–6.6	1.23	0.04	4
7.0–7.2	1.29	0.09	6
7.8–8.0	1.25	0.06	4

DISCUSSION

Because in anaerobic digestion the pH should always be in the range 6–8 (Clark and Speece, 1971; Zehnder *et al.*, 1982), the general practice is to incubate batch experiments at approx. neutral pH

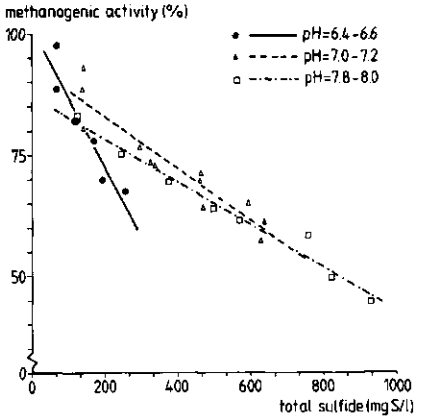

Fig. 3. The max. specific methanogenic activity (expressed as percentage of the uninhibited max. specific methanogenic activity at the concomitant pH range) of acetate-fed granular sludge incubated at 30°C at various pH ranges as a function of the total sulfide concentration. For each pH range a linear regression line has been drawn.

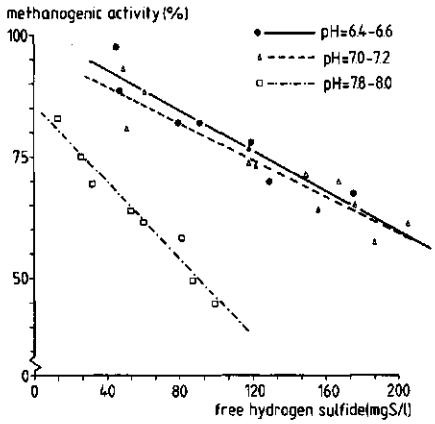

Fig. 4. The max. specific methanogenic activity (expressed as percentage of the uninhibited max. specific methanogenic activity at the concomitant pH range) of acetate-fed granular sludge incubated at 30°C at various pH ranges as a function of the free hydrogen sulfide concentration. For each pH range a linear regression line has been drawn.

values. Consequently a considerable fraction of the total sulfide will be present in the form of free hydrogen sulfide, as is shown in Fig. 1. Considering the value of the dimensionless Henry constant (representing the ratio of the concentration in the gas phase and the concentration in the liquid phase), which is 0.439 at 30°C and a partial gas pressure of 1 atm (Wilhelm *et al.*, 1977), this implies that much of the sulfide added in a toxicity assay applied for methanogenic bacteria will end up in the head space above the reactor liquid containing the biomass to be tested. Consequently in the commonly used anaerobic toxicity assays which are based on monitoring the gas production for several days (or weeks sometimes) the sulfide concentration will continuously decrease because of sulfide transport out of the system with the biogas. This phenomenom will occur in batch reactor toxicity tests from which the biogas is continuously removed (McCarty and McKinney, 1961; Koster and Lettinga, 1984) as well as in Hungate technique derived serum bottle toxicity tests from which the biogas is removed at intervals (Owen *et al.*, 1979; Parkin *et al.*, 1983; Witkowski and Jeris, 1983). In the batch anaerobic toxicity assay of Kroiss and Wabnegg (1983) the loss of sulfide from the liquid is less severe because their method makes use of a reactor head space which is 10 times bigger than the expected gas production.

Normally in batch anaerobic toxicity assays a considerable increase in pH due to the conversion of acetic acid into methane and carbon dioxide occurs. The carbon dioxide will partly diffuse to the gas phase; the carbonic acid remaining in the liquid phase is weaker than acetic acid.

Since in our serum bottle technique all gas produced during the actual period of measurements was kept in the head space which had a constant volume, the sulfide concentration in the liquid remained constant. The very low detection level (approx. 0.5 ppm) of the gaschromatographic methane determination we used made it possible to operate the toxicity assay at an acetate conversion rate of approx. 30 μmol per hour per serum bottle. Such a very small conversion in relation with the amount of acetate present guarantees a nearly constant pH during the tests. The excess pressure resulting from the gas production in a constant volume head space was only some millibars. Elevation of the pressure itself has no effect on the specific methanogenic activity (Ariga *et al.*, 1984) but it should be kept low in order to prevent a change in sulfide concentration in the liquid.

The fact that the maximum specific acetoclastic methanogenic activities measured at 2000 mg l^{-1} acetate are somewhat lower than the maximum specific acetoclastic methanogenic activities measured at 500 and 1000 mg l^{-1} acetate might be attributed to substrate toxicity, but the amount of unionized acetic acid is too low to make this very probable. At pH = 7 and an acetate concentration of 2000 mg l^{-1} the amount of unionized acetic acid present is 11 mg l^{-1}. Duarte and Anderson (1982) reported toxicity at unionized acetic acid concentrations exceeding 10–25 mg l^{-1}, whereas Kroeker *et al.* (1979) reported 30–60 mg l^{-1} as the toxicity range.

The results shown in Fig. 2(b) of the tests performed at different acetate concentrations clearly indicate that in the concentration range of 500–2000 mg l^{-1} acetate does not influence sulfide toxicity.

From Fig. 2(a) the conclusion can be drawn that 1000 mg l^{-1} is sufficient to avoid a mass transfer resistant effect with respect to the substrate on the specific acetoclastic methanogenic activity of the granular sludge. So the inhibitory effect of sulfide on the biomass activity will not be masked by a partial abolition of substrate transfer limitation. The absence of substrate transfer limitation means that all bacteria present in the granules are saturated with substrate, so that the observed specific acetoclastic methanogenic activities are the maximum achievable. The absence of substrate transfer limitation at acetate concentrations exceeding 500 mg l^{-1} is in accordance with results obtained with granular methanogenic sludge cultivated on wastewater from beet sugar producing and processing factories (Tramper *et al.*, 1984; Dolfing, 1985).

In the often cited reviews concerning toxicity in anaerobic digestion of McCarty (1964) and Kugelman and Chin (1971) an upper limit of tolerable sulfide concentrations of 200 mgS l^{-1} is advocated. This is based on research with sewage sludge digesters which completely failed when the total sulfide concentration was increased from 200 to 390 mgS l^{-1} (Lawrence *et al.*, 1964). From the results

Table 2. Concentrations of the various sulfide forms at 50% inhibition of the maximum specific methanogenic activity of granular sludge fed with acetate. The values are calculated from the linear regression lines shown in Figs 3 and 4

pH	Total sulfide (mgS l^{-1})	Free hydrogen sulfide (mgS l^{-1})	Bisulfide (mgS l^{-1})
6.4–6.6	357	246	111
7.0–7.2	810	252	558
7.8–8.0	841	90	751

shown in Fig. 3 and Table 1 it can be seen that in our experiments at 200 mgS l^{-1} a maximum specific acetoclastic methanogenic activity of 0.9–1.1 kg COD kg^{-1} VSS d^{-1} remained. Such a maximum specific methanogenic activity is well in the range of activities which have been reported for mixed cultures present in one-stage anaerobic wastewater treatment systems treating a wide variety of wastewaters (Henze and Harremoës, 1983). With the modern high-rate anaerobic wastewater treatment systems, which are characterized by a very good sludge retainment (Van den Berg and Kennedy, 1983; Callander and Barford, 1983), much lower maximum specific methanogenic activities can be allowed. In such systems the high biomass concentration makes up for the loss of activity caused by inhibition. If we assume that a maximum specific *acetoclastic* methanogenic activity of 0.5 kg COD kg^{-1} VSS d^{-1} suffices to economically operate a UASB-reactor a sulfide concentration of approx. 1000 mgS l^{-1} would be tolerable at neutral or alkaline pH values. It should be noted that in the case of sulfide toxicity in the anaerobic treatment of sulfate containing wastewater the hydrogenotrophic methanogenic bacteria will be outcompeted by the sulfate reducing bacteria, resulting in a decrease of the overall maximum specific methanogenic activity of the sludge. However, the maximum specific *acetoclastic* methanogenic activity will not be affected by the ingrowth of sulfate reducing bacteria, since these sulfate reducing bacteria do not consume acetate and have a growth yield which is comparable to the growth yield of the hydrogenotrophic methanogenic bacteria which they replace (Rinzema and Lettinga, 1986).

The results shown in Fig. 3 clearly show that an acidic pH intensifies the inhibitory effect of sulfide on acetoclastic methanogenesis, as compared to the effect of sulfide at neutral or alkaline pH values. If the relationship between the maximum specific acetoclastic methanogenic activity and the free hydrogen sulfide concentration at various pH-levels is contemplated (Fig. 4) it appears that the free hydrogen sulfide concentration cannot be used as the sole parameter to describe sulfide inhibition in anaerobic digestion, as is suggested by Kroiss and Plahl-Wabnegg (1983). A good correlation between the free hydrogen sulfide concentration and the maximum specific acetoclastic methanogenic activity exists at neutral and acidic pH values. At pH = 7.8–8.0, where the fraction of free hydrogen sulfide is only approx. 10%, the maximum specific acetoclastic methanogenic activity decreased much faster with an increasing free hydrogen sulfide concentration than at the other pH values tested. In the alkaline pH range the total sulfide concentration appears to dictate the inhibition (Fig. 3). This phenomenon might be caused either by an inhibitory effect of the bisulfide ion which becomes apparent only at increased concentrations or by an increased susceptibility for free hydrogen sulfide of the acetoclastic methanogens near the limit of their physiological pH range.

Notwithstanding the lack of an unambiguous explanation for the role of the bisulfide ion in sulfide toxicity, from our work it can be concluded that 50% inhibition of the maximum specific acetoclastic methanogenic activity of granular sludge occurred at a free hydrogen sulfide concentration of 250 mgS l^{-1} or a total sulfide concentration of 825 mgS l^{-1}, depending of the pH range (Table 2).

The similarity of the relationship between maximum specific acetoclastic methanogenic activity and total sulfide concentration at pH = 7.8–8.0 and pH = 7.0–7.2 might be attributed to the existence of a pH gradient in the granula. In the granula, apart from methane, bicarbonate is being produced. The diffusional resistance against transport of the bicarbonate out of a granule causes a bicarbonate gradient in the granule, with the highest concentration near the center. Since in methanogenic environments bicarbonate is the principal buffering agent this results in a pH gradient with the highest pH near the centre of the granule. The existence of such pH gradients has been confirmed for denitrifying biofilms (Arvin and Kristensen, 1979; Arvin and Kristensen, 1982). Some indications for the existence of a pH gradient in granular methanogenic sludge have been reported recently (Ten Brummeler *et al.*, 1985). The existence of a pH gradient in biofilms such as granular sludge might also explain the fact that in our study the effect of free hydrogen sulfide on methanogenesis appeared to be less severe than had been reported in previous studies (all concerning dispersed sludges). Another explanation might be the existence of a sulfide gradient as the result of sulfide stripping by biogas leaving the biofilm.

CONCLUSIONS

The serum bottle anaerobic toxicity assay presented in this article is a relatively simple technique for quantifying the effect of certain chemicals on acetate cleaving methanogenic bacteria. The technique enables tests to be run at a constant pH without the necessity of a pH-controlling apparatus.

The effect of sulfide on the maximum specific acetoclastic methanogenic activity of granular sludge depends on the pH. In the pH range 6.4–7.2 a good correlation was found between the unionized hydrogen sulfide concentration and the maximum specific

acetoclastic methanogenic activity. However at pH = 7.8–8.0 the maximum specific acetoclastic methanogenic activity decreased faster with an increasing concentration of unionized hydrogen sulfide than in the neutral and acidic pH ranges. At pH = 7.0–7.2 and pH = 7.8–8.0 the same correlation between maximum specific acetoclastic methanogenic activity and total sulfide concentration was found.

The average uninhibited specific maximum acetoclastic methanogenic activity of the granular sludge was 1.26 kg COD kg^{-1} VSS d^{-1}. This was slightly influenced by pH, the neutral value being optimal. Unionized hydrogen sulfide concentrations leading to a 50% decrease of the maximum specific acetoclastic methanogenic activity were found to be 250 mgS l^{-1} in the pH range 6.4–7.2 and 90 mgS l^{-1} at pH = 7.8–8.0.

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CHAPTER 4

THE EFFECT OF AMMONIA ON THE MAXIMUM SPECIFIC ACTIVITY OF GRANULAR METHANOGENIC SLUDGE

GENERAL INTRODUCTION

Since ammonia is an end-product of protein degradation it is present in almost all agro-industrial waste streams enable to anaerobic digestion. Depending on the type of raw material and the processes applied the concentration of ammonia in the waste streams may vary a lot, although the general tendency in industry to reduce the use of water will lead to higher concentrations. In this chapter the range of ammonia concentrations from non-inhibitory low to extremely high is covered.

Since even in the recent past most work concerning toxicity in anaerobic digestion was done with practical reactors or laboratory scale simulations of practical circumstances, almost all attention went to conditions leading to more or less complete failure of the process. Such research results in guidelines concerning maximum allowable concentrations, but little quantitative attention has been paid to inhibition at concentration levels at which the practical reactors or their simulations still work in an economically feasible manner, albeit less efficient or at a lower loading rate. To my best knowledge the article "The influence of ammonium-nitrogen on the specific activity of pelletized methanogenic sludge" (which is the first one included in this chapter) is the first in which the effect of increasing ammonia concentrations below the toxicity threshold level is evaluated in terms of specific activity. In that article the importance of quantitative knowledge of the effect of inhibitors at relatively low concentrations is illustrated with the example of a UASB-reactor treating diluted potato juice. Without changing the COD-loading rate a change of ammonia concentration from circa 1400 mg N/l to circa 900 mg N/l by means of further diluting the influent relieved overloading of the biomass caused by ammonia inhibition of methanogenic conversions.

If ammonia concentrations exceed 1700 - 1800 mg N/l the methanogenic population has to adapt (or acclimate) before any substantial methanogenesis can occur. In previous research at the Wageningen Agricultural University [Van Velsen, Water Research 13(1979) 995-999] the fact that methanogenic populations fed with volatile fatty acids can become adapted has been proved. In the article " Characteristics of the pH-influenced adaptation of methanogenic sludge to ammonium toxicity" (which is the second one included in this chapter) the interference of acidogenic conversions (via the pH and the volatile fatty acids concentration) with the methanogenic adaptation process is described. Moreover, the research reported in that article quantifies the methanogenic activity of the same population before and after the adaptation process. Also in that article an evaluation of the nature of the adaptation process (viz. growth of resistant strains/mutants or actual metabolic changes in the bacteria already present) is presented.

The adapted biomass obtained during the research concerning the pH-influenced adaptation was used to assess the maximum ammonia tolerance of methanogenic populations present in granular biofilms. This is described in the third article included in this chapter, titled "Anaerobic digestion at extreme ammonia concentrations". Methanogenesis proved to be possible at almost 12 g N/l; a higher value has not yet been reported. Unlike almost all other publications concerning extreme tolerance of methanogenic populations for inhibitory substances, this article not only reports the tolerance level but also quantifies the effect of extreme ammonia concentrations in terms of specific activity. However, more important than the tolerance level is the finding that even at extreme ammonia concentrations which severely inhibit methanogenesis, acidogenesis is hardly affected.

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The Influence of Ammonium-Nitrogen on the Specific Activity of Pelletized Methanogenic Sludge

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ABSTRACT

The influence of ammonium-nitrogen concentrations in the range 680–2601 mg litre^{-1} *on the specific activity of pelletized methanogenic sludge was investigated in batch experiments. A discontinuous linear negative correlation was found between ammonium-nitrogen concentration and methane production rate. The slope of the discontinuous correlation changed sharply at an ammonium-nitrogen concentration in the range 1600–1700 mg litre*$^{-1}$*. The reversibility of the effect of ammonium-nitrogen was shown in a continuously fed experiment using an Upflow Anaerobic Sludge Blanket (UASB) reactor.*

Some proof was gathered for the hypothesis that acetate-consuming methanogenic bacteria are more strongly affected by ammonium-nitrogen than hydrogen-consuming methanogenic bacteria. In gradually increasing ammonium-nitrogen concentration the methanogenic sludge can adapt to ammonium-nitrogen concentrations above 1700 mg litre^{-1} *without the occurrence of a lag-phase.*

INTRODUCTION

In anaerobic digestion organic matter is transformed into methane via a series of successive bacteriological processes. Apart from organic matter, wastewaters generally contain inorganic substances, some of which can be inhibitory at high concentrations to the bacterial populations present in

In this text ammonium-nitrogen is the sum of NH_3 and NH_4^+ expressed as N

digesters. Four metabolic groups of bacteria can be distinguished. There are, hydrolytic bacteria which break down polymers such as proteins and carbohydrates into their monomers; fermentative bacteria which ferment these monomers to organic acids, alcohols, carbon dioxide, hydrogen and ammonia; acetogenic bacteria which convert higher VFA into acetic acid and hydrogen; and methanogenic bacteria which utilize methanol, acetate and/or hydrogen and carbon dioxide to produce methane.

The methanogenic bacteria are the key organisms in anaerobic digestion. Their low growth rate, high substrate specificity and relatively high susceptibility to environmental stress frequently make the methanogenesis the most critical phase of the process, the more so because it is only after completion of this step that a satisfactory efficiency in the wastewater treatment process is achieved. So knowledge of the effect of substances inhibiting methanogenic bacteria is of vital importance for optimal application of anaerobic digestion.

The effect of any substance, irrespective of its nature and origin, on the metabolism of an organism is primarily concentration-dependent. Frequently, the effect of metabolites is stimulative in the lower concentration range whereas, in the higher concentration range, they become inhibitory (Kugelman & Chin, 1971).

A very common metabolic end product in the anaerobic digestion of protein-containing wastewater is ammonium-nitrogen which is of relatively high potential toxicity for methanogenic bacteria. In effluents originating from the digestion of a concentrated waste, high ammonium-nitrogen concentrations may occur, e.g. wastewater from a baker's yeast factory (Trulsson, 1979), liquid manure (Hobson *et al.*, 1981), settled sewage sludge (Albertson, 1961; Melbinger & Donnellon, 1971) and wastewater from the potato starch industry (Van Bellegem, 1980).

So far research concerning ammonium-nitrogen toxicity in anaerobic digestion has been mainly focused on very high ammonium-nirogen concentrations, exceeding approximately 1700 mg litre^{-1}. The reason for this is that 1700 mg litre^{-1} is considered as a threshold level above which methane formation is only possible after a certain period of acclimatization. This implies that, during the start up of a digester system working at such high ammonium-nitrogen concentrations, a considerable unbalance of the digestion process frequently occurs (Van Velsen, 1979*a*, *b*).

The start up of an anaerobic digester system generally is considered to proceed without severe difficulties at ammonium-nitrogen concentrations

below the supposed threshold level. Whether or not this really is true is open to discussion. Recently, Hulshoff Pol *et al.* (1982*b*) clearly demonstrated a significantly retarded start up (i.e. pelletization) of a USB reactor at an ammonium-nitrogen concentration of approximately 1000 mg litre^{-1} relative to USB reactors started up at 400 mg litre^{-1} and lower.

The possible adverse effect of ammonium-nitrogen at low concentrations on anaerobic digestion has never been adequately investigated so far. This paper describes experiments intended to assess the effect of ammonium-nitrogen at concentrations in the range 680–2601 mg litre^{-1} on the methanogenic activity of pelletized sludge.

METHODS

Materials

Successive batch experiments were performed in a 4-litre glass bottle placed in a temperature controlled room at 30 ± 2 °C. The reactor contents (3 litre) were mixed by a mechanical stirrer for 20 s each 20 min at 60–100 rpm.

Methane production was determined using a sodium hydroxide solution displacement system. The strength of the sodium hydroxide solution was such that all carbon dioxide was removed from the biogas.

A continuously fed experiment was performed in a 5·25 litre USB reactor (Lettinga *et al.*, 1980) in which a temperature of 14 ± 1 °C was maintained by flushing tapwater through the water-jacket surrounding the reactor. Carbon dioxide was removed from the biogas with a concentrated sodium hydroxide solution and methane production was monitored with a wet gasmeter.

The methanogenic sludge used in all experiments was pelletized sludge obtained from an industrial USB reactor treating the wastewater from a beet-sugar factory (Pette *et al.*, 1979). The predominant bacteria in this sludge are acetate-utilizing methanogenic bacteria, presumably the species *Methanobacterium soehngenii* (Hulshoff Pol *et al.*, 1982*a*) which is an acetate-decarboxylating, non-hydrogen-oxidizing methane bacterium recently isolated and characterized by Zehnder *et al.* (1980).

The substrate used in the batch experiments consisted of a mixture of acetic acid, propionic acid and butyric acid (in stock solution 200 g litre^{-1}

TABLE 1
Composition of Potato Juice

Total Solids	4·5–5·3 Wt-%
Protein and amino acids	2·2–3·0 Wt-%
Carbohydrates	0·4–0·8 Wt-%
Potassium	0·4–0·8 Wt-%
Di-phosphate	0·06–0·2 Wt-%
Chemical Oxygen Demand	approx. 54 g litre^{-1}
Kjeldahl-nitrogen	3·4–3·8 g litre^{-1}

Source: Van Bellegem & Lettinga (1975).

each). Trace elements and phosphate were added according to Zehnder *et al.* (1980).

The substrate used in the continuous experiment was diluted potato juice (i.e. the liquid fraction of mashed raw potatoes). The general composition of potato juice is shown in Table 1.

Analysis

For determining the sludge concentration, TSS and VSS of a well mixed sample were determined according to *Standard Methods for the Examination of Water and Wastewater* (1975), as was Chemical Oxygen Demand. Ammonium-nitrogen was determined by the Direct Nesslerization Method as described in the Standard Methods.

VFA were determined directly from the liquid phase of centrifuged (14 000 *g*, 15 min) samples using a gas chromatograph equipped with a 1 m × 4 mm glass column packed with Chromosorb W-AW (80–100 mesh) coated with 20% Tween 80. The column temperature was 115 °C, and that of the injection port and FID detector, 170 °C. Nitrogen saturated with formic acid was used as the carrier gas at a flow rate of 60 ml min^{-1}.

Experimental procedure

In order to maximize the methanogenic activity of the seed sludge successive VFA feedings were applied until no further increase of specific activity occurred. Then the ammonium-nitrogen inhibition experiments were started using similar VFA feedings but at increased ammonium-nitrogen concentrations achieved by adding ammonium chloride to the

TABLE 2
Results of Batch VFA-Fed Experiments with 5·10 g VSS Methanogenic Sludge at Various Ammonium-Nitrogen Concentrations

Ammonium-nitrogen ($mg\ litre^{-1}$)	*Maximum methane production rate* ($ml\ h^{-1}$)	*Activity* (%)
680	130	100
759	97	74·6
853	76	58·5
853	66	50·8
1 351	45	34·6
1 653	19	14·6
1 666	20	15·4
2 101	14	10·8
2 601	10	7·7

reactor contents a few hours before VFA were added. Any new substrate addition was made only after the potentially possible amount of methane from the preceding feed had been generated. The ammonium-nitrogen concentrations applied in the batch experiments are summarized in Table 2.

The pH of the reactor contents was kept in the range 7·6–7·95 by, if necessary, adding sodium hydroxide or HCl. On opening the bottle, anaerobic conditions were maintained by passing nitrogen through the contents.

The USB reactor was started using 3 × diluted potato juice. The load was increased stepwise and the buid up of VFA in the reactor effluent was used as the main indicator for the loading potentials of the reactor.

After the VFA in the effluent reached a value of 3–3·5 g $litre^{-1}$ (expressed as COD) the organic loading rate was maintained at the same value, whereas the dilution factor of the potato juice was increased from 3 to 4 in order to assess the effect of a decreasing ammonium-nitrogen concentration.

RESULTS

The cumulative methane productions from a batch VFA feed at various ammonium-nitrogen concentrations are shown in Fig. 1. The lower

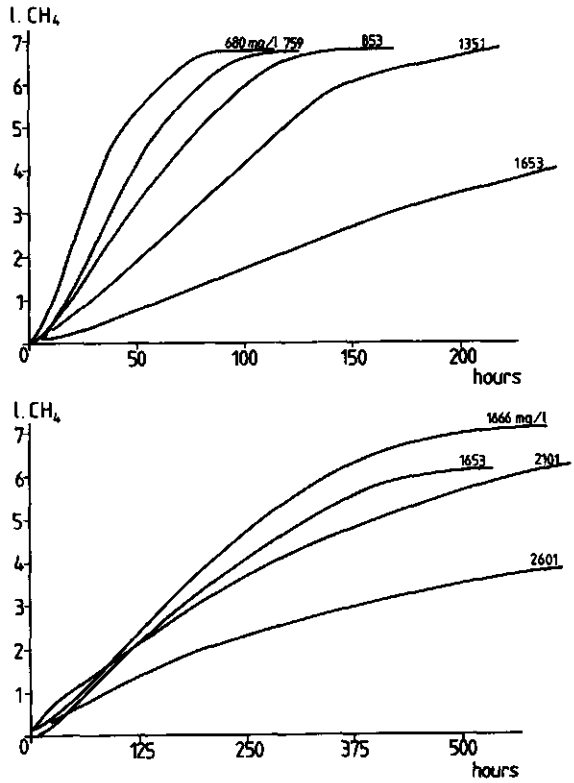

Fig. 1. The cumulative methane production as measured in a 3-litre VFA-fed batch experiment in which the NH_4^+-N concentration was stepwise increased from 680 mg litre^{-1} to 2601 mg litre^{-1}. The NH_4^+-N concentrations in the successive feedings are shown on the curves. The experiments were carried out at 30 °C with a granular sugar beet wastewater sludge.

TABLE 3

VFA Composition in the Batch Experiment at an Ammonium-Nitrogen Concentration of 2 601 mg litre^{-1}

Time (*h*)	*Acetic acid* (*mg litre*$^{-1}$)	*Propionic acid* (*mg litre*$^{-1}$)	*Butyric acid* (*mg litre*$^{-1}$)	*Methane production* (*per cent of potential*)
0	1 333	1 333	1 333	0
736	2 565	490	4	62·4
1 172	2 459	85	4	74·6

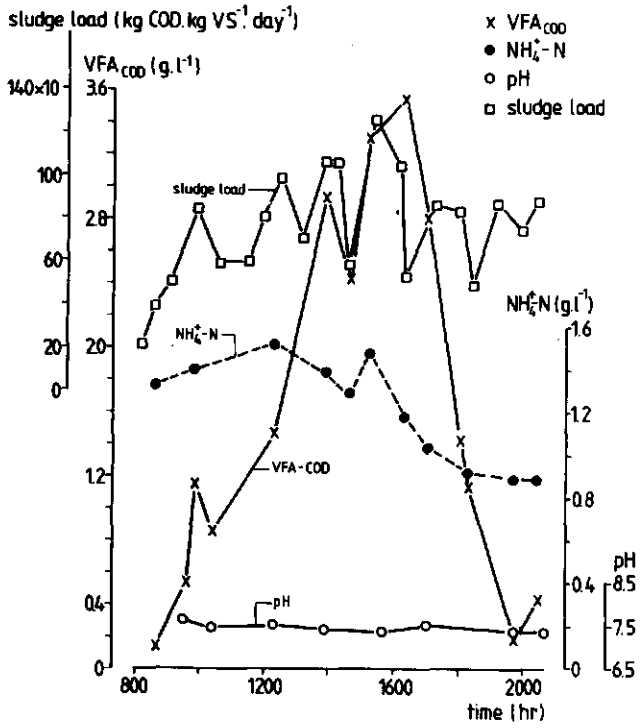

Fig. 2. Results obtained over the period 800–2100 h in a 5·25-litre UASB reactor using a diluted potato sap solution as feed. The experiment was performed at 14 °C and with a granular sugar beet wastewater sludge as seed. The dilution factor of the feed solution was increased from 3 to 4 at $t = 1550$ h.

methane production in the experiment at an ammonium-nitrogen concentration of 1653 mg litre^{-1} is due to an erroneous supply of a too small amount of VFA mixture. The experiment at an ammonium-nitrogen concentration of 2601 mg litre^{-1} was terminated after 1800 h, i.e. before all VFA were removed. About 1000 h after the start of the experiment the methanogenic activity of the sludge fell off. The sludge failed to recover during the next 800 h. Table 3 shows the build up of acetic acid during this experiment. The loading rate applied in the experiment with the USB reactor and the composition of the effluent before and after increasing the dilution factor of the potato juice used as the influent are shown in Fig. 2. In this case the predominant VFA present in the effluent was propionic acid (Fig. 3).

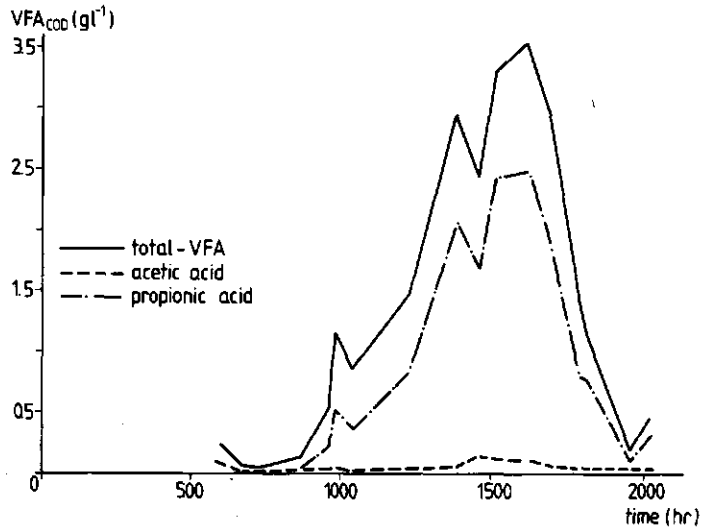

Fig. 3. VFA analysis of the effluent of a USAB reactor using a diluted potato sap solution as feed. The experiment was performed at 14 °C. At $t = 1550$ h the dilution factor of the feed solution was increased from 3 to 4 whereas the sludge load was not changed. See also Fig. 2.

DISCUSSION

The results presented in Fig. 1 show a decreasing methanogenic activity with an increasing ammonium-nitrogen concentration, which confirms the results of experiments with piggery waste (van Velsen & Lettinga, 1981). With respect to methane production rates during the period in which substrates are present in excess, there is no significant difference between the results obtained in the two consecutive experiments at ammonium-nitrogen concentrations of 1653 mg litre^{-1} and 1666 mg litre^{-1}. As any lag-phase was absent, the bacteria apparently adapted instantly to these ammonium-nitrogen concentrations. Considering the nearly equal maximum methane production rates in both experiments, this adaptation is almost complete.

It is noticeable that, in the experiment at an ammonium-nitrogen concentration of 2101 mg litre^{-1} methane production started immediately, although at a rate much less than that prevailing during the previous experiment at an ammonium-nitrogen concentration of 1666 mg litre^{-1}. The lag-phase mentioned in many reports dealing with

ammonium-nitrogen inhibition studies (Van Velsen, 1979*a*,*b*; Braun *et al.*, 1981; Van Velsen, 1981) did not occur. However, Melbinger & Donnellon (1971) also did not observe any clear lag-phase in the start up of a high-rate sewage sludge digester provided the loading rate, and accordingly the ammonium-nitrogen concentration, was raised gradually.

The absence of any clear lag-phase was attributed by Melbinger & Donnellon to the fact that, above the ammonium-nitrogen threshold level of approximately 1700 mg litre^{-1}, the imposed increase of the loading rate had not exceeded the adaptation ability of the methanogenic bacteria. The results of the experiments presented in this paper indicate that methanogenic bacteria can more or less prepare themselves for ammonium-nitrogen concentrations exceeding the threshold level while they are still at an ammonium-nitrogen concentration below the threshold level. The maximum methane production rate at various ammonium-nitrogen concentrations can be derived from the curves shown in Fig. 1 by determining the maximum slope (Table 2). (The initial slope in the 2101 curve is ignored because it concerns a very short period of time.)

As shown in Fig. 4, these data can satisfactorily be fitted to two linear relationships between methanogenic activity and ammonium-nitrogen

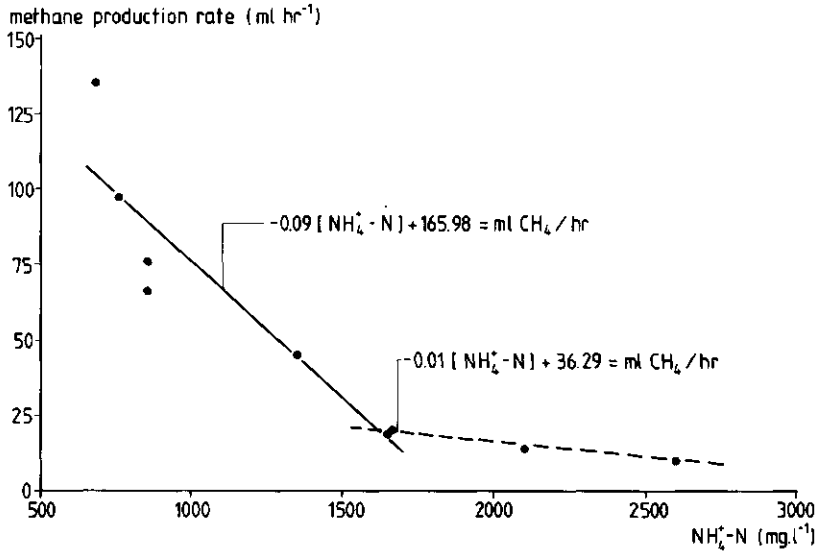

Fig. 4. The effect of the NH_4^+-N concentration on the methane production rate as measured in a 3-litre VFA fed batch experiment in which the NH_4^+-N concentration was stepwise increased from 680 mg litre^{-1} to 2601 mg litre^{-1}.

concentration, one applying to the concentration range 680 to approximately 1700 mg litre^{-1} (correlation coefficient, 0·92) and the other applying to the concentration range approximately 1700 to 2601 mg litre^{-1} (correlation coefficient, 0·99). The correlation coefficients are so satisfactory that the linear relationships, as expressed in Fig. 4, may be considered valid in the respective concentration ranges. Both relationships cross at the ammonium-nitrogen concentration of 1620 mg litre^{-1} which indeed is in the range of the ammonium-nitrogen threshold level.

The data in Fig. 4 indicate the ability of pelletized sludge to produce methane at ammonium-nitrogen concentrations well above the threshold level although the activity of the sludge is considerably lower than at ammonium-nitrogen concentrations below the threshold level. On the other hand, the sharp decline in the specific activity of the methanogenic sludge in the ammonium-nitrogen concentration range 680 to approximately 1700 mg litre^{-1} should not be absolutized, as it should be recognized that the adaptive conditions for methanogenesis have not been optimized in the batch experiments. This is particularly the case with respect to the pH, which was kept at a rather high level because free ammonia is supposed to be more toxic than the ammonium-ion (McCarty & McKinney, 1961).

The results obtained in the research presented are of considerable practical value in applying anaerobic treatment to the wastewater of the potato-starch industry. One of the most important measures for solving the water-pollution problem of this industry is decreasing the water consumption in the production process (Drenth, 1979). This implies a sharp increase in the ammonium-nitrogen concentration in the wastewater. Considering the relationship found between the methanogenic activity of the granular sludge and the ammonium-nitrogen concentration, application of anaerobic treatment needs a careful management of the process, particularly during the start up, even if a granular seed sludge is used. The results of the USB experiment shown in Fig. 2 clearly illustrate the importance of careful management of the production process with respect to the ammonium-nitrogen concentration in the resulting wastewater.

The build up of VFA, mainly propionic acid, in the period 900–1400 h indicates that the sludge load (kilograms of COD turnover per kilogram of biomass (as VSS) per day) exceeded the sludge activity under the prevailing experimental conditions. This was no longer the case when the ammonium-nitrogen concentration was decreased by raising the dilution

factor of the potato juice from 3 to 4. The response of the digestion process was almost immediate—a sharp decrease of the VFA concentration.

The results of the USB experiment show that, as far as VFA are concerned, the breakdown of propionic acid is limited during the period of overloading (Fig. 3). As propionic acid can only be degraded at extremely low hydrogen concentrations (Kaspar & Wuhrmann, 1978; Heyes & Hall, 1981) the inhibited breakdown should be attributed to an ammonium-nitrogen inhibited metabolism of the hydrogen-consuming methanogenic bacteria. Apparently, the metabolism of the acetate-consuming methanogenic bacteria is not—or much less—affected by ammonium-nitrogen at concentrations below the threshold level of approximately 1700 mg litre^{-1}. The acetate build up in the batch experiment at an ammonium-nitrogen concentration of 2601 mg litre^{-1} indicates that above the threshold level of about 1700 mg litre^{-1} ammonium-nitrogen has relatively more effect on the metabolism of the acetate-consuming methanogenic bacteria than on the hydrogen-consuming methanogenic bacteria. Since all experiments were performed with mixed bacterial populations of unknown composition it is impossible to draw valid conclusions about the quantitative effect of ammonium-nitrogen on the various kinds of methanogenic bacteria; only indications can be noted.

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Characteristics of the pH-influenced Adaptation of Methanogenic Sludge to Ammonium Toxicity

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Increasing ammonium-nitrogen concentrations caused failure of methanogenesis at 1900–2000 mg dm^{-3}. After an adaptation period characterised by an almost nil methane production, methanogenesis appeared to be possible at even higher concentrations. A kinetic analysis of methane production during the adaptation process indicated that the adaptation was the result of a metabolic change in the methanogenic bacteria already present, rather than of growth of new bacteria. A high pH value causing toxic concentrations of un-ionised ammonia during the adaptation period appeared to result in a decreased maximum specific methanogenic activity of the adapted sludge. A low pH value during the adaptation period resulted in a retarded degradation of propionic acid, probably due to inhibition of the hydrogen consuming methanogenic bacteria by undissociated volatile fatty acids, but this did not result in a decreased maximum specific methanogenic activity in the adapted sludge. The maximum specific methanogenic activity at an ammonium-nitrogen concentration of 2315 mg dm^{-3} after adaptation as a percentage of that at 1000 mg dm^{-3} before adaptation was 31, 65 and 61% for a pH during the adaptation period of 7.6, 7.25 and 7.0 respectively. Except for the sludge which was maintained at pH 7.6 during the adaptation period, after adaptation the maximum specific methanogenic activity at an ammonium-nitrogen concentration of 2315 mg dm^{-3} was higher than the maximum specific methanogenic activity at an ammonium nitrogen concentration of 1900 mg dm^{-3} before adaptation.

Keywords: Toxicity; ammonium; volatile fatty acids; adaptation; methanogenic activity; anaerobic digestion; kinetics.

1. Introduction

In anaerobic digestion organic matter is converted into methane and carbon dioxide via a series of interrelated microbial metabolisms, including the formation of organic acids. If the methane formation proceeds at a slower rate than the production of organic acids, the pH might reach values below pH6 which is fatal for the methanogenic bacteria. For this reason it is necessary to know the effect of potentially inhibitory compounds on the activity of the methanogenic population in order to maintain a stable digestion process. In anaerobic digestion of nitrogen-containing organic compounds ammonium-nitrogen is a metabolic end product.[1] Ammonium-nitrogen is an essential nutrient for methanogenic bacteria,[2–4] but at concentrations exceeding approximately 700 mg dm^{-3} an increasing concentration results in a decreasing methanogenic activity.[5] Unacclimatised or unadapted methanogenic populations will cease methanogenesis at ammonium-nitrogen concentrations exceeding the so-called toxicity threshold level. The threshold level for ammonium toxicity has been reported to be in the concentration range of 1700–1800 mg N dm^{-3},[6–8] whereas a strong inhibition of methanogenesis at ammonium-nitrogen concentrations in the range 1500–3000 mg dm^{-3} and a complete cessation of methanogenesis at ammonium-nitrogen concentrations exceeding 3000 mg dm^{-3} have also been reported.[9,10] An ammonium-nitrogen toxicity threshold level of 3300 mg dm^{-3} has been reported for a pure culture of the hydrogen-consuming methanogen

In this text ammonium is the sum of NH_3 and NH_4^+

Methanobacterium formicicum.[11] Methanogenesis at ammonium-nitrogen concentrations exceeding the initial threshold level has been reported by several authors. Digested sewage sludge acclimatised to an ammonium-nitrogen concentration of 815 mg dm^{-3} was able to produce methane from a mixture of volatile fatty acids at an ammonium-nitrogen concentration of 2360 mg dm^{-3} after a lag phase of 25 days during which methane production was nil. With the same seed material a lag phase of 45 days was needed for methanogenesis at an ammonium-nitrogen concentration of 4990 mg dm^{-3}.[12] Once adapted to high ammonium-nitrogen concentrations, the methanogenic populations can be kept in an animal waste treatment system for retention times much shorter than the time required for adaptation.[13] High-rate anaerobic wastewater treatment systems characterised by sludge immobilisation can handle very high ammonium-nitrogen concentrations. Anaerobic filters have been reported to be operated successfully at ammonium-nitrogen concentrations of 6000 mg dm^{-3} [14] or even 7800 mg dm^{-3}.[15] The specific methanogenic activities of these populations have not been determined.

In the present article the adaptation process taking place in granular methanogenic sludge exposed to a proteineous wastewater is described in detail, including a kinetic analysis. Maximum specific methanogenic activities before and after the adaptation process have been determined.

2. Experimental

2.1. Materials

The experiments were performed in Plexiglass cylindrical reactors with a working volume of 4.5 dm^3. The reactors were placed in a temperature controlled room at 30±1°C. The reactor contents were completely mixed by a mechanical stirrer every 20–30 min. Continuous mixing was not applied in order to minimise erosion of the granular sludge.

Methane production was measured using a sodium hydroxide displacement system. The strength of the sodium hydroxide solution was such that all carbon dioxide was removed from the biogas.

The methanogenic sludge used was granular sludge originating from an industrial Upflow Anaerobic Sludge Blanket (UASB) reactor treating the wastewater from a beet-sugar factory. The predominant acetotrophic methanogenic bacterium present in this sludge is a *Methanothrix* species,[16] which is an acetate-decarboxylating non-hydrogen-oxidizing methanogen.[17,18] The hydrogenotrophic species in this sludge have not been identified.

The method of preparation of the potato juice has been described elsewhere.[19] The only change was that for the present experiments the juice was prepared only a few hours before application. The average composition of potato juice is shown in Table 1.

Table 1. Average composition of potato juice[5]

Total solids	4.5–5.3% (w/v)
Proteins and amino acids	2.2–3.0% (w/v)
Carbohydrates	0.4–0.8% (w/v)
Di-phosphate	0.06–0.2% (w/v)
Chemical oxygen demand	approx. 54 g dm^{-3}
Kjeldahl-nitrogen	3.4–3.8 g dm^{-3}

2.2. Sampling

Samples were taken through a water lock in order to prevent oxygen from entering the reactors. Samples for the analysis of volatile fatty acids were filtered by suction through a membrane filter with a pore size of 0.45 μm. Samples for the analysis of ammonium-nitrogen and Kjeldahl-nitrogen were centrifuged at 14 000 ***g*** for 15 min. Filtering or centrifuging were done immediately after sampling. If immediate analysis was not convenient, the samples were stored in a deep freeze.

2.3. Analysis

Kjeldahl-nitrogen, defined as the sum of ammonium-nitrogen and nitrogen present in organic compounds, was determined according to method NEN 3235.6.5 as published by the Dutch Normalisation Institute in 1972.

Volatile fatty acids were determined using a gas chromatograph equipped with a 1 m×4 mm glass column packed with Chromosorb W-AW (80–100 mesh) coated with 20% Tween 80. The temperature of the injection port and of the detector was 170°C, the temperature of the column was 115°C. Nitrogen saturated with formic acid was used as carrier gas at a flow rate of 60 cm^3 min^{-1}.

All other analyses were carried out according to *Standard Methods*.[20]

2.4. Calculations

The un-ionised fraction of the total amount of ammonium-nitrogen (as determined by the Direct Nesslerisation method) was calculated by the following equation:

$$F_{NH_3}=(1+10^{(pK_w-pK_b-pH)})^{-1} \quad (1)$$

The values of the dissociation constant of water (K_w) and the ionisation constant of ammonia (K_b) at 30°C were obtained from literature:[21] $pK_b=4.740$ and $pK_w=13.833$.

The fraction of undissociated volatile fatty acids was calculated with the following equation:

$$F_{VFA}=\frac{10^{(pK_a-pH)}}{1+10^{(pK_a-pH)}} \quad (2)$$

From the values of the dissociation constants (K_a) obtained from literature[21] (valid only at 25°C) the values of pK_a at 30°C could be calculated[22] to be 4.676 and 4.793 for acetic and propionic acid respectively.

2.5. Experimental procedure

The sludge used in the experiments had been stored unfed at ambient temperatures for several months. Methanogenic sludge stored in these conditions has almost the same metabolic conversion potential as it had before storage, but it has to be reactivated.[23] The sludge was reactivated by applying consecutive batch feeds of a weight equivalent mixture of acetic, propionic and butyric acid together with sufficient nutrients, until no further increase of the specific methanogenic activity occurred.

In each of four similar reactors enough reactivated sludge was put in for each reactor to contain approx. 30 g of volatile solids. These four reactors were supplied with consecutive feeds of 0.56, 1.125 and 2.25 dm^3 of fresh potato juice. Any next feed was supplied only after the previous one had completely been digested. Because of the high proteins and amino acids content of the potato juice this feed pattern was bound to result in an ammonium-nitrogen concentration exceeding the threshold level for unadapted methanogens during digestion of the third feed.

Methane production and pH were monitored in all four reactors. With bicarbonate additions the pH of the reactor contents was kept in the range 7.2–7.8. Until cessation of the methane production due to ammonium-nitrogen concentrations exceeding the threshold level volatile fatty acids were monitored in two reactors only. During the period of adaptation volatile fatty acids were monitored in all four reactors. At 363.5 h after the start of the experiment with the third feed of potato juice, when inhibition of the still unadapted sludge was complete, the pH in three reactors was decreased by adding 62 ml of a 36% HCl solution.

After digestion of the third feed of potato juice the sludge (which had suffered from the inhibition to which it had to be adapted) was reactivated in a way similar to the reactivation of the stored sludge before the start of the actual experiments. After this reactivation a feed of 0.5 dm^3 of potato juice was applied to determine the specific methanogenic activity of the now adapted sludge.

3. Results and discussion

Progress curves for methane production from the third batch feed of potato juice are shown in Figure 1. During the first 75 h of the experiment the methane production rate was fairly constant, but after this period inhibition started, resulting in an almost negligible methane production rate after 350 h

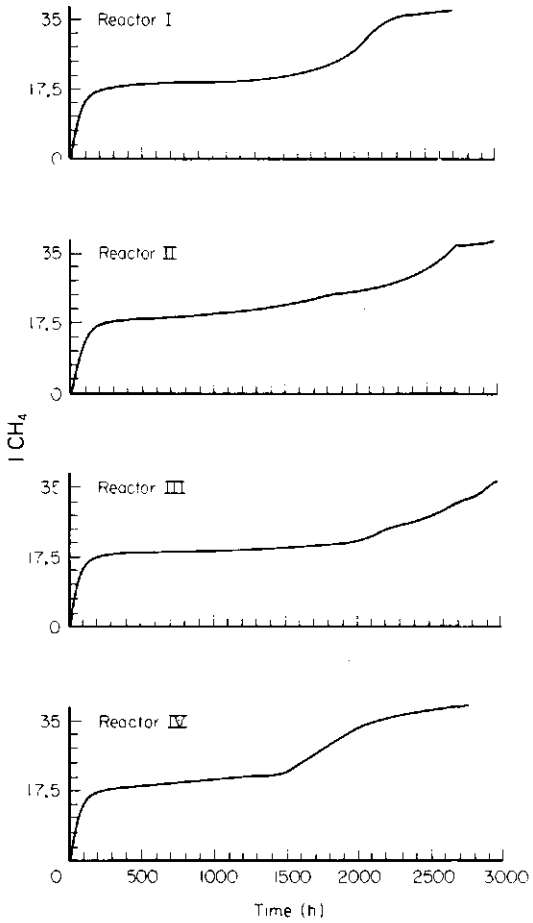

Figure 1. Cumulative methane production during digestion of the third feed of potato juice by granular methanogenic sludge at 30°C. During the experiment the digestion of proteinous compounds caused the ammonium-nitrogen concentration to exceed the toxicity threshold level. At 363 h from the start of the experiment in reactors II, III and IV the pH was lowered by means of the addition of 62 ml of concentrated HCl. See Table 3 for pH and ammonia-N concentrations during adaptation period.

from the start. Judging from the build-up of volatile fatty acids, the initial constant methane production rate was the maximum achievable. During this period the average maximum specific methanogenic activity was only 54% of the average maximum specific methanogenic activity during digestion of the second feed of potato juice. This can be explained by the increased ammonium-nitrogen concentration induced by the addition of nitrogeneous compounds with each new feed of potato juice. A similar, but more extreme, decline in maximum specific methanogenic activity with an increasing ammonium-nitrogen concentration has been reported for the digestion of a volatile fatty acids mixture by a similar type of granular methanogenic sludge.[5]

At the time that the inhibition appeared to result in a significant decreasing of the methanogenesis (approx. 165 h from the start) the concentration of Kjeldahl-nitrogen and ammonium-nitrogen were determined (Table 2). In our present experiments methanogenesis failed at ammonium-nitrogen concentrations of 1900–2000 mg dm^{-3}. This concentration range is slightly higher than the ammonium-nitrogen threshold levels reported for sewage sludge digesters[6,7] and piggery waste digesters,[8] which is probably caused by the step-wise increase in ammonium-nitrogen concentration resulting from the feed pattern applied.

The difference in reaction to inhibition of the hydrogen consuming methanogens and the acetate consuming methanogens is reflected in the volatile fatty acids patterns during the initial phase of the experiment. The concentrations of acetic acid, propionic acid and the sum of volatile fatty acids up to *n*-valeric acid are shown in Figure 2. In order to show the results of the intensive sampling program

Table 2. Concentration of Kjeldahl-nitrogen and ammonium-nitrogen at the moment (t=165 h) that a decreasing gas production indicated the beginning of the inhibition of methanogenesis by granular sludge digesting potato juice at 30°C

Reactor	Kjeldahl-N (mg dm^{-3})	Ammonium-N (mg dm^{-3})
I	2283	1975
II	2344	1938
III	2352	1975
IV	2358	2000

applied to reactors I and III during the first 80 h of the experiment properly, both ordinate and abscissa of the diagrams representing this period are less condensed than in the other diagrams of Figure 2. After the initial peak caused by overloading as a result of the batch feeding, the propionic acid concentration kept continuously increasing, whereas the acetic acid concentration was even decreasing a while. Inhibition of the hydrogen consuming methanogens almost immediately leads to a hydrogen concentration which makes it thermodynamically unfavourable to degrade propionic acid, whereas the response of the acetic acid consuming methanogens generally is much slower.[24] The case of acetic acid concentration as an early warning parameter for forthcoming upsets also holds for the present experiments. After the initial peak resulting from the already discussed overloading the acetic acid consumption rate exceeded the acetic acid production rate, but 75 h from the start of the experiment the acetic acid concentration started rising again. The methane production rate however started decreasing 125 h from the start of the experiment.

At 363 h after the start of the digestion of the third feed, when methanogenesis had almost completely stopped, the pH in the reactors II, III and IV was decreased by means of HCl addition in order to try to overcome the ammonia inhibition. Due to an apparent variation in buffer capacity the addition of hydrochloric acid resulted in three different pH ranges during the adaptation period (Table.3).

Unintendedly the pH in reactor I also decreased a little, probably as a result of a build-up of organic acids. Decreasing the pH will result in a decrease of the concentration of un-ionised (free) ammonia, which is believed to be more toxic than the ammonium ion at ammonium-nitrogen concentrations between 1500 and 3000 mg dm^{-3}.[9] Decreasing the pH as a method to avoid ammonia toxicity has been recommended by several authors.[10,25,26] At the onset of the inhibition in all reactors the pH was in the range 7.6–7.8, resulting in a free ammonia-nitrogen concentration of approx. 80 mg dm^{-3}. This is in accordance with the free ammonia-nitrogen concentrations that have been reported by De Baere *et al.*[15] and Anderson *et al.*[27] to be inhibitory to unadapted methanogenic cultures, but it is considerably lower than the value of 150 mg dm^{-3} which was reported by McCarty & McKinney[9] as toxic (viz. causing total cessation of methanogenesis).

The average pH and the free ammonia-nitrogen concentrations which occurred during the adaptation period are shown in Table 3. The free ammonia-nitrogen concentration range has been calculated from ammonium-nitrogen analysis just before the adaptation period (viz. at t=165 h) and after methanification of the feed had been completed. During the adaptation period further breakdown of organic nitrogen containing compounds caused a gradual increase of the ammonium-nitrogen concentration of approx. 10%. Although the pH adjustment caused a considerable decrease of the free ammonia-nitrogen concentrations (Table 3), it did not result in an immediate relief of the inhibition of the methanogenesis. Apparently at ammonium-nitrogen concentrations of approx. 2000 mg dm^{-3} some kind of adaptation is necessary, independent of the pH of the environment. The fact that inhibition had already been going on some time before the pH was adjusted might also partly explain the retarded recovery.

A serious drawback of decreasing the pH as a measure to overcome ammonia toxicity might be the effect it has on the toxicity of the volatile fatty acids that are present in high concentrations as a result of the ammonia toxicity. Although data concerning the toxicity of volatile fatty acids are scarce and often difficult to interpret because of a lack of distinction between the different kinds of acids it is

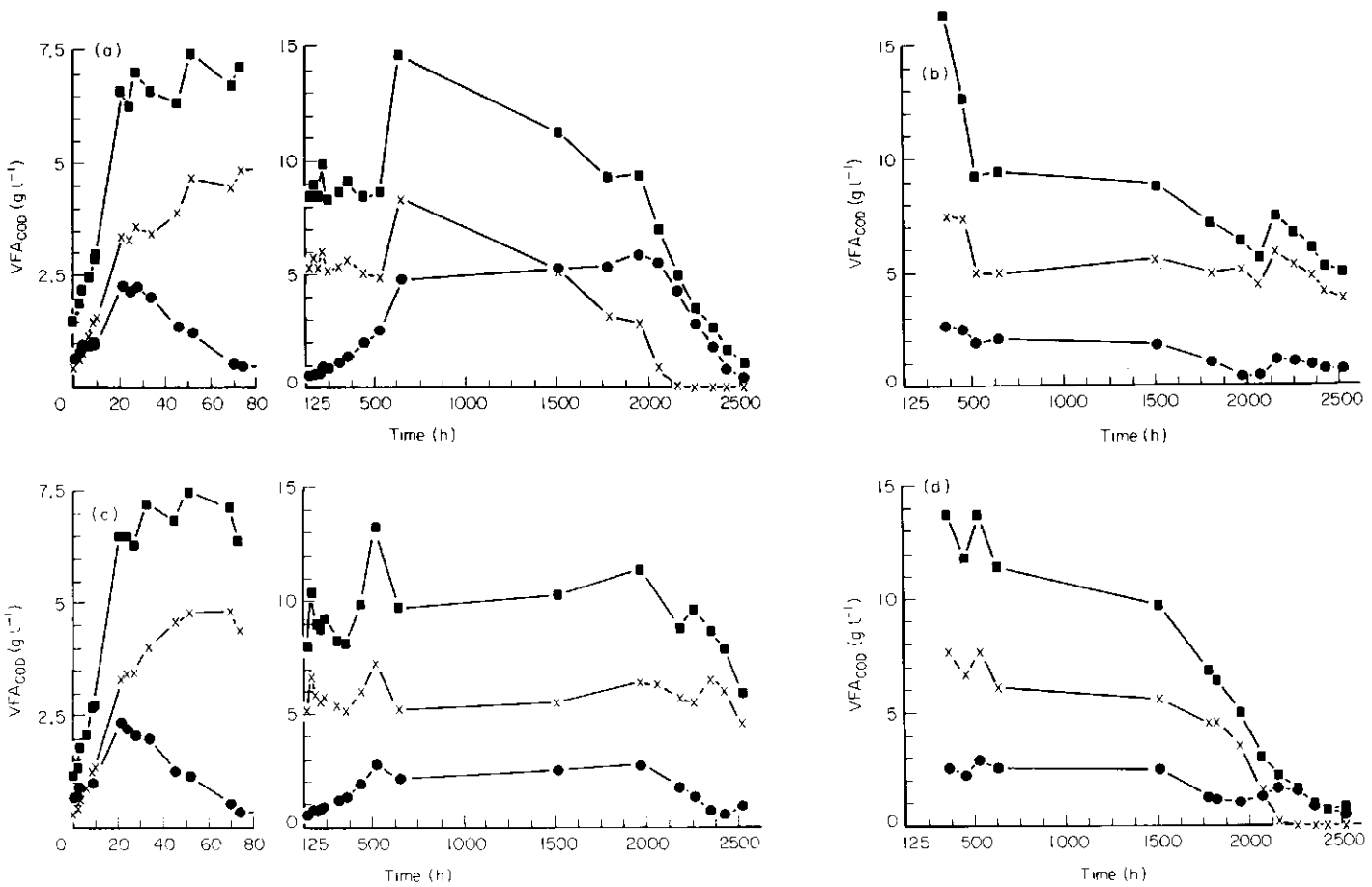

Figure 2a–d. Concentrations of acetic acid (●), propionic acid (×) and the sum of volatile fatty acids up to valeric acid (■) during digestion of the third feed of potato juice by granular methanogenic sludge at 30°C. The concentrations are expressed in terms of chemical oxygen demand (COD). The COD equivalent of acetic and propionic acid is 1.067 and 1.514 g COD g^{-1} respectively. During the experiment, digestion of proteinous compounds caused the ammonium-nitrogen concentration to exceed the toxicity threshold level. At 363 h from the start of the experiment in reactors II, III and IV the pH was lowered by means of the addition of 62 ml of concentrated HCl. See Table 3 for pH and ammonia-N concentrations during adaptation period. (a) Reactor I; (b) reactor II; (c) reactor III; (d) reactor IV.

Table 3. Average pH and calculated free ammonia-nitrogen concentration during the period of adaptation to ammonium-toxicity of granular methanogenic sludge digesting potato juice at 30°C

Reactor	pH	Free ammonia-N (mg/dm^{-3})[a]
I	7.6	61–68
II	7.0	16–18
III	7.0	16–18
IV	7.25	28–33

[a] Range based on initial and final ammonium-N.

commonly accepted that the un-ionized molecules represent the toxic form.[28-31] In Figure 3 the effect of pH on the fraction of un-ionised molecules of ammonia, acetic and propionic acid is shown. Data derived from literature by Kroeker *et al.*[29] indicated a toxic concentration of volatile fatty acids (expressed as acetic acid) of 10 mg dm^{-3}, whereas their own results indicated 30–60 mg dm^{-3} as the toxic level. Duarte and Anderson[31] found that in acetate-fed chemostats substrate-inhibition started at 10–25 mg dm^{-3} un-ionised acetic acid. Hobson and Shaw[11] demonstrated that the hydrogenotrophic *Methanobacterium formicicum* is not inhibited by an un-ionised acetic acid concentration as high as 28 mg dm^{-3} (the highest concentration they tested). The average concentrations of un-ionised acetic and propionic acid in each reactor during the adaptation period are shown in Table 4.

Comparison of the data shown in Tables 3 and 4 makes clear that during the adaptation period three different situations with respect to toxicity occurred. In reactor I the free ammonia-nitrogen concentration was near the toxic level, in reactors II and III the un-ionised volatile fatty acids were at a more or less toxic level and in reactor IV neither un-ionised ammonia-nitrogen nor un-ionised volatile fatty acids were at a toxic level. It should be noted that in all reactors the (un-ionised) volatile fatty acids were still at concentrations far below the toxic levels reported in literature at the time inhibition was complete (viz. methanogenesis had become nil). So the high concentration of volatile fatty acids is a result of the inhibition, not the cause.

In the reactors II and III, which had the lowest pH during the adaptation period, the propionic acid concentration started decreasing rather slowly between 2000 and 2500 h from the start of the experiment. In the reactors I and IV, which had a higher pH during the adaptation period, the propionic acid concentration started decreasing rather slowly between 2000 and 2500 h from the

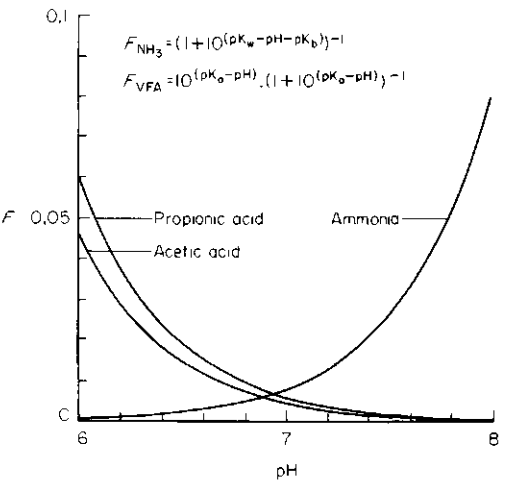

Figure 3. The relationship between pH and the fraction F of un-ionized molecules for ammonia, acetic and propionic acid at 30°C.

Table 4. Average concentration of the main un-ionised volatile fatty acids during the period of adaptation to ammonium toxicity of granular methnogenic sludge digesting potato juice at 30°C

Reactor	Free acetic acid (mg dm^{-3})	Free propionic acid (mg dm^{-3})
I	5.1	5.8
II	9.3	24.6
III	9.7	24.6
IV	6.4	15.1

start of the experiment. In the reactors I and IV, which had a higher pH during the adaptation period, the propionic acid concentration started decreasing much earlier and had become less than 100 mg dm^{-3} at 2150 h after the start of the experiment. This difference cannot be contributed to the effect of the chloride added with the pH adjustment, because reactor IV received the same amount of HCl as the reactors II and III. So it can be concluded that a pH below approx. 7.25 causing a more or less toxic concentration of un-ionised volatile fatty acids during the adaptation period had a detrimental effect on the degradation of propionic acid. Since these experiments were carried out with undefined mixed cultures, it is not possible to distinguish between an effect on the propionic acid degrading bacteria directly, or an indirect effect via the hydrogenotrophic methanogens. An

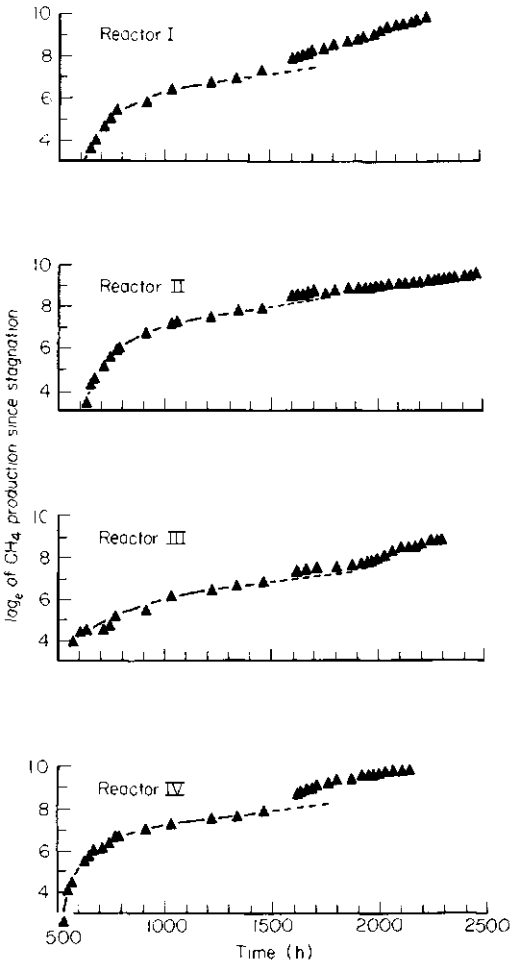

Figure 4. Natural logarithm of the methane production by granular methanogenic sludge exposed to ammonium toxicity at 30°C since the start of the adaptation period as a function of time. Each triangle represents a gas production reading. The amount of methane produced before total inhibition (viz. when gas production rate is negligible) was 19.0 l for reactor I, 18.6 l for reactor III and 18.8 l for reactors II and IV respectively.

indication of an inhibited hydrogen consumption during the adaptation period was obtained from the occurrence of acetone, which could qualitatively be determined during volatile fatty acids analysis in all samples taken during the adaptation period. In anaerobic environments where the hydrogen produced by fermentative bacteria is not instantly removed by syntrophic bacteria, acetone can be formed by certain *Clostridia*.[32]

The production of methane in anaerobic digestion can be described by Monod kinetics of bacterial growth.[33,34] If Monod kinetics are valid, a plot of the (natural) logarithm of the methane production versus time results in a curve ending in a straight line with a slope equal to the maximum growth rate.[35,36] In Figure 4 the natural logarithms of the methane production since the start of the adaptation period (viz. the amount of methane exceeding 18.6 dm^3 for reactor III, 18.8 dm^3 for reactors II and IV and 19.0 dm^3 for reactor I) is plotted as a function of time for each reactor. Initially the curve is of the predicted shape, but in all cases the linear relationship is distorted after some time. This means that the adaptation of the methanogenic population cannot be attributed exclusively to growth of a resistant (perhaps mutant) type of bacteria, but that the original population has adapted by a metabolic change during the period of stagnation of the methane production. From this kinetic analysis it can also be concluded that the recovery of the methanogenic bacteria was completed at approx. 1500 h from the start of the digestion of the third feed of potato juice, because in all logarithmic plots at this time the diversion of the linear relationship started. This implies that the time needed for changing the metabolism in such a way that the initially toxic ammonium-nitrogen concentration can be tolerated was approx. 1200 h at all conditions tested. From the methane production progress curves however, it can be seen that although the time needed for recovery was more or less the same at all conditions tested, there really was an effect on the remaining activity once methanogenesis had started again. This becomes even more evident if the maximum specific methanogenic activity of the granular sludge is contemplated. The maximum specific activity of the sludge (deduced from the maximum methane production rate and the sludge volatile solids content) in each reactor during digestion of the second feed of potato juice, during digestion of the third feed of potato juice prior to the adaptation period and during the digestion of a 0.5 dm^3 feed of potato juice after completion of the adaptation is shown in Table 5. After adaptation the maximum specific methanogenic activity in reactor I was only 31% of the maximum specific methanogenic activity during digestion of the second feed of potato juice. In reactors II and III 61% of the uninhibited maximum specific methanogenic activity remained; in reactor IV this was slightly higher: 65%. Except for reactor I, in all cases the maximum specific methanogenic activity after adaptation is higher than it was just before the start of the adaptation period, although the ammonium-nitrogen concentration after adaptation was a little bit higher.

It may be concluded that a pH value causing more or less toxic free ammonia nitrogen concentrations during the adaptation period had a severe effect on the remaining maximum specific methanogenic activity after adaptation, whereas a pH value causing more or less toxic concentrations of free volatile fatty acids during the adaptation period had some effect on the recovery pattern,

Table 5. Maximum specific methanogenic activities (expressed as kg COD equivalent of methane produced per kg VSS of sludge per day) before and after adaptation to ammonium-nitrogen concentrations exceeding the initial threshold level

Approx. ammonium-N ($mg\ dm^{-3}$)		Reactor			
		I	II	III	IV
1000		0.64	0.71	0.76	0.71
1900[a]		0.39	0.34	0.42	0.37
before / after	adaptation				
2300		0.20	0.44	0.47	0.46

[a] Immediately before total inhibition.

but did not have an essential effect on the remaining maximum specific methanogenic activity after adaptation. The free volatile fatty acids appeared to inhibit the propionic acid degradation, probably through inhibition of the hydrogen-consuming bacteria. The hydrogen-consuming population in anaerobic digesters has been found to operate at less than 1% of the maximum possible rate, whereas the acetate consumption is about half saturated.[37] The large over-capacity with respect to hydrogen consumption might be the explanation for the fact that inhibitory concentrations of free volatile fatty acids during the adaptation period did not result in an insufficient hydrogen consumption rate in the adapted sludge, whereas the acetate consumption rate in the adapted sludge was clearly influenced by inhibitory ammonia-nitrogen concentrations during the adaptation period.

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Anaerobic Digestion at Extreme Ammonia Concentrations

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ABSTRACT

The anaerobic digestion of potato juice, used as a model complex wastewater, was investigated at extreme ammonia concentrations. Even at 11·8 g ammonia-N/litre methanogenesis occurred, but at 16 g ammonia-N/litre methanogenic activity had become nil. The adaptation potential of the granular sludge used in this study was 6·2, which means that after the adaptation process in which the sludge gained the ability to produce methane at ammonia concentrations exceeding the initial toxicity threshold level, the maximum tolerable ammonia concentration was 6·2 times higher than the initial toxicity threshold level.

After more than three weeks of exposure to 9·1 g ammonia-N/litre the maximum specific methanogenic activity of granular sludge at 5·2 g ammonia-N/litre was found to fit well in the general relationship between ammonia concentration and methanogenic activity established in the present research. This indicates that even at extremely high concentrations ammonia toxicity is reversible to a great extent.

It was also found that contrary to methanogenesis, the production of volatile fatty acids from the complex substrate was hardly affected by high ammonia concentrations.

INTRODUCTION

Potential inhibition of methanogenic bacteria by ammonia (here the sum of NH_3 and NH_4^+) plays a role in almost all medium- and high-strength agricultural wastes, because ammonia is the end-product of anaerobic

digestion of proteins. Starting at approximately 700 mg ammonia-N/litre the maximum specific activity of methanogenic bacteria has been found to decrease with increasing ammonia concentrations (Koster & Lettinga, 1984). In unadapted methanogenic bacterial sludge, methanogenesis ceases when the ammonia concentration is raised to approximately 1700–2000 mg N/litre (van Velsen, 1979; Koster & Lettinga, 1984).

One of the first reports dealing with the possibility of adapting methanogenic bacteria to ammonia by exposing them to slowly increasing concentrations was the sludge digestion study of Melbinger & Donnellon (1971). They succeeded in operating a stable high-rate sludge digester at ammonia concentrations up to 2700 mg ammonia-N/litre, whereas at that time 1700 mg ammonia-N/litre was considered to be the upper limit. At present, adaptation of methanogenic bacteria to a wide variety of potentially inhibitory substances has been reported (Speece, 1983; Speece & Parkin, 1983; Parkin & Miller, 1983). Once adapted, methanogenic bacteria can be active at concentrations exceeding the initial inhibitory concentrations by several times (Parkin & Miller, 1983). Adaptation of methanogenic bacteria to ammonia has been investigated in detail (van Velsen, 1979; Koster, 1986). Parkin *et al.* (1983) reported successful operation of anaerobic filters at ammonia concentrations of 6000 mg ammonia-N/litre, while De Baere *et al.* (1984) were able to operate an anerobic filter at 7800 mg ammonia-N/litre. However, in these studies, the specific methanogenic activity of the biomass was not reported, and therefore it is not possible to make a distinction between a successful operation as a result of adaptation of the biomass and the effect of a high biomass retention in the filters.

This paper deals with the results of a study concerning the anaerobic digestion of potato juice by granular methanogenic sludge at extremely high ammonia concentrations. Potato juice was used because it is high in protein and is the main constituent of the wastewater of potato-processing industries. The effect of inhibitors is often reported in terms of maximum allowable concentrations, which refer to these concentrations where only a specified fraction (often 50%) of the specific methanogenic activity (often expressed as methane production per volume or weight unit of biomass) is left. In our opinion, however, the maximum allowable concentration of an inhibitor should not merely be related to its effect on the specific microbial activity, but the biomass retention capacity and the concomitant biomass concentration of the reactor system to be applied for anaerobic treatment of the waste in which the toxicant is present should also be considered. A high biomass content can, to a certain extent, make up for the loss of specific activity.

Since in all our experiments the amount of biomass was known, the tolerance of methanogenic bacteria to increasing ammonia concentrations

can be given in terms of remaining maximum specific activity expressed as gram COD equivalent of methane produced per gram volatile sludge solids (VS) per day. Since the biomass used for assessing ammonia inhibition was granular sludge originating from an Upflow Anaerobic Sludge Bed (UASB) reactor, the quantitative relation between maximum specific methanogenic activity and ammonia concentration established in this study can be used to estimate the upper ammonia concentration at which a UASB-reactor can still be operated economically.

METHODS

All experiments concern batch tests carried out in plexiglass cylindrical reactors with a working volume of 4·5 litres. The reactors were placed in a temperature controlled room at 30 ± 1°C. Intermittent stirring was applied for mixing of the reactor contents. Continuous stirring was not used in order to minimize damage to the granular structure of the sludge. Methane production was measured by displacement of a sodium hydroxide solution of a strength sufficient to absorb all CO_2 from the biogas. The methanogenic sludge used in the present work originated from an industrial UASB-reactor treating beet-sugar factory wastewater.

At 1000 mg ammonia/N-litre the maximum specific methanogenic activity of this sludge was 0·73 g COD/(g VS.day). At 1900–2000 mg ammonia-N litre the sludge completely failed to produce methane. After an adaptation period characterised by an almost nil methane production, methanogenesis appeared to be possible at even higher concentrations. The adaptation process (which lasted approximately 2800 h, including a 1200 h period of zero activity) has been described elsewhere (Koster, 1986). It should be noted that although adapted sludge is able to produce methane at ammonia concentrations at which prior to adaptation activity was nil,

TABLE 1
Average Composition of Potato Juice

	(g/litre)
Total solids	45–53
Proteins and amino acids	22–30
Carbohydrates	4–8
Potassium	4–8
Di-phosphate	0·6–2
Chemical Oxygen Demand (COD)	approx. 54
Kjeldahl-nitrogen	3·4–3·8

adaptation did not make the sludge insusceptible to ammonia inhibition. Each reactor contained approximately 19 g VS of sludge.

At the start of each experiment the sludge was allowed to settle for approximately 24 h so that all the sludge, including very small particles eroded from the granules, was retained. Next, 0·5 litre of the supernatant was removed and 0·5 litre of fresh potato juice was added. The average composition of potato juice as reported by van Bellegem & Lettinga (1975) is shown in Table 1. The nutrient composition of potato juice is sufficient for anaerobic digestion, so extra nutrients were not added in our experiments. The COD content was the only parameter of the potato juice used in our experiments that was exactly known. The method of preparation of the potato juice is described elsewhere (Koster & Lettinga, 1985).

Simultaneously with the potato juice addition, the ammonia concentration was adjusted to the desired level by addition of ammonium chloride. In all experiments except one the ammonia concentration was higher than in the preceding experiment with the same sludge in the same reactor. Before one experiment, the ammonia concentration was lowered by replacing extra supernatant with tap water from which oxygen had been removed by flushing with nitrogen.

The sequence of performance of the experiments, each with its pH-range and average ammonia concentration is shown in Table 2. Due to the

TABLE 2

Characteristics of the Experiments Performed with 2315 mg Ammonia-N/litre Adapted Granular Sludge Digesting Potato Juice at 30°C

Reactor[a]	*Chronological order of experiment*	*COD-input (g/litre)*	*Ammonia concentration (mg N/litre)*	*pH*	
				Minimum	*Maximum*
II	1	30·5	2296	7·68	7·70
II	2	32·8	3532	7·40	7·78
II	3	26·3	4051	7·47	7·81
II	4	27·2	9139	7·40	7·68
II	5	30·4	5229	7·40	7·60
III	1	30·5	2300	7·65	7·69
III	2	32·8	4563	7·40	7·70
III	3	26·3	5734	7·39	7·75
III	4	27·2	11831	7·38	7·57
IV	1	30·5	2372	7·64	7·70
IV	2	32·8	4111	7·38	7·77
IV	3	26·3	4992	7·43	7·80
IV	4	27·2	15963	7·27	7·31

[a] The reactor numbers correspond with those in the adaptation study (Koster, 1986).

degradation of nitrogen-containing compounds, the ammonia concentration increased by approximately 400 mg ammonia-N/litre during the experiments. The pH was not constant during the experiments due to an initial build-up of volatile fatty acids. pH-control was not applied in order to avoid any inhibitory effects of high salt concentrations which might result from that. Sampling and analytical procedures have been described elsewhere (Koster & Lettinga, 1984).

RESULTS AND DISCUSSION

From each experiment a cumulative methane production curve was obtained. The maximum slope of that curve divided by the amount of biomass present in the reactor, gives the maximum specific methanogenic activity of the biomass under the specific experimental conditions. Table 3 presents the assessed maximum specific methanogenic activity at different ammonia concentrations. At 15 963 mg ammonia-N/litre a continuousl declining methanogenesis was observed during the first 200 h of the experiment, but after that time methanogenesis became nil. The experiment was continued for another 400 h, but since recovery did not occur it was

TABLE 3

The Assessed Maximum Specific Methanogenic Activity of 2 315 mg Ammonia-N/litre Adapted Granular Sludge with Potato Juice as Feed at Various Ammonia Concentrations

Ammonia concentration (mg N/litre)	*Maximum specific methanogenic activity g COD/(g VS. day)*
2 323 ± 43[a]	0·46 ± 0·015[a]
3 532	0·28
4 051	0·23
4 111	0·21
4 563	0·30
4 992	0·16
5 229[b]	0·12
5 734	0·13
9 139	0·07
11 831	0·04

Except where noted, the ammonia concentration was increased between experiments.

[a] Mean ± standard deviation of the three experiments in series 1.

[b] Reactor contents diluted from previous experiment at 9 139 mg N/litre.

terminated. Thus the highest concentration at which methanogenesis was found to be possible was 11 831 mg ammonia-N/litre. In a previously reported adaptation study (Koster, 1986) the unadapted sludge failed to produce methane at a concentration of 1900 mg ammonia-N/litre.

The present results show that, after being adapted (i.e. after having gained the ability to produce methane at ammonia concentrations exceeding the initial toxicity threshold level), the same sludge was able to produce methane at 11 831 mg ammonia-N/litre. This means that the adaptation potential (defined as the ratio of the maximum allowable inhibitor concentration after adaptation and the pre-adaptation toxicity threshold concentration) of this particular granular sludge was 6·2. Parkin & Miller (1983) reported a lower adaptation potential of 1·8–3·2. In the discussion of the adaptation potential of their methanogenic culture, Parkin & Miller stated that especially in the case of ammonia the procedure they used should yield a conservative estimate of the adaptation potential.

The maximum allowable ammonia concentration established in the present experiments fits well within the data reported by Parkin & Miller (1983). However, it is 1·5 to 2 times higher than the maximum ammonia concentrations at which anaerobic filters could be operated without decrease in process performance (Parkin *et al.*, 1983; De Baere *et al.*, 1984). It should be noted that the values for maximum specific methanogenic activity shown in Table 3 only provide information concerning the biochemical properties of the granular sludge. The successful operation of any anaerobic wastewater treatment system depends, however, not only on the biochemical characteristics of the sludge, but also on the settling ability of the sludge and the design of the reactor, especially with respect to biomass retainment. For an economically attractive application of a UASB-reactor the retained (granular) sludge should have a maximum specific methanogenic activity of at least 0·1–0·15 kg COD/(kg VS . day). With maximum specific methanogenic activities of 0·1–0·15 kg COD/(kg VS . day) a UASB-reactor which has an average sludge content of 40 kg VS/m^3 can be operated at loading rates comparable to those achievable in aerobic systems. These considerations imply that for a successful operation of a UASB-reactor a concentration of 5000–7500 mg ammonia-N/litre would be the maximum allowable.

In Fig. 1 the maximum specific methanogenic activity of the granular sludge at different ammonia concentrations is shown as a percentage of the average maximum specific methanogenic activity in the first series of experiments. The maximum specific methanogenic activity at 2323 mg ammonia-N/litre is used as a reference, because 2323 mg ammonia-N/litre is the lowest concentration at which the maximum specific methanogenic activity was determined, after adaptation of the sludge to ammonia concentrations exceeding the initial toxicity threshold level of 1900–2000 mg

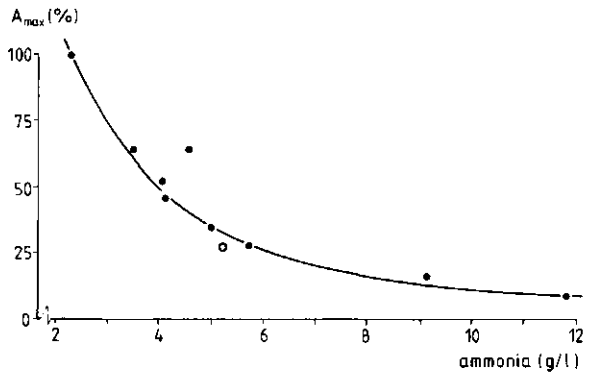

Fig. 1. The decline of the maximum specific methanogenic activity (A_{max}) with increasing ammonia concentrations. The open circle represents the result of an experiment carried out with sludge which had been previously exposed for more than three weeks to 9139 mg ammonia-N/litre.

ammonia-N/litre. It should be noted that at this concentration the maximum specific methanogenic activity was only 63% of that at 1000 mg ammonia-N/litre (i.e. below the initial toxicity threshold level). It is obvious that even after adaptation to ammonia toxicity, the methanogens still are inhibited by increasing ammonia concentrations.

The experiment carried out at 5229 mg ammonia-N/litre was preceded by an experiment which lasted more than three weeks, with the same sludge at 9139 mg ammonia-N/litre. Figure 1 clearly shows that the assessed maximum specific methanogenic activity at 5229 mg ammonia-N/litre almost fits within the relationship between maximum specific methanogenic activity and ammonia concentration. Hence it may be concluded that even after prolonged exposure to extreme ammonia concentrations ammonia toxicity is more or less reversible. This is in accordance with results of other studies concerning exposure periods from 1 h up to four days (Speece & Parkin, 1983; Parkin *et al.*, 1983). This reversibility indicates that even at such high concentrations ammonia may inhibit certain life functions of the methanogens, but does not kill the whole population.

In the third series of experiments, the volatile fatty acids concentration was monitored in addition to the methane production. This enables estimation of the ammonia inhibition of the acidogenic bacteria, because at any moment the sum of the methane produced and the total amount of volatile fatty acids present in solution (both expressed as COD equivalent) represent the amount of substrate-COD that has already been converted by acidogenic bacteria. Figure 2 shows this, together with the methanogenesis, as a percentage of the substrate input. The gap between the two curves represents the build-up of volatile fatty acids. In all experiments the largest part of the volatile fatty acids build-up was from acetic acid and, though to a

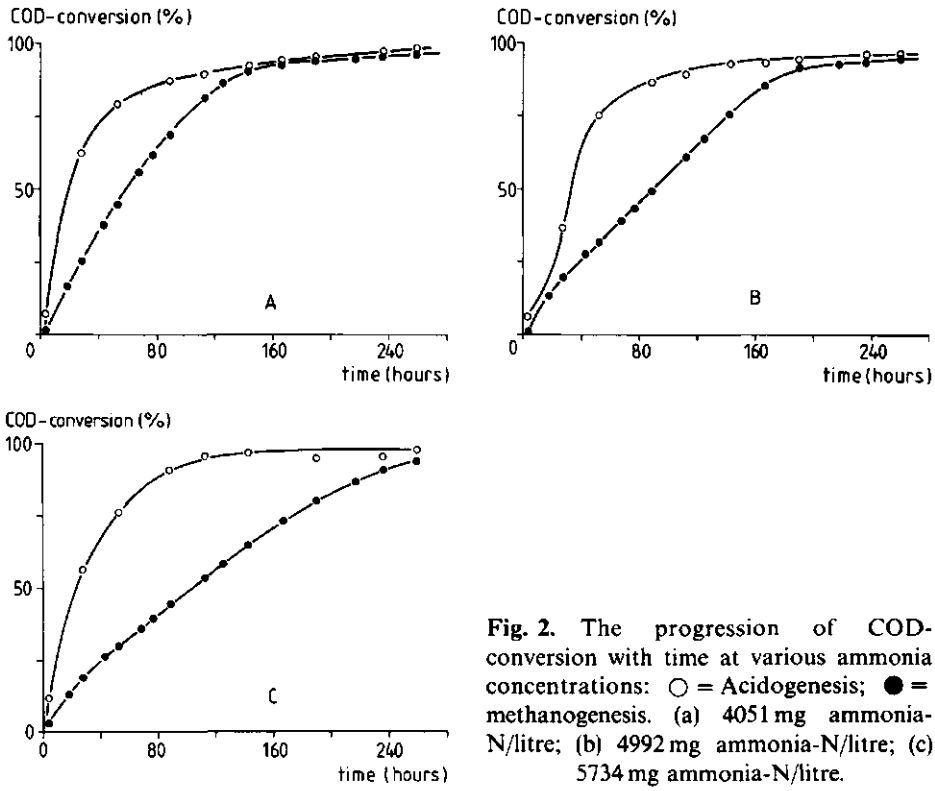

Fig. 2. The progression of COD-conversion with time at various ammonia concentrations: ○ = Acidogenesis; ● = methanogenesis. (a) 4051 mg ammonia-N/litre; (b) 4992 mg ammonia-N/litre; (c) 5734 mg ammonia-N/litre.

much lesser extent, propionic acid. The results shown in Fig. 2 indicate that the acidogenic population in the granular sludge was hardly affected by increasing ammonia concentrations in the range 4051–5734 mg ammonia-N/litre, while the methanogenic population lost 56·5% of its activity under these conditions. The practical implication of this finding is that an increase of the concentration of ammonia and/or easily degradable nitrogenous compounds in (partially) unacidified influent of an anaerobic wastewater treatment system will result in a volatile fatty acids build-up. In the case of insufficient buffering capacity of the reactor contents, such a volatile fatty acids build-up might result in a pH-drop below the range in which methanogenic bacteria can survive. Only in relatively under-loaded systems would the build-up of volatile fatty acids be small or even absent.

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CHAPTER 5

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Ammonia inhibition of the maximum growth rate (μ_m) of hydrogenotrophic methanogens at various pH-levels and temperatures

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Summary. A method for the quantitative measurement of the maximum growth rate (μ_m) of hydrogen-consuming methanogenic populations was applied to assess the toxicity of ammonia[1] under various pH and temperature conditions. The maximum uninhibited growth rate of the hydrogenotrophic population present in sludge from an industrial anaerobic wastewater treatment system appeared to be 0.126 h^{-1} at pH = 7 and 37° C. At 350 mM ammonia the maximum growth rate had decreased to almost half that value. At a temperature of 29° C the maximum growth rates in the ammonia range tested appeared to be approximately 60% of that at 37° C, while increasing ammonia concentrations caused a similar maximum growth rate decline. At 37° C an increase of the pH to 7.8 appeared to enhance ammonia inhibition of the maximum growth rate. Increased propionate concentrations (tested up to 60 mM) appeared to have no effect on ammonia inhibition.

Introduction

The anaerobic digestion of nitrogenous compounds results in the formation of ammonia. Ammonia is a nutrient for bacteria involved in the anaerobic digestion process, but at concentrations exceeding approximately 50 mM it inhibits methanogenesis (Koster and Lettinga 1984). Since methanogens are the most sensitive bacteria amongst the complex population involved in anaerobic digestion, knowledge of their reactions to inhibitors such as ammonia is of vital importance for the successful operation of anaerobic waste or wastewater treatment processes. Even a temporary inhibition of the methanogenic stage of anaerobic digestion can be fatal for the whole process, because the acid-producing bacteria are not, or are much less, inhibited. The organic acids that build up during inhibition of methanogenesis can cause a fatal drop in pH that stops methane production. Although only 30% of the methane produced in the anaerobic digestion of complex substrates is formed via hydrogen (Jeris and McCarty 1965), hydrogenotrophic methanogens play a crucial role in anaerobic digestion by keeping the hydrogen concentration low enough to make it thermodynamically possible for propionate and butyrate to be converted into the methanogenic substrates acetate and hydrogen (Wolin and Miller 1982). The hydrogen partial pressure (which in a stable digestion process does not exceed 10^{-4} bar) is the parameter that most promptly indicates process upsets. Within a fraction of a second hydrogen accumulation inhibits propionate degradation, whereas acetate accumulation affects the anaerobic digestion process by reducing the pH, which takes place on a time scale of about 1 day (Gujer and Zehnder 1983).

Sprott and Patel (1986) have provided some data on the effect of ammonia on the maximum growth rate (μ_m) of pure cultures of hydrogenotrophic methanogens. The occurrence of these species used in anaerobic wastewater treatment systems is not known. Sprott and Patel carried out their experiments at a fixed pH and temperature. In this article research concerning the effect of ammonia on the maximum growth rate of the mixed hydrogenotrophic methanogenic population present in sludge from an industrial anaerobic wastewater treatment system is presented. Since NH_3 is supposed to be the most inhibitory

[1] The term ammonia is used to indicate the sum of NH_3 and NH_4^+ chemical species

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ammonia species, the effect of temperature and pH (which both affect the ionization of ammonia) on ammonia inhibition has also been researched.

Materials and methods

Principal considerations. For the correct measurement of the maximum growth rate of bacteria utilizing gaseous, low water-soluble substrates like hydrogen, the transport rate of the substrate from the gas phase to the liquid phase containing the bacteria should exceed the substrate consumption rate. The method for growth rate measurements applied in this study therefore combined a high hydrogen transfer potential with a very slow hydrogen consumption rate. This could be realized by putting a very small inoculum of the bacteria to be tested in a reactor in which maximum contact between gas phase and liquid phase was provided.

Experimental set-up. A 500 ml Plexiglas reactor was provided with 200 ml liquid during measurements. The liquid was continuously agitated by means of a magnetic stirrer at 825 rpm. Through a syringe needle of 0.53 mm internal diameter, which ended close to the stirring flea, a constant flow of gas [$H_2/CO_2 = 4/1$ (vol/vol)] was passed. Traces of oxygen were removed from the gas by clean copper chips at 300° C. The exit gas flow rate was measured with a wet test gas meter. Between the reactor and the gas meter a sample port for gas analysis was placed. Additions to the reactor contents could be made with a syringe via a rubber septum in the lid. A water jacket allowed thermostating of the reactor contents.

Medium. The medium (made up in demineralized water) contained per litre: 1 ml trace element solution after Zehnder et al. (1980); 6 ml of a stock solution of 195 g/l NH_4Cl and 25 g/l $MgCl_2 \cdot 6H_2O$; 2 ml stock solution of 150 g/l $Na_2S \cdot 15H_2O$; 50 ml 0.5 M NaH_2PO_4/K_2HPO_4 buffer; 0.2 g yeast extract; 20 ml digester fluid.

In order to avoid a pH-change caused by dissolved CO_2 the medium was provided with the amount of bicarbonate in balance with a 20% CO_2 atmosphere at 1 bar at a certain pH according to the equation:

$$[HCO_3^-] = 0.2 k_1 H 10^{pH} \quad (1)$$

in which H is the Henry coefficient describing the dissolving of CO_2 and k_1 is the dissociation constant of H_2CO_3 (which is dissolved CO_2). Values of H and k_1 were adapted from Stumm and Morgan (1970).

Assay procedure. At the start of an assay the medium was composed by combining all stock solutions except for the sodium sulphide in such an amount of demineralized water that, after addition of the inoculum, the liquid volume would be 200 ml. The ammonia concentration was adjusted to the desired level by adding NH_4Cl. In order to remove all oxygen this solution was flushed with H_2/CO_2 gas at 5 l/h for 1 h. After lowering the gas flow to the rate desired to obtain CH_4 concentrations within the detection range, the sulphide solution was added. Fifteen minutes later the reactor was inoculated with 5—20 ml of the supernatant of a settled suspension of ground granules obtained from the anaerobic wastewater treatment system of a sugar factory. The granule-free suspension had been digested at 30° C until its methane production became nil. In the acutal experiments methane production from substrates other than H_2/CO_2 could therefore be excluded.

After inoculation the reactor was operated overnight without being monitored. The next day the methane concentration in the exit gas was monitored during approximately 6 h. Immediately afterwards the reactor was opened and the pH and temperature of the contents were measured and samples were taken for ammonia analysis.

Calculation of μ_m. We assume that the methane production rate (dM/dt) is proportional to the amount of biomass (X):

$$dM/dt = kX_t \quad (2)$$

The amount of biomass at time t (X_t) can be described by the following equation:

$$X_t = X_0 e^{\mu t} \quad (3)$$

in which μ is the growth rate and X_0 is the amount of biomass in the inoculum.

All methane produced during a certain time interval will leave the reactor in the exit gas. A mass balance for this situation gives:

$$dM/dt = QC_m \quad (4)$$

in which Q is the gas flow rate and C_m is the methane concentration in the exit gas. Combination of Eqs. (2)—(4) for substrate-saturated conditions gives a relation between exit gas methane concentration and maximum growth rate:

$$C_m = (k/Q) X_0 e^{\mu_m t} \quad (5)$$

This equation can be linearized by taking the natural logarithm of both parts:

$$\ln C_m = \ln(kX_0/Q) + \mu_m t \quad (6)$$

If the gas flow rate is kept constant, the factor $\ln(kX_0/Q)$ is a constant and the plot of $\ln C_m$ against time will be a straight line with slope μ_m.

Chemicals. Yeast extract was provided by Gist-brocades (Delft, The Netherlands). The gas was provided by Hoekloos (Schiedam, The Netherlands). Digester fluid was obtained by centrifuging sewage sludge which had been digested for several months at 30° C (volatile fatty acids below 1 mg/l) for 15 min at 16000 *g*. All other chemicals were of analytical grade and were provided by Merck (Darmstadt, FRG).

Analyses. Volatile fatty acids were determined by gas chromatography as described elsewhere (Koster and Lettinga 1985). The methane concentration in the gas was also determined by gas chromatography using a 1.5 × 2 mm internal diameter stainless steel column packed with Mol Sieve 5A (mesh 60—80). The temperatures of the injection port, column and FI detector were 150, 120 and 250° C respectively. Helium at 30 ml/min was used as carrier gas. The sample volume was 250 µl.

Results and discussion

An example of the result of an assay is shown in Fig. 1, where the natural logarithm of each mea-

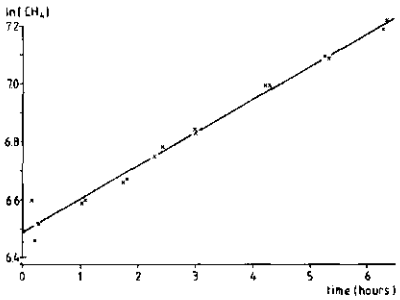

Fig. 1. Progress curve of the natural logarithm of methane concentration (measured as ppm) in the exit gas of a reactor operated at 37.3°C, pH 6.9 and 35.5 mM ammonia

sured CH_4 concentration in the exit gas is plotted against time. In this case the straight line fitted through the data has a slope of 0.114 (h^{-1}) with a 95% confidence interval of 0.001 (h^{-1}). The perfect fit is also shown by the very high correlation coefficient of 0.99. The fact that, even in the case of a fast-growing hydrogenotrophic population, the methane concentration in the exit gas kept increasing exponentially shows that the substrate transfer rate from gas to liquid was not limiting. If it had been, the methane concentration would have become constant, resulting in a horizontal line in a half-logarithmic plot. The mean of the uninhibited maximum growth rates determined at pH 7 and 37°C is 0.126 (h^{-1}). This value fits in with the range of data reported in the literature (Table 1).

In Fig. 2A the results of a series of experiments at 37°C and pH 7, each with a different ammonia concentration, are shown. It appears

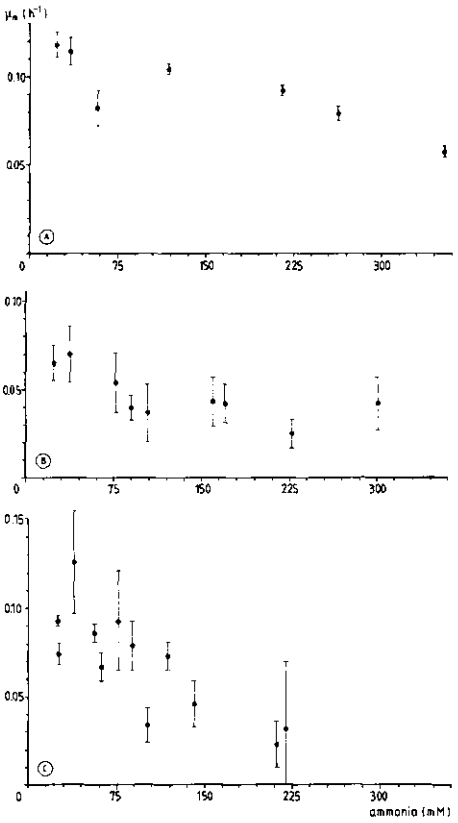

Fig. 2 A—C. The maximum growth rate of a hydrogenotrophic methanogenic population as a function of the ammonia concentration. The *bar* represents the 95% confidence interval. **A** pH = 7.0 ± 0.1 and 37° ± 0.5°C; **B** pH = 7.0 ± 0.1 and 29° ± 0.5°C; **C** pH = 7.8 ± 0.1 and 37° ± 1.0°C

Table 1. Maximum growth rates of pure culture isolates and undefined, mixed, populations of hydrogenotrophic methanogenic bacteria

	μ_m (h^{-1})	Temperature (°C)	pH	Reference
Methanobacterium bryantii M.o.H.	0.029	37—39	7.1	Dubach and Bachofen (1985)
Methanobacterium bryantii M.o.H.G.	0.029	37—39	7.1	Dubach and Bachofen (1985)
Methanobrevibacter arboriphilus AZ	0.139	33—40	6.6—7.4	Dubach and Bachofen (1985)
Methanomicrobium paynteri	0.144	40	7.0	Dubach and Bachofen (1985)
Methanogenium olentangyi RC/ER	0.064	37	nr[a]	Dubach and Bachofen (1985)
Methanoplasma elizabethii	0.016	37	7.2	Rose and Pirt (1981)
Methanobacterium strain AZ	0.144	33	7.0	Zehnder and Wuhrmann (1977)
Methanospirillum hungatei	0.053	37	nr	Robinson and Tiedje (1984)
Methanobacterium formicicum	0.082	40	7.2	Schauer and Ferry (1980)
Enrichment culture	0.044	37	7.8	Shea et al. (1968)
Fresh digested sewage sludge	0.05	33	7.0	Zehnder (1978)
Digested sewage sludge	0.102	37	nr	Wise et al. (1978)
Biomass from industrial wastewater digester	0.126	37	7.0	This study

[a] Not reported

Table 2. The fraction (F) of ammonia present as NH_3 at various pH and temperature combinations

pH	Temperature (°C)	F (%)
7.0	37	1.27
7.0	29	0.75
7.8	37	7.51

that the maximum growth rate decreased with increasing ammonia concentration. This might indicate that *Methanobrevibacter arboriphilus, M. smithii* and *Methanobacterium* strain G2R were not predominant in the culture used in the present study. These species have been reported to grow equally well in the presence of 3—400 mM ammonia at 35°C and pH 6.8 (Sprott and Patel 1986). An analysis of the strains present in our culture would be necessary to be sure whether or not these strains were present. The culture used in our experiments was less sensitive to ammonia than a pure culture of *Methanobacterium formicicum* used by Hobson and Shaw (1976), in which growth at 38°C and pH 7.1 was partly inhibited by 177 mM ammonia, while 235 mM completely inhibited growth. The trend shown in Fig. 2A is in accordance with the findings of Sprott and Patel (1986) with pure cultures of *Methanospirillum hungatei, Methanosarcina barkeri* and *Methanobacterium bryantii* incubated at 35°C and pH 6.8, which grew 2—3.5 times slower with 300 mM ammonia than with 3 mM.

It is generally acknowledged that NH_3 is more toxic than NH_4^+ (Kugelman and Chin 1971; Anderson et al. 1982). Therefore the effect of a lower temperature or a higher pH on ammonia inhibition has also been researched. The results are shown in Fig. 2B and C. The effect of pH and temperature on ammonia dissociation is shown in Table 2. The calculation used to obtain the data shown in Table 2 is explained elsewhere (Koster 1986). As would be expected for biological systems, the maximum growth rate decreased with decreasing temperature. Within a similar range of ammonia concentrations at a similar pH, all data obtained at 29°C (Fig. 2B) were much lower than those obtained at 37°C (Fig. 2A). Even at 37°C and pH 7.8, where the fraction of NH_3 was ten times higher than at 29°C and pH 7, the maximum growth rates appeared to be higher up to 150 mM ammonia.

The effect of pH and temperature can best be judged from the slope of the linear relation between maximum growth rate and ammonia concentration at different pH and temperature combinations. In Table 3 the parameters of the relationship obtained by means of linear regression are shown. In view of the values of the respective correlation coefficients, it can be concluded that at pH 7 a decrease in temperature from 37°C to 29°C resulted in a slightly diminished susceptibility of the hydrogenotrophic methanogens for increasing ammonia concentrations, while this positive effect is overshadowed by the overall decrease in maximum growth rate. An increase in pH from 7.0 to 7.8 hardly affected the maximum growth rate at uninhibitory ammonia concentrations. However, at pH 7.8 and 37°C the slope of the relationship between ammonia concentration and maximum growth rate appeared to be almost three times steeper than at pH 7.0 and 37°C (Table 3). This confirms the concept that NH_3 is a more powerful inhibitor than NH_4^+.

All experiments were performed with sludge that had never before experienced inhibitory ammonia concentrations. In our present work the hydrogenotrophic methanogenic population was found to grow well (although inhibited) at ammonia concentrations as high as 350 mM without requiring an adaptation period. All the available reports about unadapted methanogenic populations which temporarily cease methanogenesis at ammonia concentrations in the range 120—140 mM (Albertson 1961; Melbinger and Donnellon 1971; van Velsen 1979; Koster and Lettinga 1984; Koster 1986) and resume methanogenesis only after an adaptation period concern mixed populations in which methane is produced from hydrogen as well as from actate. Our present findings indicate that probably in those studies the

Table 3. Parameters of the linear regression function μ_m (h^{-1}) = A − B*ammonia (M) for three sets of experiments at different pH and temperature combinations

pH	Temp. (°C)	A	B	Number of data	Correlation coefficient
7.0±0.1	37±0.5	0.114	0.141	7	−0.84
7.0±0.1	29±0.5	0.061	0.110	9	−0.70
7.8±0.1	37±1.0	0.105	0.369	12	−0.80

adaptation period was required for the acetoclastic methanogens only. The finding that hydrogenotrophic methanogens are less susceptible to ammonia toxicity than acetoclastic methanogens is in accordance with earlier findings (Koster and Lettinga 1984; Sprott and Patel 1986).

If hydrogenotrophic methanogens are inhibited, the resulting build up of hydrogen in a digester treating complex wastes may result in a thermodynamic blockage of propionate degradation (Gujer and Zehnder 1983). Therefore, we also researched whether increased propionate concentrations interfere with ammonia inhibition of hydrogenotrophic methanogens. The results of these experiments with propionate concentrations which are representative for those often found in inhibited or overloaded digesters are shown in Fig. 3. The propionate concentrations were determined at the beginning and at the end of each experiment. It was found that there was no significant difference between concentrations before and after an experiment. Obviously the hydrogen concentration in the liquid phase was high enough to prevent propionate degradation. Although is has been suggested that propionate can inhibit methane fermentations (Andrews 1969; Hobson and Shaw 1976) hydrogenotrophic methanogens were not inhibited by propionate in our experiments, even in the presence of high ammonia concentrations.

From the results obtained in the present research some conclusions concerning the operation of anaerobic waste and wastewater treatment systems can be drawn. The lowest maximum growth rate found in our experiments was $0.023\ h^{-1}$, at pH 7.8 and 37° C. This maximum growth rate allows for a minimum cell retention time of 30.1 h. This means that, even under the most severe conditions tested, wash-out of hydrogenotrophic methanogens from digesters treating slurries such as manure or sewage sludge (which are operated as completely mixed tank fermentors without cell retention) will not occur. Such digesters are generally operated for 20—30 days retention time (Meynell 1982). Wash-out from industrial anaerobic wastewater treatment systems also will not occur, since these systems are all provided with highly sufficient cell retention devices allowing for cell residence times which far exceed those of sludge digesters (Lettinga et al. 1984).

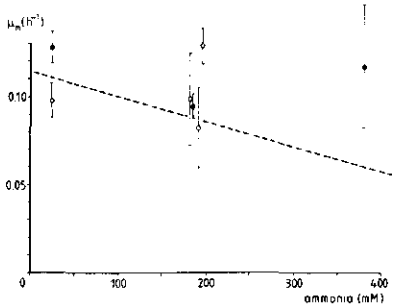

Fig. 3. Maximum growth rate of a hydrogenotrophic methanogenic population at 37.5° ± 0.5° C and pH 7.1 ± 0.2 at various ammonia concentrations with a background of 12.1 ± 0.4 mM (●) or 56.6 ± 5.0 mM (○) of propionate (mean ± standard deviation). The *dotted line* represents the linear regression line for data obtained under similar conditions with the exception of propionate (see Fig. 2A). The *bar* represents the 95% confidence interval

It has also been established that the inhibition of hydrogenotrophic methanogens by ammonia is not accelerated by the build up of propionate, which in practice would undoubtedly occur as a result of such an inhibition. Therefore, as long as it does not cause a pH drop below the physiological limits of the methanogenic bacteria, a propionate build up is tolerable. Probably the most important practical implication of the present work can be derived from Tables 2 and 3, which indicate that changing the ionization state of ammonia by means of decreasing the pH might be a good option to overcome ammonia inhibition. If the ammonia inhibition experienced is not too severe, the pH drop caused by build up of organic acids such as propionate as a result of hydrogen accumulation caused by ammonia inhibition might probably be sufficient. In that way the digestion process would be self-regulating to a certain extent.

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CHAPTER 6

AMMONIA INHIBITION OF METHANOGENESIS DURING ANAEROBIC DIGESTION OF CONCENTRATED MANURE SLURRIES

ABSTRACT

Methanogenesis during anaerobic digestion of liquid poultry manure and several mixtures of liquid poultry manure and cow manure was investigated using lab-scale semi-continuously fed reactors. During anaerobic digestion of liquid poultry manure the ammonia concentration was 9.8 g N/l at a pH in the range 7.9-8.0. At 30 ^{o}C and a hydraulic retention time of 73.3 days the amount of methane that could be produced per m^3 of liquid poultry manure was 31.8 Nm^3. This is considerably higher than the values reported in the literature, which can be attributed to the very high solids content of the manure investigated (16.1 % TS). However, the methane production rate per unit reactor volume was only 0.433 $Nm^3/(m^3.day)$. This is lower than all available literature data, which concern situations in which 2 - 4 times shorter hydraulic retention times were applied. The long hydraulic retention time and the low reactor specific methane production are due to the extreme ammonia concentration, which also caused a large build-up of propionic acid.

Dilution of liquid poultry manure with cow manure resulted in much better methanogenesis during anaerobic digestion of the mixtures. The 2:3 mixture of poultry and cow manure could be digested without any serious volatile fatty acids build-up at a hydraulic retention time of 37 days resulting in a methane production of 17.6 Nm^3 per m^3 mixture. For the 3:2 and the 4:1 mixtures these values were 24.3 Nm^3/m^3 at 38.5 days and 29.7 Nm^3/m^3 at 43.5 days respectively. The highest reactor specific methane production rates that could be obtained within the experimental period of approximately 300 days were 0.47, 0.62 and 0.69 $Nm^3/(m^3_r.d)$ with the 2:3, 3:2 and the 4:1 mixture of poultry and cow manure respectively.

The increasing ammonia concentration (from 5.9 to 8.1 gN/l at an average pH of 7.9) with an increasing fraction of poultry manure in the mixture caused some inhibition of methanogenesis, which was reflected in the necessity for higher hydraulic retention times in order to achieve complete (viz. without build-up of volatile fatty acids) digestion.

Although decreasing the temperature resulted in much lower concentrations of un-ionised ammonia (generally presumed to be the most inhibitory ammonia species), this could not improve the rate of methanogenesis in any of the cases tested in the present study.

INTRODUCTION

Anaerobic digestion is a biotechnological process for the production of methane-rich biogas which depends on the combined activity of a miscellaneous microbial population, consisting of diverse genera and species of obligate and facultative anaerobic bacteria. This complex microbial population can roughly be divided into two groups: acid producers and acid consumers. The acid consumption is done by the methanogenic bacteria in close co-operation with acetogenic bacteria. The methanogens are the most sensitive of all bacteria involved in anaerobic digestion for environmental stress, and inhibition of methanogens may lead to an unbalance between acid production and acid consumption hence causing detrimental environmental conditions from which the population as a whole can never recuperate (Koster, 1988).

Ammonia, which is the end-product of anaerobic digestion of proteins and amino acids, is present in all digesters treating organic waste or wastewater. Ammonia inhibits methanogenic bacteria at concentrations exceeding several hundreds mg/l in case of un-adapted microbial populations (Koster & Lettinga, 1984), but even after a prolonged period of zero methanogenesis adaptation to much higher concentrations is possible (Van Velsen, 1979; Koster, 1986).

The nitrogen content in Dutch liquid poultry manure is such that ammonia concentrations of approximately 10 g N/l may be expected during digestion. Such high ammonia

concentrations are correlated with high solids concentrations. It has been argued on the basis of a kinetic model that methane formation from manure is inhibited by increasing concentrations of organic solids in the manure to be digested (Hashimoto, 1982 & 1984). However, in the research of Zeeman et al. (1985) it has clearly been shown that a poor digestion performance of a concentrated manure must be attributed to the ammonia concentration and not the high volatile solids concentration.

The high solids content of liquid poultry manure allows anaerobic digestion only in CSTR-like digesters with a suspended biomass. Immobilized methanogenic populations have been reported to perform methanogenesis at ammonia concentrations of 6000 mg N/l (Parkin et al., 1983) and 7800 mg N/l (De Baere et al., 1984). In a batch experiment with adapted granular methanogenic sludge it has been found that methanogenesis can occur even at an ammonia concentration of 11.8 g N/l (Koster & Lettinga, 1988).

The present article deals with the influence of ammonia on methanogenesis during anaerobic digestion of liquid poultry manure and mixtures of liquid poultry manure and cow manure at concentrations exceeding 6000 mg N/l. The effect of ammonia on any bacterial conversion other than methanogenesis (which probably exists) was not subject of the research described in this article.

MATERIALS AND METHODS

experiments

First the characteristics of methanogenesis during anaerobic digestion of liquid poultry manure at temperatures of 20, 25, 30 and 39 ^{o}C respectively were assessed. These experiments were followed by a series of experiments concerning methanogenesis with mixtures of liquid poultry manure and cow manure at 30 ^{o}C. This series of semi-continuously fed experiments was concluded with a short period of operation at 20, 25 and 30 ^{o}C respectively, during which the methane production rate immediately after addition of feed was monitorred to obtain parameters from which the effect of a decreasing temperature could be evaluated.

reactors

The experiments were carried out with a series of five identical perspex double-walled reactors, connected to thermostated water baths. Each reactor was supplied with a central 3 blade stirrer. The upper blade was U-shaped, with both ends extending out of the liquid in order to prevent the build-up of a foam layer. The lower blade was placed near the bottom in order to prevent formation of a solid grit layer. The reactors were stirred at 150 rpm during 30 seconds at 45 minutes intervals. With the long retention times applied this means that the reactors can be considered as completely mixed tanks. Each reactor had a volume of 6 liters and contained 5 liters of digesting fluid.

seed materials

At the start of the experiments with mere liquid poultry manure 4 reactors (one at each temperature) were seeded with a sample of the reactor contents from an experimental 5 m^3 digester treating solid poultry manure slurried by addition of piggery wastes at the IMAG experimental farm at Duiven. The ammonia concentration in the seed material was 9.5 g N/l.

The reactors used for the digestion experiments with poultry/cow manure mixtures were seeded with mixtures of a sample of the reactor contents of a full scale digester treating liquid poultry manure and occasional additions of piggery wastes and slaughterhouse flocculation sludges and a sample of the reactor contents of an experimental full scale digester treating liquid cow manure. The mixing ratio of the two seed materials equalled that of liquid poultry manure / liquid cow manure in the feed. The ammonia concentration in the reactor contents of the digester treating mainly liquid poultry manure was 10.2 g N/l, and 4.9 g N/l in the reactor contents of the cow manure digester.

feed

On Monday, Wednesday and Friday after vigorous mixing of the reactor contents, part of the mixed liquid was replaced by fresh manure. The amount of liquid to be replaced was determined by the required hydraulic retention time. On Friday 1.5 times the amount of Monday or Wednesday was replaced. The composition of the liquid poultry manure and of the poultry/cow manure mixtures used in the experiments is shown in tables 1 and 2 respectively.

Table 1 Composition of the liquid poultry manure

volatile fatty acids (g COD/l)	
acetic acid	22.4
propionic acid	12.7
iso-butyric acid	1.26
butyric acid	14.0
iso-valeric acid	1.95
valeric acid	0.35
total	52.6
dissolved COD (g/l)	61.85
total-COD (g/l)	179.6
ammonium-nitrogen (g/l)	7.9
total solids (% w/w)	16.1
organic solids (% w/w)	10.2

Table 2 Composition of the poultry/cow manure mixtures

	weight ratio poultry:cow		
	2:3	3:2	4:1
volatile fatty acids (g COD/l)			
acetic acid	10.6	14.6	19.4
propionic acid	7.4	9.7	12.2
iso-butyric acid	0.9	1.1	1.3
butyric acid	5.8	9.2	12.8
iso-valeric acid	1.6	1.8	2.2
valeric acid	0.2	0.2	0.6
total	26.4	36.7	48.4
dissolved COD (g/l)	41.4	60.1	77.8
ammonium-nitrogen (g/l)	4.4	5.0	6.4
total solids (% w/w)	11.1	12.8	14.4
organic solids (% w/w)	7.5	8.4	9.3

experimental set-up

The reactors fed with mere liquid poultry manure were operated at a fixed hydraulic retention time of 73.3 days, except the reactor at 20 ^{o}C in which the hydraulic retention time was 100 days. These hydraulic retention times were chosen on the basis of earlier experiences with the 5 m^3 pilot scale reactor from which seed material was obtained (Hoeksma, 1986).

The experiments with poultry/cow manure mixtures were started at a hydraulic retention time of 200 days. The hydraulic retention time was shortened as soon as the concentration of volatile fatty acids (acetic up to valeric) had reached a certain low level. Initially a value equivalent to a chemical oxygen demand (COD) of 500 mg /l was used as such a reference point. Later this criterium was changed to 1000 mg COD/l, with the restriction that a build-up of propionic acid was not to be allowed.

At the termination of the experiments with poultry/cow manure mixtures the reactor specific methane production in each reactor was assessed at 20, 25 and 30 ^{o}C by means of frequently measuring the methane production with wet test gas meters during approximately 8 hours immediately after feeding. The methane production rate was calculated by means of linear regression analyses of the relation between time and cumulative methane production. In all cases the regression coefficients exceeded 0.99.

analyses

Well homogenized samples were centrifuged for 40 minutes at 13000 *g*. Next the supernatant was filtered over a paper filter (S&S, black belt) and centrifuged again for 40 minutes at 13000 *g*. The clear supernatant was the basis for all further analyses except solids content.

Volatile fatty acids were determined gaschromatographically in a 0.45 μm membrane filtered sample after 5 times dilution in 10 % formic acid. The filtrate was injected on a 2m x 2mm (i.d.) glass column packed with 10 % Fluorad on Supelco-port 100-120 mesh. Formic acid saturated nitrogen was used as carrier gas at a flow rate of 30 ml/min. The temperature of the injection port, the column and the FI-detector was 220, 130 and 240 ^{o}C respectively.

Chemical oxygen demand (COD) was determined according to Standard Methods (anon., 1975).

Ammonia was determined by the direct Nesslerization method in a 500 times dilution in demineralized water.

Total solids were determined as the remaining weight of a well homogenized sample after drying at 105 ^{o}C in a crucible at least overnight. The dried solids were incinerated at 600 ^{o}C during 45 minutes in order to assess the ash fraction.

The biogas produced in a reactor was conveyed via a column packed with lime pellets into an aluminium covered plastic bag. The lime absorbed all CO_2 and H_2S so that the gas collected in the bags was wet methane. The amount of gas produced was measured daily by pumping the bag contents through a wet test gas meter. The readings were corrected to a temperature of 0 ^{o}C.

RESULTS AND DISCUSSION

The time course of the manure specific methane production (viz. the volume of methane produced per volume of manure) and the reactor specific methane production (viz. the methane production rate per volume of reactor contents) in the digestion of liquid poultry manure at a hydraulic retention time of 73.3 days at 30 ^{o}C is shown in figure 1. The time course of the volatile fatty acids concentration (expressed as COD equivalents in g/l) during the same experiment is shown in figure 2. It is obvious that during the first 40 days of operation digestion was not yet stable, since the methane production as well as the volatile fatty acids concentration continuously rised. After approximately 40 days the total volatile fatty acids concentration started to decrease slightly and beyond 50 - 60 days from the start both the total volatile fatty acids concentration and the methane production rate were no longer subject to short term changes.

As is shown in figure 2, the anaerobic digestion of liquid poultry manure at 30 ^{o}C and 73.3 days hydraulic retention time resulted in rather high concentrations of volatile fatty acids in the digester contents. Although generally in anaerobic digestion of manure stable conditions such a high build-up of volatile fatty acids is absent, increased volatile fatty acids concentrations in digesters fed with poultry manure have been reported often (Converse et al., 1981; Gosch et al., 1982; Hills & Ravishanker, 1984; Hobson et al., 1980; Pechan et al., 1987; Ripley et al., 1984; Safley et al., 1987). Few of these authors provide

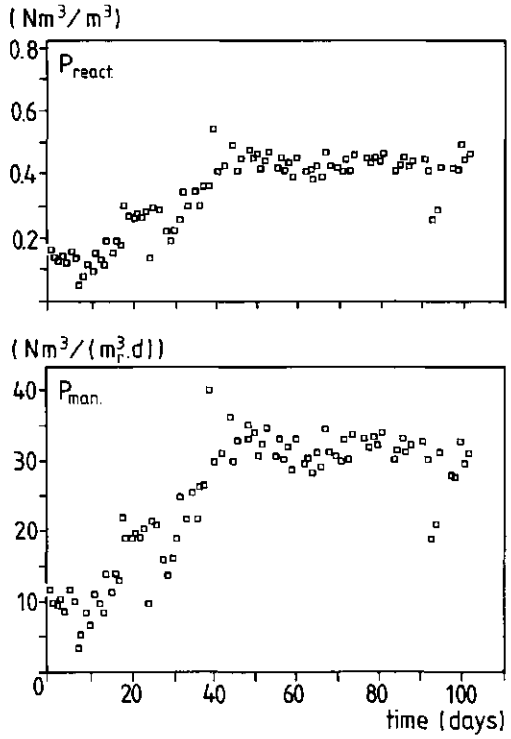

Fig. 1 *The manure specific methane production (P_{man}) and the reactor specific methane production (P_{react}) during anaerobic digestion of mere liquid poultry manure at 30 °C and 73.3 days hydraulic retention time*

data on the type of volatile fatty acids present. Pechan et al. (1987) reported a build-up of 6.34 g COD/l that consisted of 26 % acetic acid and 26 % propionic acid. Ripley et al. (1984) reported a volatile fatty acids build-up Of 4.8 g/l (expressed as HAc) consisting for approximately 94 % of acetic acid. In the present experiments the volatile fatty acids build-up (after an initial period of unstable digestion) consisted mainly of propionic acid. A build-up of propionic acid indicates an overloading of the hydrogenotrophic methanogens, resulting in a hydrogen build-up which makes it thermodynamically imposible for bacteria to degrade propionic acid (Thauer et al., 1977). The propionic acid concentration at the end of the experiment with liquid poultry manure at a hydraulic retention time of 73.3 days at 30 °C was 7.86 g/l. From the influent flow rate and the propionic acid concentration of the liquid poultry manure it can be calculated that in case of absence of either production and consumption the propionic acid concentration at that time should have been 6.39 g/l. Obviously, instead of a net degradation there had been a production of propionic acid due to fermentation processes. The butyric acid concentration at the end of the experiment was 0.013 g/l. This implies that butyric acid degradation had taken place, otherwise the concentration would have been 5.86 g/l. With respect to the obvious overloading of the hydrogenotrophic methanogens in the present experiments it should be noted that the butyric acid degradation, which can take place at hydrogen levels slightly higher than those allowing propionic acid degradation (Koster, 1988), indicates that hydrogenotrophic methanogenesis did occur. If the experiments would have been continued longer, the hydrogenotrophic methanogenic population might have become able to keep the hydrogen level sufficiently low to permit not only butyric acid degradation but propionic acid degradation as well. Especially in the case of ammonia inhibited anaerobic manure digestion steady states will not be reached within 3 - 4 volume turnovers (Hashimoto, 1986).

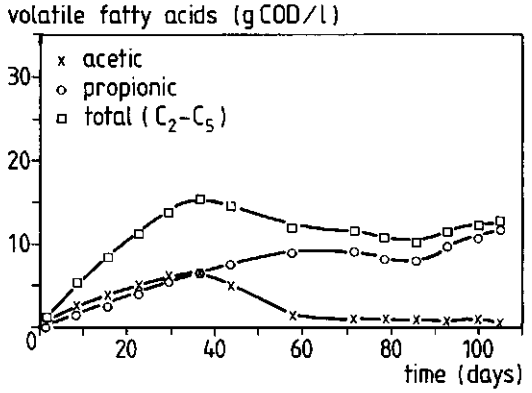

Fig. 2 *The time course of the volatile fatty acids spectrum in the digester fluid during anaerobic digestion of mere liquid poultry manure at 30 °C and 73.3 days hydraulic retention time*

The reactor specific methane production and the manure specific methane production calculated from the period of more or less stable digestion (beyond day 50) are shown in table 3, together with some literature data for anaerobic digestion of liquid poultry manure. Table 3 also contains data concerning pH and ammonia concentration in the digesting mass. In the three reactors operated on liquid poultry manure the ammonia concentration was determined 26 times; an ammonia concentration (± standard deviation) of 9778 ± 475 mg N/l was assessed. The pH in the reactors was in the range 7.9 - 8.0. The literature data presented in table 3 are a selection of the data obtained from a computer aided literature search concerning the period 1975 onwards in the databases AGRIS and AGRICOLA. Data concerning reactor types other than CSTR or influents with less than approximately 10 % total solids have been omitted.

A comparison of the results from the present study with literature data shows that the reactor specific methane production as established in the present research is relatively low, whereas the manure specific methane production is relatively high. The high manure specific methane production obviously is the direct result of the fact that the manure used in the present research is much more concentrated. However, the high concentration of the manure (viz. the high total and volatile solids content) is correlated with a high concentration of ammonia in the digester. In fact the ammonia concentrations applied in the present work are at least 3 gN/l higher than those reported in the literature for liquid poultry manure digestion. This can explain why in the present experiments with liquid poultry manure digestion was incomplete even at a hydraulic retention time exceeding 2 - 4 times most hydraulic retention times reported in the literature.

Because of the fact that at a hydraulic retention time of 73.3 days anaerobic digestion of liquid poultry manure at 30 °C had turned out to be impossible without a considerable build-up of volatile fatty acids, in the next series of experiments the reactors were started at a hydraulic retention time of 200 days. One reactor in this series was planned for digesting the same liquid poultry manure as used in the preceeding series of experiments. Even at 200 days hydraulic retention time it was not possible to avoid volatile fatty acids build-up to simolar levels as in the preceeding experiments at 30 °C and 73.3 days hydraulic retention time. Start-up of this reactor was commenced several times with the same result, viz. a build-up of volatile fatty acids within weeks. Therefore the results of that particular reactor are not included here. The other three reactors, which were fed with poultry/cow manure mixtures (table 2) performed well from the start.

With all three mixtures the manure specific methane production gradually decreased within approximately 9 weeks to a more or less stable level. Gas production from the seed material could be the cause of this "extra" methane production.

The reactor specific methane production increased from the start of the experiments. This can be explained by the decreasing hydraulic retention time. At operating

Table 3 Operating parameters of mesophilic anaerobic digestion of slurried poultry droppings in completely mixed tank reactors

temperature (°C)	influent: total solids (%)	influent: organic solids (%)	digester fluid: ammonia (mg N/l)	digester fluid: pH	retention time (days)	methane production: reactor volumetric ($m^3/(m^3_r.day)$)	methane production: manure volumetric (m^3/m^3_{man})	reference
35	9.98	7.20	4122	7.97	29.2	0.510	14.90	Webb & Hawkes, 1985
35	9.96	6.84	4274	7.78	14.6	1.092	15.94	"
n.r.	9	n.r.	1000-1500	8-8.5	80	0.41	32.8	Mahadevaswamy & Venkataraman, 1986
34.3	11.9	8.2	5690	8.07	40.3	0.461	18.59	Pechan et al., 1987
35	13	9.1	>6000	n.r.	20	1.32	15.96	Hobson et al., 1980
32	12.56	9.57	n.r.	n.r.	17	1.454	24.42	Scheurer & Maurer, 1985
32	12.56	9.57	n.r.	n.r.	30	0.963	28.83	"
35	13.35	10.01	5900	8.04	20	0.865*	17.31*	Gosch et al., 1982
n.r.@	10.1	7.4	3980	7.87	30	0.62*	18.6*	"
35	9.73	6.38	6840	8.01	36	0.390	14.03	Converse et al., 1981
35	11.68	7.58	8070	7.80	46	0.444	20.44	"
35	12.52	8.18	8990	7.86	45	0.515	23.19	"
35	11.58	8.02	6780	8.08	38	0.625	23.75	"
35	11.34	8.10	4760	7.80	36	0.752	27.08	"
30	16.1	10.2	9778	7.9-8.0	73.3	0.433 ± 0.023**	31.78 ± 1.69**	this study

n.r. : not reported | @ : probably 35 °C | * : gas volume corrected to 1 bar and 0 °C | ** : gas volume corrected to 0 °C

conditions allowing complete digestion (viz. volatile fatty acids are not allowed to build-up) an increasing reactor specific methane production will be the result of an increasing influent flow rate.

The first parts of the experiments have not been used in the further evaluation of the reactor performance with the different manure mixtures. This concerns the first 128 days for the 2:3 mixture (poultry:cow), the first 95 days for the 3:2 mixture and the fist 150 days for the 4:1 mixture. The reason for this is the extra gas production from the seed material during the first 9 weeks of the experiment, and the fact that the initial aim was to obtain digestion without volatile fatty acids build-up at low hydraulic retention times, rather than to assess accurately the gas production parameters at each hydraulic retention time. During that period feeding rates changed frequently, sometimes even within a few days. During the period used for evaluating the reactor performance each hydraulic retention time was maintained long enough to obtain sufficient indication that the digestion process was stable. Process stability was assumed if the volatile fatty acid concentration stayed below 500 mg COD/l (later: 1000 mg COD/l and no build-up of propionate) for several weeks. Although Hashimoto (1986) postulated that in manure digestion at inhibitory ammonia concentrations process failure may manifest after prolonged periods of time, for reasons of convenience the present strategy was preferred over the conventional strategy in which a series of reactors has to be operated, with each reactor at a fixed hydraulic retention time for periods of at least 3 hydraulic retention times.

Table 4 summarizes the reactor specific methane production and the manure specific methane production at various hydraulic retention times. A one way analysis of variance (ANOVA) for each of the three series of experiments showed that the reactor specific methane productions obtained at the various hydraulic retention times differ significantly ($P << 0.001$). Table 4 shows a clear trend of higher reactor specific methane productions with shorter hydraulic retention times. A one way ANOVA analysis of the manure specific meth-

Table 4 Methane production at various hydraulic retention times during anaerobic digestion of mixtures of poultry and cow manure at 30 °C.

poultry:cow	hydraulic retention time (days)	methane production * reactor specific ($Nm^3/(m^3_r.day)$)	manure specific (Nm^3/m^3_{man})
2:3	50	0.352 ± 0.043 (16)	17.62 ± 2.09 (16)
	45.5	0.398 ± 0.057 (32)	18.12 ± 2.59 (32)
	41.7	0.414 ± 0.040 (12)	17.28 ± 1.69 (12)
	40	0.433 ± 0.059 (24)	17.30 ± 2.35 (24)
	37	0.466 ± 0.029 (17)	17.24 ± 1.08 (17)
3:2	55.6	0.450 ± 0.043 (22)	25.04 ± 2.40 (22)
	52.6	0.459 ± 0.057 (16)	24.15 ± 3.01 (16)
	45.5	0.540 ± 0.070 (32)	24.58 ± 3.17 (32)
	41.7	0.573 ± 0.069 (36)	23.89 ± 2.87 (36)
	38.5	0.623 ± 0.033 (16)	24.00 ± 1.25 (16)
4:1	55.6	0.581 ± 0.084 (36)	32.31 ± 4.65 (36)
	50	0.579 ± 0.101 (11)	28.97 ± 5.03 (11)
	47.6	0.627 ± 0.081 (23)	29.84 ± 3.84 (23)
	43.5	0.686 ± 0.024 (17)	29.86 ± 1.03 (17)

* = mean ± standard deviation (number of data).

ane productions obtained in each series of experiments showed that the difference in the values shown in table 4 is not significant for the 3:2 mixtures ($P = 0.55$) and the 2:3 mixture ($P = 0.56$). For the experiment with the 4:1 mixture the manure specific methane production at a hydraulic retention time of 55.6 days is significantly different ($0.01 < P < 0.05$) from the manure specific methane productions at the other hydraulic retention times, which were found to be not significantly different ($P = 0.77$). After 150 days of operation methane production from the seed material can be ruled out as explanation. A rather hypothetical explanation is that at the start of the experiment the hydrolytic potential in the digesters was relatively high due to the high hydrolytic activity in the cow manure digester from which part of the seed material originated. According to this hypothesis the enzymes from the cow manure digester worked in their new environment, but were not fully replenished after wash-out due to ammonia inhibition of the exo-enzymes producing bacteria, therefore resulting in a gradually decreasing hydrolysis towards a steady-state level conform the new environmental conditions. For the 4:1 mixture this new level would not have been reached until the change to a hydraulic retention time of 50 days. If this hypothesis is treue, it can also partly explain the excesive methane production from all mixtures during the first 9 weeks. Unfortunately literature data supporting the hypothesis of temporarily increased hydolytic activity due to seeding with digested cow manure could not be found. Probably the most plausible explanation is that there is some effect of shortening the hydraulic retention time on the hydrolysis and subsequent acidogenesis. The experimental set-up hardly allowed build-up of volatile fatty acids. Only occasionally a short (viz. lasting only a few days) rise of the volatile fatty acids concentration beyond the performance criterium occurred after shortening the hydraulic retention time. In all cases (except the already discussed change of the hydraulic retention time for the 4:1 poultry/cow manure mixture from 55.6 to 50 days) almost all volatile fatty acids were converted into methane without a noticable effect of the hydraulic retention time on the manure specific methane production. This indicates that in those cases the hydrolysis of solids did not change with the hydraulic retention time.

Since the nitrogen concentration in liquid poultry manure is much higher than in liquid cow manure the ammonia concentration in the reactors differed according to the mixture composition. Table 5 shows the results of ammonia analysis in all reactors.

Table 5 Ammonia concentrations (mg N/l) during anaerobic digestion of mere liquid poultry manure and poultry/cow manure mixtures

poultry:cow	mean concentration	standard deviation	number of data
2:3	5856	360	31
3:2	6791	457	32
4:1	8050	449	29
1:0	9778	475	26

Ammonia is an important contributor to the buffering system of manure digesters (Georgacakis et al., 1982). In the present experiments ammonia contributed 355 meq (2:3 mixture) to 590 meq (1:0 mixture) to the buffering capacity, with the result that there was hardly any difference in pH between the three reactors ($P < 0.25$ in a one way ANOVA analysis). The average pH was 7.9, with a maximum variation of 0.1 unit.

The inhibitory effect of ammonia is reflected in the fact that it was impossible to achieve stable anaerobic digestion of mere liquid poultry manure without a considerable build-up of volatile fatty acids. Since the seed material had been obtained from a well-working practical scale digester operated on a similar substrate, the build-up of volatile fatty acids cannot be attributed to incomplete adaptation of the methanogens. Apparently, when digesting poultry/cow manure mixtures the lower ammonia concentrations allowed an-

aerobic digestion without high volatile fatty acids build-up. In these experiments the inhibitory action of ammonia became evident in the longer periods required between hydraulic retention time changes with higher ammonia concentrations. As expected a higher ammonia concentration caused a decrease of the volatile fatty acids degradation rate so that it took longer before the volatile fatty acids concentration (which temporarily rose after each hydraulic retention time decrease) was at the level at which a new hydraulic retention time decrease could be imposed. In the experiment with the 2:3 poultry/cow manure mixture it took a period of 128 days before a hydraulic retention time of 50 days was possible, whereas for the experiments with the 3:2 and the 4:1 mixture these periods were 151 and 215 days respectively.

The inhibitory effect of ammonia also becomes clear by comparing the results of the experiments with the poultry/cow manure mixtures with literature data concerning cow manure digestion. Based on results of extensive research concerning anaerobic digestion of Dutch cow manure (Zeeman et al., 1984), it can be calculated that at 30 ^{o}C the cow manure used in the present experiments would have yielded a manure specific methane production of 6.84 - 9.12 Nm^3/m^3 and a reactor specific methane production (at a hydraulic retention time of 20 days) of 0.342 - 0.456 $Nm^3/(m^3.d)$. Comparing these figures with table 4 makes clear that the addition of poultry manure resulted in much higher methane yields per volume of manure. However, the reactor specific methane production that could be obtained with the 2:3 mixture of poultry and cow manure is in the same order of that obtainable with pure cow manure. This is probably caused by the fact that the addition of poultry manure increased the ammonia concentration in the digesting slurry, therefore causing the necessity of longer hydraulic retention times. If higher volatile fatty acids concentrations had been allowed, the hydraulic retention time probably could have been shorter; in that case the reactor specific methane production would have been higher. The reactor specific methane productions obtained with the other two mixtures were higher than that obtainable with pure cow manure. Apparently the negative effect of the longer hydraulic retention times is eliminated by the much higher methane production per unit of volume of the mixture.

In case of methanogenesis in presence of potentially inhibitory compounds, the effective inhibitory concentration rather than the total concentration of these compounds is important (Koster, 1988). Since unionised ammonia is known to be a much stronger inhibitor for methanogenesis than the ammonium ion, temperature should influence ammonia toxicity. The reason for this is that at higher temperatures, a larger fraction of the total amount of ammonia will be present in the unionised form. This probably is the main reason why anaerobic digestion of liquid poultry manure at 39 ^{o}C was impossible. The volatile fatty acids concentration in the reactor rised continuously to a level of over 40 g/l COD equivalent by the end of the experiment after 105 days. Only 5.5 % of the total amount of COD that had been put into the reactor was converted into methane. The methane production at 39 ^{o}C amounted only to 12 % of that at 30 ^{o}C, while both reactors were seeded with the same material and operated at the same hydraulic retention time. The hypothesis of temperature accelerating ammonia inhibition of methanogenesis via increasing the fraction of unionised ammonia has been used previously to explain the poor results of thermophilic piggery wastes digestion as compared to mesophilic digestion (Van Velsen et al., 1979; Van Velsen & Lettinga, 1981). Nevertheless there are some reports of anaerobic digestion of liquid poultry manure at 50 ^{o}C (Steinsberger and Shih, 1984; Shih, 1987) and 55 ^{o}C (Huang and Shih, 1981), but these manures were 2-3 times less concentrated than the manure used in the present experiments, so the total ammonia concentration (which was not reported) must also have been much lower.

There is also one report claiming that decreasing the temperature alleviated the inhibitory effect of ammonia during piggery wastes digestion (Braun et al., 1981). The effect of decreasing the temperature on the amount of unionised ammonia in the reactors treating liquid poultry manure or mixtures of liquid poultry manure with cow manure is shown in table 6. The values in this table were calculated according to a method explained elsewhere (Koster, 1986). All values are well above 150 mg N/l, the concentration reported by McCarty & McKinney (1961) to cause total cessation of methanogenesis. However, it

should be recognized that very likely they worked with unadapted bacteria. Adaptation can increase the ammonia tolerance of methanogenic bacteria enormously (Koster, 1986).

Table 6 Unionised ammonia concentrations (mg N/l) at pH 7.9 in mere liquid poultry manure and poultry/cow manure mixtures during anaerobic digestion at various temperatures. (Total ammonia: see table 5)

poultry:cow	temperature 20 °C	25 °C	30 °C	39 °C
2:3	180	253	353	612
3:2	208	293	409	709
4:1	247	348	485	841
1:0	300	422	589	1021

In order to assess the effect of lower temperatures on ammonia inhibited methanogenesis during anaerobic digestion of liquid poultry manure two reactors were operated at 20 and 25 °C respectively. The operating parameters of these reactors, together with those of the reactor operated at 30 °C (which is also discussed earlier in this chapter) are summarized in table 7. In a CSTR-like operation, anaerobic digestion of liquid poultry manure at 20 °C clearly has no perspective at all. Even at a very high hydraulic retention time the reactor specific methane production as well as the manure specific methane production were found to be much lower than at higher temperatures. The volatile fatty acids concentration continuously rose during the 20 °C experiment, and amounted to 18.5 g COD/l when the experiment was terminated after 67 days of operation. At 25 °C the methane production rate per unit of reactor volume, as well as the quantity of methane produced per unit of volume of liquid poultry manure stayed far behind the results obtained with the similarly operated digester at 30 °C. In both digesters the volatile fatty acids build-up (which was more or less constant after 50 - 60 days) consisted mainly of propionic acid (see figure 2). The total volatile fatty acids build-up amounted to 25.7 and 11.6 g COD/l at 25 and 30 °C respectively. If in the anaerobic digestion of liquid poultry manure lowering of the concentration of unionised ammonia via decreasing the digestion temperature would have a stimulating effect on methanogenesis, apparently this was overshadowed in the present experiments by the rate decreasing effect of lowering the temperature.

Table 7 Methane production from anaerobic digestion (with volatile fatty acids build-up, see fig. 2) of liquid poultry manure at various temperatures and hydraulic retention times (HRT)

temp. (°C)	HRT (days)	methane production* reactor specific ($Nm^3/(m_r^3.day)$)	manure specific (Nm^3/m_{man}^3)
20	100	0.042 ± 0.008 (58)	4.17 ± 0.83 (58)
25	73.3	0.236 ± 0.023 (33)	17.32 ± 1.66 (33)
30	73.3	0.433 ± 0.023 (35)	31.78 ± 1.69 (35)

* = mean ± standard deviation (number of observations).

The results of the tests performed at the end of the experiments with mixtures of liquid poultry manure and cow manure at decreased temperatures are summarized in table 8. The values of the reactor specific methane production assessed in these tests at 30 °C (table 8) are somewhat higher than those assessed during the semi-continuously fed experiments (table 4). This is probably due to the fact that the activity measurements were based

Table 8 Temperature effect on the reactor specific methane production ($Nm^3/(m_r^3.day)$) assessed immediately after feed addition to reactors operated on poultry/cow manure mixtures

	poultry:cow		
temperature	2:3	3:2	4:1
20	0.256	0.234	0.337
25	0.365	0.409	0.516
30	0.577	0.660	0.816

data at 20 and 25 °C are mean of two experiments

on the gas production rates during the period of 8 hours immediately after feeding, whereas at other times the average gas production rate over a period of 2 or 3 days was used. At the end of such a relatively long period the substrate for methanogenesis became depleted, as a result of the operation strategy which was aimed at avoiding any build-up of methanogenic substrates. The different assesment methods make it impossible to compare table 8 with other tables.

As was the case with the liquid poultry manure digestion experiments, the activity measurements of anaerobic digestion of poultry/cow manure mixtures at various temperatures show that a temperature decrease cannot increase the methane production rate at high ammonia concentrations.

Contrary to the present findings, Braun et al. (1981) claimed a stimulating effect of a decreasing temperature in ammonia inhibited methanogenesis of swine manure. This difference may be caused by the higher ammonia concentrations in the present experiments.

In order to evaluate the interference of the total ammonia concentration with the effect of the temperature induced decrease of the unionised ammonia concentration on the activity of the methanogenic bacteria, an Arrhenius equation was applied:

$$P_{react} = Ae^{-\frac{E}{RT}}$$

in which: P_{react} = reactor specific methane production
A = frequency factor
E = activation energy
R = gas constant
T = absolute temperature

Since A and E can be considered as constants over short temperature ranges a graph of the natural logarithm of P_{react} against 1/T should yield a straight line which slope corresponds to the activation energy. Such graphs are shown in figure 3. The activation energy was highest for the 3:2 mixture of poultry manure and cow manure and lowest for the 2:3 mixture. So the expectation that a decreased temperature would have less effect at higher ammonia concentrations than at lower ammonia concentrations because of the antagonistic effect of a decreasing temperature on ammonia inhibition could not be proved.

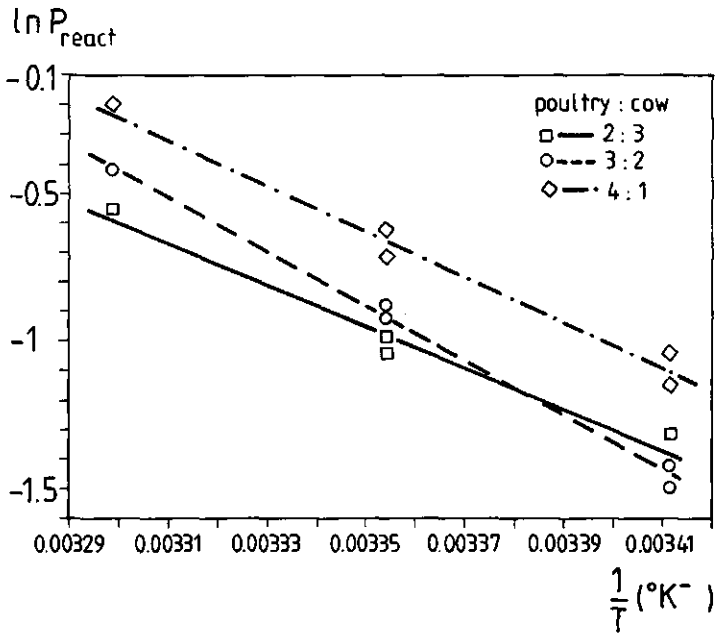

Fig. 3 *Arrhenius plot of the methanogenic activity (expressed in terms of reactor specific methane production) of digester contents from digesters treating various mixtures of liquid poultry manure and cow manure*

CONCLUSIONS

Even at a hydraulic retention time of 200 days anaerobic digestion of mere liquid poultry manure was found to be impossible without a serious build-up of volatile fatty acids. Although the volatile fatty acids build-up amounted to 11.6 g COD/l (representing a potential amount of methane of approximately 4 Nm^3 per m^3 of manure), the amount of methane that could be produced from liquid poultry manure at 30 ^oC and a hydraulic retention time of 73.3 days still amounted to 31.8 Nm^3/m^3. As a consequence of the very high ammonia concentration during digestion of liquid poultry manure, the hydraulic retention time has to be rather long. Such a long hydraulic retention time obviously results in a reactor specific methane production (only 0.433 $Nm^3/(m^3.day)$ in the present research) which is lower than the values reported in the literature for less concentrated poultry manure types, because these can be digested at 2-4 times shorter hydraulic retention times.

"Dilution" of the liquid poultry manure with cow manure resulted in manure mixtures which still contained over 10 % total solids. As a result of the significantly decreased ammonia concentrations much shorter hydraulic retention times could be applied. The experiments at 30 ^oC with "diluted" liquid poultry manure were started at 200 days hydraulic retention time, and after a 300 days experimental period stable digestion without volatile fatty acids build-up could be accomplished at 37, 38.5 and 43.5 days hydraulic retention time with the 2:3, 3:2 and 4:1 poultry/cow manure mixture respectively. Then the reactor specific methane production was 0.47, 0.62 and 0.69 $Nm^3/(m^3.day)$ respectively.

Compared to digestion of mere cow manure, the addition of liquid poultry manure appears to be only beneficial if the amount added is large enough to cause such an extra methane production that it compensates for the longer hydraulic retention time that is required for maintaining stable digestion in the presence of the increased ammonia concentrations. In the present experiments the reactor specific methane production from the

2:3 mixture of liquid poultry manure and cow manure was the same order as reported by Zeeman et al. (1984) for cow manure similar to the cow manure used in the present experiments. The reactor specific methane production from the 3:2 and the 4:1 mixture was much higher.

Experiments with digestion of mere liquid poultry manure at various temperatures as well as activity tests at various temperatures with the digesters treating the manure mixtures indicate that a decreasing temperature did not have any net stimulating effect on methane production rates, although a reduced ammonia toxicity at lower temperatrures could be expected.

The present experiments prove that adapted biomass allows anaerobic digestion at extreme conditions. Previously 150 mgN/l of unionised ammonia was considered to be the upper limit of tolerance for well performing digesters. In the present experiments anaerobic digestion of the 4:1 mixture proceeded well at 589 mg N/l of unionised ammonia.

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SUMMARY OF THE THESIS

Anaerobic digestion is the biological conversion of organic material into methane-rich biogas as the result of the close interaction of many bacterial species. The microbiology of anaerobic digestion including the chemical and physical requirements is discussed in the first part of chapter 1. The bacteria involved in anaerobic digestion can roughly be divided into two groups: acid producers and (methane producing) acid consumers. For a stable digestion the acid production rate and the rate of acid consumption (= methanogenesis) should be balanced. The acid producing biomass tolerates much lower pH values than the methanogenic biomass (with the symbiotic acetogens) and is much less sensitive for environmental stress such as lower temperatures or the presence of toxicants. Therefore the problem of toxicity in anaerobic digestion in fact is the problem of inhibition of methanogenesis.

For a long time anaerobic digestion has been regarded as extremely vulnerable for toxicants of any kinds, leading to the belief that it was unsuitable for industrial application as waste water treatment process. This might have been true for the initial reactor designs which all used suspended biomass at relatively short biomass retention times, but as chapter 1 shows, the modern high-rate digesters based on biomass immobilization and very long biomass retention times can handle waste waters with toxicants very well. The long biomass retention times enable adaptation of the biomass to the presence of toxicants, either by biochemical adjustments of the cells ("true" adaptation) or by the in-growth of bacteria that are able to degrade or at least detoxify the toxicants. Moreover, the immobilized biomass grows in biofilms, which provide micro-environments (especially with respect to pH) at which toxicants are less effective and often contain viable biomass that becomes activated only when part of the normally active biomass is inhibited.

At present the most widely applied modern high-rate anaerobic reactor design used in waste water treatment systems is the Upflow Anaerobic Sludge Bed (UASB) reactor, in which the biomass is immobilized in more or less compact granules. This thesis contains results of several research projects concerning the exposure of granular methanogenic biomass particles from UASB reactors to toxicants. In these granules the predominant acetoclastic methanogens are of the *Methanothrix* type; the population of hydrogenotrophic methanogens is rather unidentified.

In chapter 2 research is described in which the effect of four saturated long-chain fatty acids (caprylic, capric, lauric and myristic) and one unsaturated long-chain fatty acid (oleic) on acetoclastic methanogenesis in granular sludge was investigated in batch toxicity assays. Lauric acid appeared to be the most versatile inhibitor: inhibition started at 1.6 mM and at 4.3 mM the maximum specific 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 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 where mixtures of long-chain fatty acids are more likely than individual species, synergism can be expected to enhance the inhibition of methanogenesis. A background concentration of lauric acid below its own minimal inhibitory concentration strongly enhanced the toxicity of capric acid and (to an even greater extent) myristic acid. Chapter 2 also contains an example of the fact that the effective concentration of toxicants can drastically be lowered by the formation of insoluble salts. Research concerning lauric acid inhibition of methanogenesis proved that addition of calcium which forms insoluble calcium laurate salts can reverse that inhibition, provided the addition is applied within several minutes after the exposure of the granular sludge to toxic lauric acid concentrations has been started. The rapid interaction of lauric acid with the methanogenic sludge causes the need for almost instanteneous addition of calcium ions when exposure to lauric acid occurs. Of the methane producing potential of granular methanogenic sludge 50 % was lost after only 7.5 minutes of exposure in an almost calcium free environment. Addition of calcium ions after an exposure period of 6 hours had no effect at all.

The effective concentration of dissociable toxicants can be influenced by temperature as well as pH. Chapter 3 shows that at higher pH values, where the unionized fraction is smaller, sulfide is much less toxic. The effect of sulfide on the formation of methane from acetate in granular UASB sludge has been determined using a newly developed batch anae-

robic toxicity assay which allows operation at a constant pH without loss of sulfide during the test period via the off-gases. It was also established that the tolerance for sulfide toxicity by granular sludge exceeds the commonly used guidelines several times. This is probably due to the existence of a pH gradient towards alkalinity in the granular biofilms. Chapter 5 shows that the susceptibility for ammonia of the hydrogenotrophic methanogens in granular UASB sludge is higher at pH 7.8 than at pH 7.0, whereas a decrease in temperature from 37 ^{o}C to 29 ^{o}C results in less susceptibility. Although a decrease in temperature diminished the impact of ammonia, that effect is overshadowed by the general negative effect of decreasing the temperature on the growth rate. Similar results concerning the effect of temperature on ammonia toxicity were obtained in experiments with anaerobic manure digestion, where the over-all effect of a decreased temperature was found to be a decreased methanogenic activity (chapter 6).

The conclusions in chapter 1 that toxicity in anaerobic digestion is very much concentration dependent and that methanogenic biomass has an enormous adaptation potental which once adaptation is completed enables methanogenic biomass to tolerate extreme toxicant concentrations is illustrated in chapter 4 for the case of ammonia. The influence of ammonia in the concentration range 680 - 2600 mg N/l on the maximum specific methanogenic activity of granular UASB sludge showed a negative linear relationship. The slope of that linear relationship changed sharply in the concentration range 1600 - 1700 mg N/l, which is generally believed to be the toxicity threshold level above which methanogenic activity is only possible after adaptation. It was shown that gradually increasing the ammonia concentration allowed adaptation without occurrence of a lag-phase with zero methanogenesis. Step-wise increasing the ammonia concentration caused failure of methanogenesis in unadapted granular UASB sludge at approximately 1950 mg N/l. After an adaptation period, characterised by an almost nihil methane production, methanogenesis appeared to be possible at much higher ammonia concentrations, up to 11.8 g N/l. A kinetic analysis of the methane production during the adaptation process indicated that the increased methanogenic activity was not due to growth of other methanogens than the already predominant population. The methane producing potential of the sludge after adaptation was found to be influenced by the pH during the adaptation period. A high pH during adaptation (highest pH tested was 7.6) was found to result in a lower maximum specific methanogenic activity than lower pH values, although the sludge exposed to the lowest pH (7.0) suffered from a retarded hydrogen consuming ability leading to propionic acid build-up. Except for the sludge which was maintained at pH 7.6 during the adaptation period, after adaptation the maximum specific methanogenic activity at an ammonia concentration of 2315 mg N/l was higher than that at an ammonia concentration of 1900 mg N/l before adaptation. The adaptation potential of the granular UASB sludge was 6.2, which means that after the adaptation process in which the sludge gained the ability to produce methane at ammonia concentrations exceeding the initial toxicity threshold level, the maximum tolerable ammonia concentration was 6.2 times higher than the initial toxicity threshold level.

Chapter 4 also shows that ammonia toxicity is reversable. This was proved with continuously fed UASB reactor treating diluted potato juice. At an unchanged organic loading rate the volatile fatty acids build-up that occurred at 1400 mg N/l disappeared when the ammonia concentration was lowered to 900 mg N/l. The reveribility of ammonia toxicity was also shown in batch experiments at much higher concentrations. After more than 3 weeks of exposure to 9.1 g N/l the maximum specific methanogenic activity of granular UASB sludge at 5.2 g N/l fitted well in the general relationship between ammonia concentration and maximum specific methanogenic activity established for the range 2.3 - 11.8 g N/l.

Chapters 4 and 5 both provide evidence that acetoclastic methanogens are much more vulnerable for ammonia toxicity than hydrogenotrophic methanogens.

Another example of the extreme tolerance of methanogenic biomass for toxicants is the anaerobic digestion of highly concentrated ('thick') manure slurries described in chapter 6. Anaerobic digestion of liquid poultry manure at ammonia concentrations of approximately 9.8 g N/l was found to be possible only when propionic acid build-up is allowed. However, dilution of the liquid poultry manure with cow manure allows digestion even at retention times of 30 - 40 days. The ammonia concentrations in the experimental digesters ranged from 5.9 - 8.1 g N/l at an average pH of 7.9 due to the high alkalinity of the manure mixtures applied.

SAMENVATTING VAN HET PROEFSCHRIFT

Anaërobe gisting is de biologische omzetting door een groot aantal nauw samenwerkende bacteriesoorten van organisch materiaal in methaanrijk biogas. De microbiologie van de anaërobe gisting met inbegrip van de chemische en fysische behoeftes van de betreffende organismen wordt besproken in hoofdstuk 1. De bacteriën betrokken bij anaërobe gisting kunnen grofweg in twee groepen verdeeld worden: zuurvormers en zuurconsumeerders. De laatste produceren methaan. Voor een stabiele gisting is evenwicht tussen de snelheden van zuurproduktie en -consumptie vereist. De zuurvormende bacteriën verdragen een veel lagere pH dan de methaanvormende bacteriën en de in nauwe symbiose daarmee levende acetogene bacteriën en zijn bovendien minder gevoelig voor stress-situaties zoals een lagere temperatuur of de aanwezigheid van gifstoffen. Daarom is het probleem van toxiciteit (giftigheid) in anaërobe gisting in feite een probleem van giftigheid voor de methaanvormende bacteriën.

Vanwege de gevoeligheid voor gifstoffen is lange tijd gedacht dat anaërobe gisting niet kon worden toegepast voor industriële afvalwaterzuivering. Deze opvatting is terecht voor de allereerste procesontwerpen die alle waren gebaseerd op vrij groeiende biomassa met relatief korte biomassa verblijftijden in de reactoren. In hoofdstuk 1 wordt echter aangetoond dat de moderne reactorontwerpen, die alle uitgaan van immobilisatie van de biomassa om zodoende naast een hoge biomassaconcentratie ook zeer lange biomassa verblijftijden te realiseren, zeer goed in staat zijn om afvalwaters met gifstoffen te behandelen. De lange biomassa verblijftijd maakt adaptatie aan de aanwezigheid van gifstoffen mogelijk. Die adaptatie kan bestaan uit het veranderen van de biochemische karakteristiek van de cellen zodat ze bestand raken tegen de gifstof of het ingroeien van bacteriën in de biomassa die in staat zijn de gifstoffen te ontgiften dan wel volledig af te breken. Bovendien groeit de geïmmobiliseerde biomassa veelal in biofilms, die zorgen voor micromilieus waarin veel gifstoffen minder effectief zijn dan in het milieu in de vloeistof rondom de biofilm, iets wat met name geldt voor de pH. Bovendien bevatten de biofilms vaak een overschot aan bacteriën die pas echt actief worden als de bacteriën die normaal het meeste werk doen geheel of gedeeltelijk zijn uitgeschakeld.

De UASB reactor (UASB = Upflow Anaerobic Sludge Bed = Opstroom Anaëroob Slibbed) is momenteel de meest toegepaste reactor in anaërobe afvalwaterzuiveringssystemen. Het slib in een UASB reactor is meestal geïmmobiliseerd in compacte korreltjes. Dit proefschrift bevat resultaten van diverse onderzoeksprojekten waarin dergelijk korrelslib is blootgesteld aan gifstoffen. In dat korrelslib is *Methanothrix* sp. de dominante acetaat consumerende methanogene bacteriesoort. De populatie van waterstof consumerende methanogene bacteriën in korrelslib is tamelijk ongedefinieerd.

In hoofdstuk 2 wordt onderzoek beschreven naar het effect van vier verzadigde hogere vetzuren (capryl-, caprine-, laurine- en myristinezuur) en één onverzadigd hoger vetzuur (oliezuur) op methanogenese uit acetaat in korrelsib. Van de onderzochte vetzuren is laurinezuur de krachtigste gifstof: bij 1,6 mM begon de remming en bij 4,3 mM was er nog maar 50 % van de maximale specifieke methaanvormingsactiviteit over. Caprylzuur is nauwelijks giftig, terwijl oliezuur bijna even giftig is als laurinezuur. Hoewel adsorptie van de hogere vetzuren aan de celwand een belangrijke rol zou kunnen spelen in het remmingsmechanisme blijkt de remmingsintensiteit niet gecorreleerd met de dosering per hoeveelheid cellen, maar met de totaal aanwezige concentratie van de gifstof. Bij vetzuurmengsels is er een aanzienlijke kans dat de giftigheid van de vetzuren versterkt wordt door elkaars aanwezigheid. Een achtergrondconcentratie van laurinezuur onder het niveau waarop het begint te remmen versterkt de giftige werking van caprinezuur en myristinezuur. In hoofdstuk 2 wordt verder een duidelijk voorbeeld gegeven van het feit dat door de vorming van onoplosbare zouten de werking van gifstoffen afgezwakt kan worden. Door het doseren van calciumionen wordt de giftige werking van laurinezuur opgeheven indien de dosering binnen enkele minuten na de blootstelling aan laurinezuur plaats vindt. Bij dosering van calciumionen 7,5 minuten na het begin van blootstelling aan laurinezuur bleek nog slechts 50 % van de maximale specifieke methaanvormingsactiviteit over te zijn. Toevoegen van calciumionen na 6 uur bleek geen enkel effect meer te hebben.

De effectieve concentratie van dissocieerbare (ioniseerbare) gifstoffen kan beïnvloed worden door de temperatuur en de pH. In hoofdstuk 3 wordt aangetoond dat bij hogere pH waarden, waar de fractie ongedissocieerd waterstofsulfide geringer is, sulfide veel minder

giftig is. Het effect van sulfide op de methaanvorming uit acetaat door korrelslib is bepaald met behulp van een speciaal ontwikkelde meetmethode die het mogelijk maakt om zonder verlies van sulfide via het gevormde biogas bij een constante pH te werken. Korrelslib heeft een vele malen hogere tolerantie voor sulfide dan op basis van de literatuurgegevens verwacht werd. Dat komt omdat die gegevens gebaseerd zijn op dispers groeiende bacteriën, terwijl in het korrelslib sprake is van biofilms. De biofilm zorgt voor een hogere pH in de slibkorrel dan in de omringende vloeistof. Ook voor ammonia geldt dat de remmende werking sterk beïnvloed wordt door temperatuur en pH. Hoofdstuk 5 laat zien dat waterstof consumerende methanogene bacteriën bij pH 7,8 veel gevoeliger voor ammonia zijn dan bij pH 7,0, terwijl een temperatuurdaling van 37 ^{o}C naar 29 ^{o}C in een verminderde gevoeligheid resulteert. Hoewel de remming door ammoniak wordt afgezwakt door een temperatuurdaling geven resultaten beschreven in hoofdstuk 5 en in hoofdstuk 6 aan dat in het algemeen bij ammonia toxiciteit van een temperatuurdaling geen toenemende groei en activiteit verkregen kan worden, omdat het algemene effect van temperatuurdaling (afname van groei en activiteit) klaarblijkelijk overheerst.

De conclusie in hoofdstuk 1 dat giftigheid in anaërobe gisting in hoge mate afhangt van de concentratie en dat de methaanvormende bacteriemassa zodanig kan adapteren dat zelfs extreme concentraties gifstof nog verdragen worden wordt geïllustreerd in hoofdstuk 4. De invloed van ammonia op de methaanvorming door korrelslib in het concentratiegebied 680 - 2600 mg N/l wordt beschreven door een negatief lineair verband. De helling van dat lineaire verband verandert scherp bij 1600 - 1700 mg N/l, het concentratiegebied dat algemeen beschouwd wordt als het niveau waarboven zonder adaptatie geen methanogenese mogelijk is. Er is aangetoond dat een geleidelijk opvoeren van de concentratie ammonia adaptatie mogelijk maakt zonder lag-fase. Het stapsgewijs opvoeren van de ammoniaconcentratie bleek te leiden tot het bijna geheel wegvallen van de methaanvormingsactiviteit bij circa 1950 mg N/l. Na een adaptatieperiode, gekenmerkt door vrijwel afwezige methaanproduktie, bleek het mogelijk om methaanvorming te bewerkstelligen bij ammoniaconcentraties tot 11,8 g N/l. Een kinetische analyse van de methaanproduktie tijdens de adaptatieperiode heeft uitgewezen dat het herstel van de methanogene populatie te danken was aan een soort "ombouwen" van de reeds aanwezige cellen en niet aan het ingroeien van nieuwe, beter aangepaste cellen. Tevens is aangetoond dat het van belang is de pH tijdens de adaptatieperiode te beheersen. Een hoge pH (in de proef: 7,6) resulteert in een lagere maximale specifieke methaanvormingsactiviteit na adaptatie. Slib dat aan een lage pH wordt blootgesteld tijdens adaptatie (in de proef: 7,0) blijkt enige last van verminderde waterstofomzetting te hebben. Het slib dat bij pH 7,2 was geadapteerd bleek het beste te functioneren na de adaptatieperiode. In alle gevallen, behalve het bij pH 7,6 geadapteerde slib, bleek na adaptatie bij een ammoniaconcentratie van 2315 mg N/l de maximale specifieke methaanvormingsactiviteit hoger te zijn dan bij 1900 mg N/l voor adaptatie. Na adaptatie bleek de maximaal verdraagbare ammoniaconcentratie 6,2 maal hoger te zijn geworden.

In hoofdstuk 4 wordt ook aangetoond dat het effect van ammoniatoxiciteit niet blijvend is. Bij een gelijkblijvende organische belasting bleek het afnemen van het ammoniagehalte van 1400 mg N/l naar 900 mg N/l in een UASB reactor gevoed met verdund aardappelvruchtwater te resulteren in het verdwijnen van het door ammoniaremming veroorzaakte ophopen van propionzuur. Dat het remmingseffekt van ammonia niet blijvend is werd ook aangetoond in een experiment waarin na 3 weken blootstelling aan een ammoniaconcentratie van 9,1 g N/l de maximale specifieke methaanvormingsactiviteit bij 5,2 g N/l nog precies bleek te passen in het algemene verband tussen maximale specifieke methaanvormingsactiviteit en ammoniagehalte in het concentratiegebied 2,3 - 11,8 g N/l.

De hoofdstukken 4 en 5 geven beide aanleiding voor de conclusie dat de acetaat consumerende methanogene bacteriën veel gevoeliger zijn voor remming door ammonia dan de waterstof consumerende.

Een voorbeeld van de extreme giftolerantie van methanogene biomassa wordt geleverd door de in hoofdstuk 6 beschreven experimenten met mestslurries. Kippemestvergisting bij een ammoniaconcentratie van 9,8 g N/l is niet mogelijk zonder propionzuurophoping, maar verdunning van de kippemest met koemest resulteert in goedlopende vergisting. Stabiele gisting bij verblijftijden van 30 - 40 dagen, met ammoniaconcentraties van 5,9 tot 8,1 g N/l en een pH die als gevolg van de hoge alkaliteit van de mest zelfs 7,9 bedroeg bleek mogelijk.

CURRICULUM VITAE

De auteur van dit proefschrift is op 8 februari 1958 geboren te Stavenisse (thans gemeente Tholen). In 1976 werd het diploma Atheneum B behaald aan de Christelijke Scholengemeenschap Johannes Calvijn te Rotterdam.

In hetzelfde jaar werd begonnen met de studie aan de Landbouwhogeschool (later Landbouwuniversiteit) te Wageningen. In juni 1977 werd het examen van de N-propaedeuse met lof behaald. Vervolgens begon de specialisatie in de studierichting Milieuhygiëne, waarbij het accent werd gelegd op de oriëntatie Waterzuivering. Het kandidaatsdiploma werd in januari 1981 met lof behaald. In de daarop volgende doctoraalstudie werden een verzwaard hoofdvak Waterzuivering en de bijvakken Technische Microbiologie en Proceskunde uitgevoerd. Tevens werden drie stages verricht. Eén stage was bij de projektgroep "Biotechnologische verwerking van agrarisch afval" van het Instituut voor Bewaring en Verwerking van Landbouwprodukten (IBVL) te Wageningen. De andere twee stages werden met ondersteuning van het L.E.B.-fonds verricht in Schotland bij de "Engineering Division" van het North of Scotland College of Agriculture te Bucksburn en het "Microbial Biochemistry Department" van het Rowett Research Institute te Aberdeen. De ingenieursbul werd in augustus 1983 met lof behaald.

Van oktober 1983 tot augustus 1985 was de auteur werkzaam als toegevoegd onderzoeker in tijdelijke dienst bij de vakgroep Waterzuivering van de Landbouwuniversiteit Wageningen, waar hij tewerkgesteld was bij de onderzoeksgroep "Anaërobe waterzuivering". Vanaf augustus 1985 is hij als stafmedewerker verbonden aan dezelfde vakgroep met als taakveld "Valorisatie en verwerking van organische afvalstoffen (mest, slib, huisvuil)".

Vanaf 21 november 1989 zal de auteur werkzaam zijn als plaatsvervangend hoofd bedrijfsbureau van Ecotechniek bv te Utrecht, met als voornaamste taak het projektmanagement betreffende research en ontwikkeling van het Ecosun mestverwerkingsproces.