Conversion of Methanogenic Substrates in Anaerobic Reactors

Metals, Mass Transport, and Toxicity

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About the Cover: Scanning electron microscopy (SEM) image of a cross-sectioned anaerobic granular sludge. Darker zones indicate zones with higher biomass concentrations.

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Propositions

1. The required amount of a trace nutrient does not reflect its importance

   This dissertation

2. Contrary to the conclusions of Zaiat et al. 1997, Zaiat et al. 1996, and Kato 1994, external mass transfer resistance can normally be neglected in anaerobic biofilms.

   This dissertation

   Kato, M. T. 1994. The anaerobic treatment of low strength soluble wastewaters. Ph.D. Thesis. Wageningen Agricultural University, The Netherlands.

   Zaiat, M., L. G.T. Vieira, and E. Foresti. 1997. Intrinsic kinetic parameters of substrate utilization by immobilized anarobic sludge. Biotehnol. Bioeng. 53:220-225.

   Zaiat, M., L.G.T. Vieira, and E. Foresti. 1996. Liquid-phase mass transfer in fixed-bed of polyurethane foam matrices containing immobilized anaerobic sludge. Biotechnol. Techniques. 10:121-126.

3. The statement of Beer de, et al. (1992) that pH effects are more important than internal mass transfer resistance on the conversion of acetate by methanogenic granules is incorrect because an accurate description of internal mass transfer phenomena in granular sludge can be obtained without including pH effects.

   This dissertation

   Beer de, D., J.W. Huisman, J.C. van den Heuvel, and S.P.P. Ottengraf. 1992. The effect of pH profiles in methanogenic aggregates on the kinetics of acetate conversion. Water Res. 26(10):1329-1336.

4. The conclusion that higher concentrations of formaldehyde can be tolerated when added continuously (Bhattacharyra and Parkin 1988, and Qu and Bhattacharya 1997) is quite doubtful because according to our findings formaldehyde irreversible toxicity is independent on the mode of addition (slug or continuous).

   This dissertation

   Bhattacharya, S. K., and G.F. Frankin. 1988. Fate and effect of methylene chloride and formaldehyde in methane fermentation systems. J. WPCF. 60:531-536.

   Qu, M., and S.K. Bhattacharya. 1997. Toxicity and biodegradation of formaldehyde in anaerobic methanogenic culture. Biotechnol. Bioeng. 55:727-736.

5. The observed increase of hydrogen in the gas phase (Hickey et al. 1987), and the inhibition of propionate and butyrate conversion by formaldehyde (Omil et al. 1999), does not imply the selectively inhibition of hydrogenotrophic methanogens by formaldehyde.

   Hickey, R.F., J. Vanderwielen, M.S.Switzenbaum. 1987. The effects of organic toxicants on methane production and hydrogen gas levels during the anaerobic digestion of waste activated sludge. Water Res **21**:1417-1427.

   Omil, F., D. Mendez, G. Vidal, R. Mendez, and J.M. Lema. 1999. Biodegradation of formaldehyde under anaerobic conditions. Enzyme and Microbial Technol. **24**:255-262.

6. The metabolic characterization of anaerobic sludge should become an integral component of toxicity studies.

   This dissertation

7. The analytical and methodological procedure of a piece of research should be design in such a way that at the end, the results obtained may look like an "X-ray" plaque of the experiment, ready to be interpreted.

8. The degree of success depends on attention to details. To get back in the laboratory is a necessary component of scientific survival.
   Ralph S. Wolfe, Pioneer of biochemistry of methanogenesis

9. Knowledge gives true freedom

10. Many people live with, for, and by definitions

11. Love, sport and music are essential nutrients, whether they are needed in micro or macro scale is people dependent

12. By the process of aging, humans should not lose the intrinsic curiosity of childhood

13. The motivation of youth should not die...be 18...till you die.

14. If you are going to shoot...shoot, don't talk
    From the film: Il buono, il bruto e il cattivo.

Propositions belonging to the thesis entitled "Conversion of methanotrophic substrates in anaerobic reactors, metals, mass transfer, and toxicity"

Graciela Gonzalez-Gil
Wageningen, 30 January 2000.

Abstract

Gonzalez-Gil, G. (2000). **Conversion of methanogenic substrates in anaerobic reactors, metals, mass transfer, and toxicity**. Doctoral Thesis. Wageningen University, The Netherlands.Pp.157.

The EGSB systems represents an attractive option to extend further the use of anaerobic technology for wastewater treatment, particularly with respect to waste streams originating from chemical industries. Frequently chemical waste streams are unbalanced with respect to nutrients and/or micronutrients and furthermore these streams may contain toxic-biodegradable compounds. To reduce toxicity high recycle ratios may be applied as in the case of EGSB reactors however, this at the same time may adversely affect the substrate conversion rates due to mass transport limitations. These aspects were considered in this research. The main objectives of the work described in the thesis were to assess the kinetic impact of (i) nickel and cobalt limitations on the methanogenic degradation of methanol, (ii) the relative importance of mass transport phenomena in methanogenic granular sludge, and (iii) to characterize the toxicity and biodegradation of formaldehyde in the anaerobic conversion of methanotrophic substrates. Particularly in the case of anaerobic systems, the ubiquitous presence of sulfides resulting from sulfate reduction and organic matter mineralization will lead to strong metal precipitation as metal sulfides. These precipitated metals are not directly available for the biomass. It is shown here that precipitation-dissolution kinetics of metal sulfides may play a key role in the bioavailability of essential metals. We furthermore showed that nutrient limitations can be overcome if the essential metals are added continuously at a proper rate so that their availability in solution can fulfill the requirement for biomass activity and growth. The metal dosing rates utilized range from 0.05 to 0.2 μmol/h corresponding to metal to methanol ratios of 0.1-0.4 (μmol/g methanol-COD) and these values agree well with the calculated metal requirements based on the biomass yield and Ni and Co content of methylotrophic methanogens (*Methanosarcina* sp.) grown on methanol. With respect to mass transport phenomena, it was found that at liquid upflow velocities exceeding 1 m/h liquid-film (external) mass transfer limitations normally can be neglected for acetate degrading methanogenic granular sludge. On the contrary, a clear increase in apparent K_S-value was found at increasing mean granule diameters. Herewith we have clearly shown that anaerobic biofilms can be internally transport limited. In addition we also demonstrated that substrate transport in the biofilm can be described by diffusion, and that there was no evidence of convective flow due to biogas production in the anaerobic granules. Regarding formaldehyde conversion and toxicity, we demonstrated that methanogenesis from formaldehyde mainly occurred after intermediate formation of methanol and formate. Furthermore it was shown that the characteristics of formaldehyde toxicity were independent of the methanotrophic substrate used (methanol or acetate). Formaldehyde toxicity was in part reversible since once the formaldehyde concentration was extremely low or virtually removed from the system, the methane production rate was partially recovered. Since the degree of this recovery was not complete, we conclude that formaldehyde toxicity was also irreversible. The irreversible toxicity likely can be attributed to biomass formaldehyde-related decay. Independent of the mode of formaldehyde addition (slug or continuous), a certain amount of formaldehyde irrevocably reduced the methane production rate to a certain extent, hence the irreversible toxicity was dependent on the total amount of formaldehyde added to the system. This finding suggest that in order to treat formaldehyde containing waste streams a balance between formaldehyde-related decay and biomass growth should be attained. We furthermore showed that the biomass diversity may play a key role in the outcome of toxicity tests. Therefore we recommend that metabolic characterization of methanogenic sludge should be an integral component of toxicity studies and we describe a newly developed methodology that can be used for that purpose.

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1

General Introduction

Introduction

Anaerobic wastewater treatment may represent the core method for a sustainable environmental protection and resource conservation technology. The concept of anaerobic wastewater treatment only has been implemented at wide scale since the early eighties using the high-rate anaerobic reactors developed in the seventies. Presently more than 1700 anaerobic reactors are in operation worldwide, treating a wide variety of waste streams [7].

Advances of anaerobic technology

Compared to conventional aerobic processes, the present high-rate anaerobic technology offers the following advantages: (i) About 10 times higher organic loading rates can be applied with good removal efficiencies even at low temperatures [55, 74], (ii) investment, pumping and energy-associated operating costs are substantially reduced [36, 89], (iii) waste is converted to an useful fuel, methane (CH_4), which in many cases can be further used in-situ [100], (iv) low production of well stabilized excess biological solids [53] and (v) anaerobic sludge can be stored unfed for long periods of time while conserving reasonably good metabolic capacity [101].

Breakthrough for implementation

The key for the successful implementation of anaerobic wastewater treatment was the successful development of high-rate reactors. High-rate reactors are characterized by their capacity to uncouple solid and liquid retention times, resulting in high concentrations of biomass. The high biomass concentrations are accomplished either by means of bacterial immobilization on an inert carrier as applied in fluidized bed (FB) and fixed film (FF) reactors, or by self-immobilization of biomass in the form of granules in upflow anaerobic and expanded granular sludge bed reactors (UASB and EGSB, respectively).

Generally, anaerobic systems are operated at mesophilic temperatures and they are applied for the treatment of high, medium and low strength, noncomplex waste streams like those from the agro and food industries. Due to its relatively plain construction, easy operation, and maintenance, the UASB reactor developed by Lettinga et al. [56] presently is the most widely applied. About 61% of the anaerobic reactors are of the UASB-type [7].

How high rate anaerobic reactors work

In the UASB and EGSB systems, the wastewater is pumped from the bottom into the reactor. While the wastewater moves upwards, the organic contaminants are removed from the water in the anaerobic sludge bed (Figure 1.1). The sludge bed in EGSB-systems and frequently in

UASB reactors as well, is composed of a high concentration of granules of diameters ranging from 0.1 to 5 mm (Figure 1.1*a*) [82].

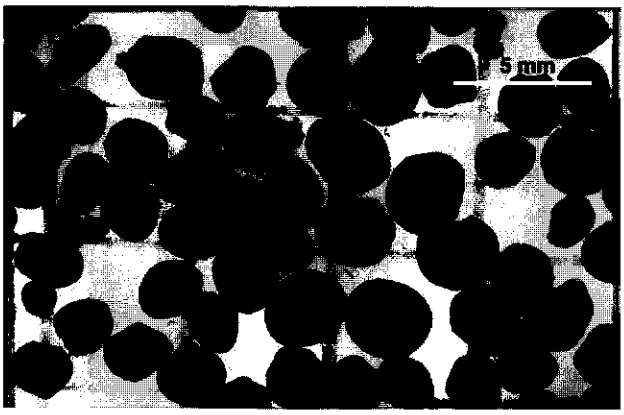
An anaerobic granule (Figure 1.1*b*) is a methanogenic ecosystem consisting mainly of several groups of self-immobilized bacteria (Figure 1.1*c*). Although it is still not well understood how granular sludge is formed [82], it is clear that the substrate(s) present in the influent waste stream primarily determines the dominant bacterial composition of the granules [23].

The bacteria in the granules purify the wastewater by converting the organic pollutants into a mixture of methane (CH_4) and carbon dioxide (CO_2). The conversion process is accomplished via a complex metabolic network. The product from a reaction carried out by one group of bacteria serves as substrate for the subsequent microbial group (Figure 1.1). When the different groups work in a concerted action, a balance is established between the rates of formation and consumption of intermediates, so that there is no accumulation of the intermediates [80, 81, 91] and very high conversion rates can be accomplished.

In summary (see Figure 1.1), polymers like polysacharides, proteins, nucleic acids, and lipids are hydrolized to monomers like sugars, amino acids, purines pyrimidines, fatty acids, and glycerol by primary fermenting bacteria. These bacteria further ferment the resulting monomers to acetate, carbon dioxide, hydrogen and reduced products like alcohols, lactate, and volatile fatty acids. For the degradation of the fermentation products like fatty acids longer than two carbon atoms, alcohols longer than one carbon atom, and branched chain and aromatic fatty acids, the secondary-fermenting bacteria or obligate hydrogen-producing acetogenic bacteria are needed. These secondary-fermenters convert their substrates to acetate, carbon dioxide, hydrogen, and perhaps formate, which are subsequently used by methanogenic archaea. Methanogenic archaea are specialized in the conversion of a limited number of substrates [103]. They can only use acetate, CO_2+H_2, formate and/or other one carbon compounds such as methanol, methylthiols and methylamines [8].

The advances achieved in the knowledge of the biochemistry and microbiology of anaerobic microorganisms have supported the improved engineering of anaerobic treatment systems. Further fundamental knowledge of the process, as well as new reactor designs and/or improvements should contribute to the implementation of anaerobic technology to the treatment of more complex wastewaters.

a



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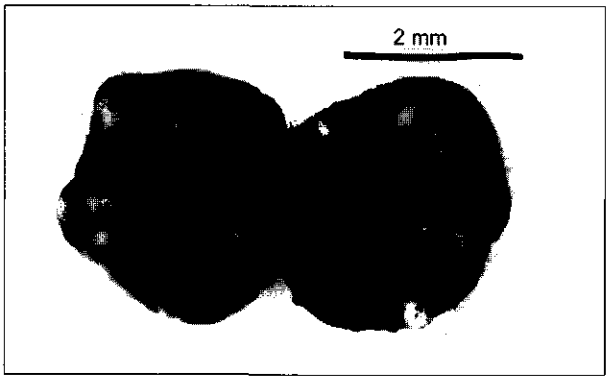

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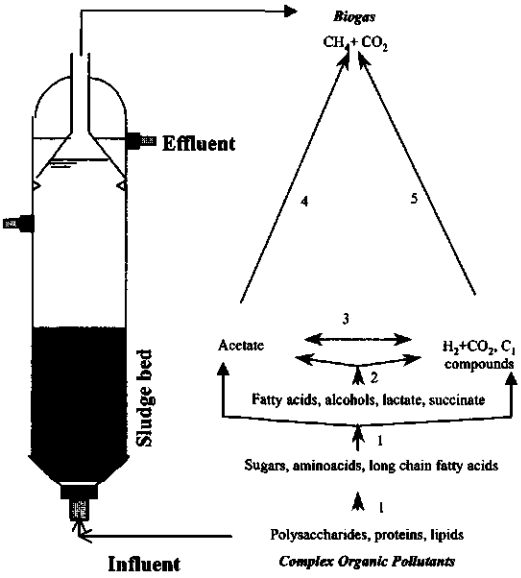

Figure 1.1 *Carbon flow to methane and carbon dioxide in EGSB/UASB reactors. Bacterial groups involved:* 1. *Primary fermentive bacteria.* 2. *Secondary fermentative H_2-producing ('syntrophic') bacteria.* 3. *Homoacetogenic bacteria.* 4. *Acetotrophic methanogens* 5. *H_2 and formate-oxidizing methanogens. [29, 69]. Photos:* (***a***) *Granular sludge taken from the sludge bed of a full scale EGSB reactor.* (***b***) *Cross-sectioned granule.* (***c***) *Scanning electron micrograph of the same granule showing the diverse bacterial morphology.*

Feasibility of anaerobic treatment for waste streams from the chemical industry

The introduction of anaerobic processes for the treatment of complex wastewaters originating from the chemical industries was seriously hampered by the *a priori* conclusion that anaerobic bacteria were more susceptible to toxic compounds than aerobic microorganisms [89], and that many industrial chemicals were poorly biodegradable under anaerobic conditions. Recently, however, it has been shown that most compounds are biodegradable in anaerobic environments [11, 13, 32, 33, 48, 50, 57, 76, 84], and that the susceptibility to toxicity of methanogenic bacteria is highly comparable to that of aerobic heterotrophs [9]. These findings have resulted in an increased interest in anaerobic treatment of waste streams from the chemical industries [20, 34, 51].

Limitations of the conventional UASB-system

Particularly for the treatment of effluents from the chemical industries, the process characteristics of UASB systems may be insufficient for a wider application of anaerobic technology. This applies particularly for the applicable maximum liquid upflow velocities (V_{up}) which are in the range of 0.5 to 2 m/h for UASB reactors [54, 69].

Due to the configuration of the UASB-system higher values for V_{up} frequently can not be applied without considerable biomass loss and decrease of the removal efficiencies.

The organic concentration of some industrial wastes is so high (e.g. molasses, wine-distillery, yeast), that process instability may occur due to accumulation of volatile fatty acids (VFA) [10, 102]. In this case, ultra-high loading rates can be applied when high recycle rates are used which obviously result in higher V_{up} values. This effect is only true if plug-flow pattern is assumed in the reactor.

High V_{up} values are also needed in order to make possible the treatment of toxic-biodegradable compounds found in many chemical waste streams. High recycle rates may also decrease the sensitivity of the process to the presence of non-degradable toxic components. Additionally, in industries like alcoholic and soft drink bottling, fruit and vegetable canneries, malting and brewing [43, 44, 49], the organic pollution may be so low that the natural mixing of the system as a result of gas production becomes limited. In this case, higher V_{up} values are required to increase the mixing and enhancing the contact between the wastewater and the sludge to improve the capacity of the process.

The EGSB-system as a solution for chemical and dilute waste streams

The above mentioned limitations of the UASB-system could be overcome by the FB-system [40, 61]. Fluidized bed reactors are considered ultra-high rate processes mainly because of the supposed high hold up of viable biomass which is believed to result from the high specific surface area of the carrier material for biomass attachment. Contaminated water is passed upward at a velocity sufficiently high to expand or fluidize the bed particles. The required V_{up} velocities are mainly achieved by recirculation. Due to the high concentration of the biomass-carrier particles, very high biomass concentrations ranging from 10 to 40 Kg VSS/m^3 can be retained in the reactor [61] despite the imposed high V_{up}. It has been mentioned that loading rates as high as 315 Kg COD/m^3.d with 79% removal efficiencies can be achieved with fluidized bed systems [41].

Based on theoretical considerations [75], the process characteristics of FB-systems, would permit (i) ultra-high loading rates with high removal efficiencies, (ii) homogeneous distribution of biomass and substrate preventing dead zones, thus allowing the treatment of dilute wastewaters [39], while (iii) at high recycle rates, it would also be possible to treat the complex wastewaters from chemical industries.

Although the ultra-high loaded FB concept is available for a number of years, this technology so far, had very limited success. The reasons for this range from the relatively high investment, operational and maintenance costs to the complexity of operation[26].

The phenomenon of granule formation and maintenance in UASB reactors has been exploited to extend further the application of anaerobic technology. By introducing effluent recirculation and applying an improved gas-solid separator [54], the UASB system has been modified resulting in the development of the ultra-high rate EGSB reactor. As a matter of fact, installed full-scale FB plants have been modified into EGSB reactors. Ultra high loading rates are achieved using granular biomass and therefore no carrier material is required for operation [104]. EGSB systems are tall and slender reactors with a relatively small footprint, and they generally can accommodate very high volumetric loading rates. Compared to FB reactors, EGSB reactors are operated at lower upflow velocities to expand (and not to fluidize) the granular sludge bed. This operational variation is due to the difference in density between the carrier material (2 to 3 Kg/dm^3 [41]) in FB, and granular sludge (1.05 to 1.1 Kg/dm^3 [71]). As a result, the energy requirements are low and biofilm damage due to abrasion characteristic of the FB are greatly overcome. Since EGSB systems can accept high liquid upflow velocities, high recirculation rates can be applied. This provides dilution of the influent wastewater, which makes the system very attractive for the treatment of industrial streams with toxic biodegradable compounds [104].

Specific factors for the treatment of chemical wastes in EGSB systems

Frequently, wastes originating from chemical industries are unbalanced with respect to nutrients and/or micronutrients. Furthermore, these industrial effluents may contain toxic biodegradable compounds and EGSB systems may represent an attractive option for their treatment as described above. However, since high recycle ratios are applied to reduce toxicity, this at the same time may adversely affect substrate conversion rates due to mass transport limitations.

The aspects of the anaerobic treatment of chemical wastes were the specific subjects of the work described in this thesis, and will be addressed in detailed below.

Trace metals

An important factor to consider for the anaerobic treatment of waste streams from chemical industries is that (contrary to the food and agro industry wastewaters), often they are deficient in macronutrients, as well as in trace metals. This is of importance since methanogens highly depend on the presence and availability of elements like iron, cobalt, nickel, molybdenum and/or tungsten.

In general, anaerobic processes exhibit a more varied requirement for trace metals than aerobic systems. The reason lies on the metal composition of the enzyme complexes that catalyze many of the anaerobic reactions. Figures 1.2 and 1.3 show simplified representations of the metabolic pathways of methanogenesis from methanol and acetate respectively.

The hypothetical metabolic pathways as well as the enzymology of methanogenesis from different substrates have been presented in detail [8, 12, 19, 73, 95, 96]. Important to notice is that, independent on the methanogenic substrate, all of the pathways have in common the reduction of methyl-coenzyme M to methane. An enzyme complex catalyzes this reaction, the methyl-coenzyme M reductase, which contains as the active site of the enzyme, a cofactor named F_{430}. F_{430} is a yellow nickel-containing compound [22].

Another fact that illustrates the importance of trace metals in anaerobic systems is that most of the methane produced in nature originates from acetate where the key enzyme complex involved in the degradation is the carbon monoxide dehydrogenase (CODH). CODH is a metallo-enzyme [52, 58] that cleaves acetate as the activated form (i.e. acetyl CoA) into CO, CoA, and a methyl group. The same enzyme complex further transforms CO into CO_2 and transfers the methyl group to coenzyme M. CODH contains two metal enzyme components: (i) the nickel/iron-sulfur (Ni/Fe-S), and (ii) the corrinoid/iron-sulfur (Co/Fe-S).

The metals involved in the methanogenesis from methanol and acetate are depicted in Figure 1.2 and Figure 1.3, respectively. The names and the metal composition of the enzymes are indicated as well.

Previous research indicates that metal deficiencies can limit the performance of anaerobic digestion. Particularly the benefits of micronutrient supplements for the methanization of industrial wastewaters have been recognized [90, 93]. However the reported concentrations of trace metals for optimal anaerobic treatment vary by several orders of magnitude [93] and extrapolation from research done so far becomes impossible as the available information is rather unclear in relation to kinetic data and "optimum" metal concentrations. This is illustrated by the fact that for similar batch systems reported in literature, metal (Ni or Co) dosage can vary from no dosage to dosage of 40 μM [60, 85, 86, 88]. It should be noted that most of the information about metal deficiencies and enhancements reported in literature is fortuitous [87] since metal deficiencies were not the main subject of these studies. Only few papers have directly addressed the topic [18, 21].

Particularly in the case of anaerobic systems, the ubiquitous presence of sulfides resulting from sulfate reduction and organic matter mineralization will lead to strong metal precipitation as metal sulfides. Precipitated metals are not directly available for biomass.

The rational supply of trace metals during the treatment of waste streams is of paramount importance for several reasons. It is clear that the addition of trace metals may stimulate methanogenesis, however, at higher concentrations, heavy metals may form unspecific complex compounds in the cell [62]. This could lead to toxic effects that inhibit methanogenesis. Methanogenesis from acetate was found to be stimulated by the addition of 2 μM of Ni, Co, and Mo, but inhibition occurred at concentrations higher than 5 μM [18]. Overdose of metals not only may cause inhibition but may also lead to strong metal precipitation as sulfides. Anaerobic sludge with high metal contents is considered a chemical waste. The costs associated with trace metal dosage and the treatment of sludge with high metal contents may contribute significantly to the operational costs of anaerobic reactors. In an ideal situation, the rates of metal supply and biomass uptake for metabolic processes should be comparable.

Methanol is an important component of several chemical waste streams [21] and is used frequently as co-substrate for the co-metabolic removal of a variety of compounds [14, 65]. The kinetics of methanol conversion under different Ni and Co doses conditions are addressed in Chapter 2.

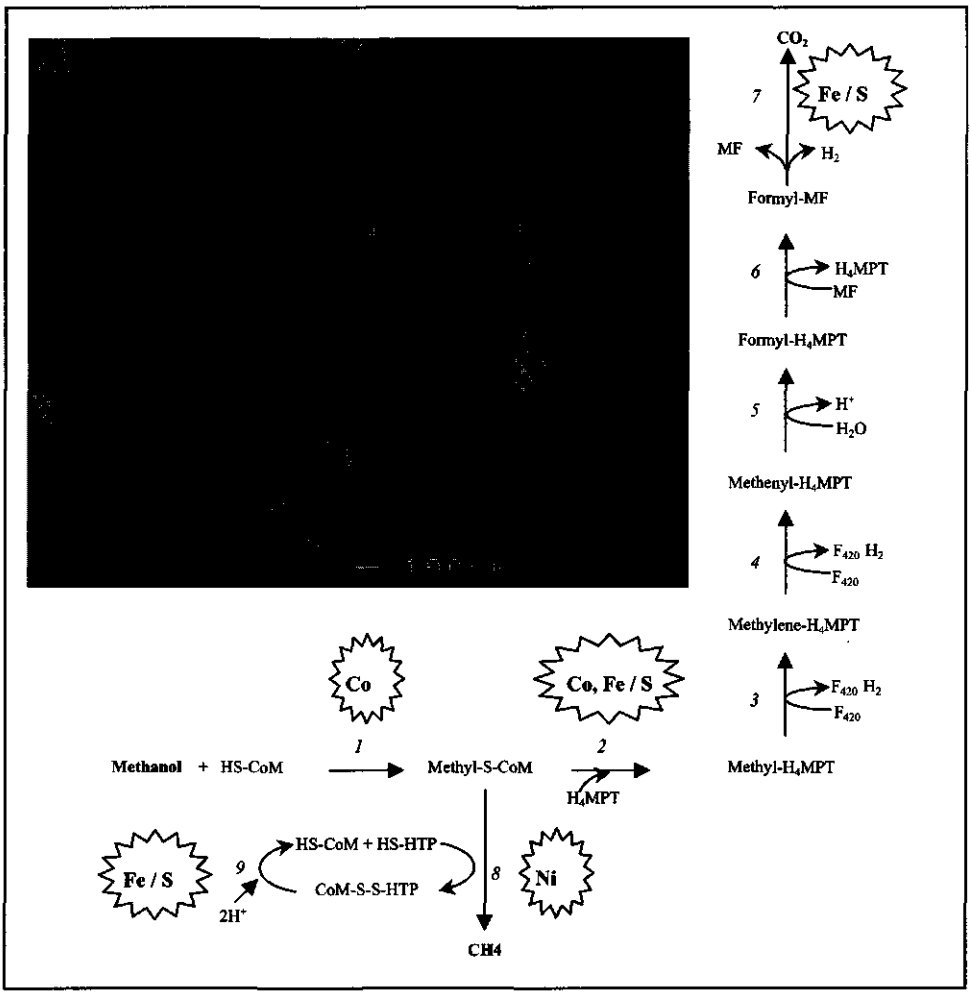

Figure 1.2 *Schematic representation of the theoretical pathway of direct methanogenesis from methanol. This conversion is conducted by Methanosarcina type bacteria shown in the scanning electron microphotograph. Constitutive sulfur and metals of the enzymes are indicated in the respective metabolic steps. MF Methanofuran, H_4MPT tetrahydromethanopterin, HS-CoM coenzyme M (2-mercapto-ethanesulfonate), HS-HTP 7-mercaptoheptanoylthreonine phosphate, CoM-S-S-HTP hetherodisulfide of HS-CoM and HS-HTP. The enzymes involved are:* (*1*) *5-hydroxybenzimidazolyl methyltransferase and Methylcobalamin:HS-CoM methyltransferase.* (*2*) *Methyl-H_4MPT:HS-CoM methyltransferase.* (*3*) *Methylene-H_4MPT reductase.* (*4*) *3 Methylene-H_4MPT dehydrogenase.* (*5*) *3 Methenyl-H_4MPT cyclohydrolase.* (*6*) *Formyl-MF:H_4MPT transferase.* (*7*) *Formylmethanofuran dehydrogenase.* (*8*) *Methyl-CoM reductase, containing the F_{430} (a nickel porphinoid) as cofactor .* (*9*) *Hetherodisulfide reductase. F_{420} is a coenzyme.*

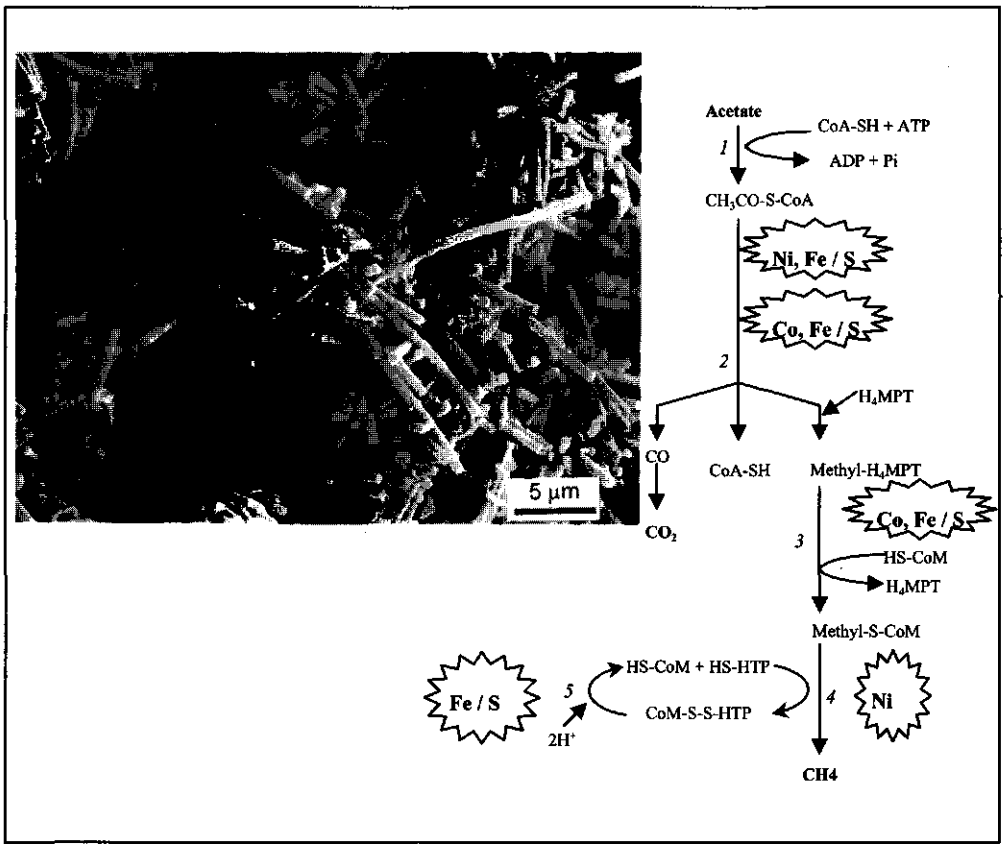

Figure 1.3 *Schematic representation of the hypothetical pathway of methanogenesis from acetate. Only the genera Methanosarcina and Methanosaeta are capable of catalyzing this conversion. A Methanosaeta colony is shown in the scanning electron microphotograph. Constitutive sulfur and metals of the enzymes are indicated in the respective metabolic steps. CH_3COO-S-CoA Acetyl coenzyme A, H_4MPT tetrahydromethanopterin, HS-CoM coenzyme M (2-mercapto-ethanesulfonate), HS-HTP 7-mercaptoheptanoylthreonine phosphate, CoM-S-S-HTP hetherodisulfide of HS-CoM and HS-HTP. The enzymes involved are:* ***(1)*** *Acetate kinase and phosphotransacetylase (in Methanosarcina) or acethyl CoA synthetase (in Methanosaeta).* ***(2)*** *CO dehydrogenase.* ***(3)*** *Methyl-H_4MPT:HS-CoM methyltransferase.* ***(4)*** *Methyl-CoM reductase.* ***(5)*** *Hetherodisulfide reductase.*

Mass transfer

Low substrate concentrations must be maintained in well-mixed reactors with low effluent concentrations. These conditions may prevail in EGSB systems due to the high recirculation rates applied. At low substrate concentrations, thick biofilms may give rise to mass transfer limitations, resulting in an overall limitation of reactor capacity.

The impact of diffusion and mass transfer resistance on substrate utilization has been studied extensively in aerobic systems where clearly oxygen can be externally and internally mass transport limited due to its poor solubility in water [27, 99]. However, in case of anaerobic systems controversies exist with respect to (i) under which conditions mass transfer may limit the overall treatment capacity, (ii) whether increased liquid upflow velocities (V_{up}) enhance mass transport in granular sludge, and to (iii) the relative importance of external and internal mass transport limitations in anaerobic granular biomass.

Due to the conditions prevailing in EGSB systems, mass transfer phenomena may play an important role. This topic is addressed in more detail in Chapter 3.

Toxicity

The EGSB technology likely will enable the treatment of complex and toxic wastewaters such as those from the chemical industry. The success of the treatment process will highly depend on the knowledge of the characteristics and the mechanism of toxicity and/or biodegradability of the toxic compound involved. This knowledge will lead to more efficient design, control and monitoring strategies.

In anaerobic systems, toxicity is reflected in a decrease on the methane production rate and a concomitant decrease in the treatment efficiency. The toxicity of a compound is related to its physical/chemical properties, and the conditions prevailing in the reactor system. All these factors will determine the availability and reactivity of the toxic compound with the target biomass.

Before the treatment system is implemented, the following needs to be known: (i) if methanogenesis can recover during or after exposure of the toxic compound(s), and (ii) whether or not the toxic compound(s) can be mineralized.

In Chapters 4 and 5, the characteristics of toxicity and biodegradability of formaldehyde were investigated. The specific procedures used can also be applied for the assessment of the toxic effects of other compounds that may be present in industrial waste streams.

Formaldehyde production and uses

Formaldehyde, HCHO, is commercially available in aqueous solution containing 37% formaldehyde or as paraformaldehyde, a solid polymer, that contains 91 to 99% formaldehyde. Formaldehyde is an important industrial and research chemical. With an annual production of 3.8 million tons in 1995, it is ranked among the top 25 chemicals produced in the world (Chemical and Engineering news, 1996). The main use of formaldehyde is in the chemical production of urea and phenolic resins. About 80% of the slow-release fertilizer market is based on urea-formaldehyde containing products. Formaldehyde is also used in the production of acetal resins and permanent-press finishes of cellulosic fabrics [24]. It is furthermore used as a chemical intermediary to make explosives and cosmetics. Furthermore, formaldehyde is a widely used disinfectant.

Interactions between formaldehyde and microorganisms

The role of formaldehyde in eubacteria, fungi, and yeast. Despite its toxicity, formaldehyde is an intermediate in the metabolic pathways of many methylotrophic microorganisms that grow on single-carbon compounds as sole carbon or energy source. Formaldehyde can either be assimilated via one or more pathways into central metabolites, or oxidized further to carbon dioxide generating energy in the form of $NADH_2$. Formaldehyde oxidization is performed either by sequential action of formaldehyde dehydrogenase and formate dehydrogenase [45], or by using a cyclic pathway based on enzymes of the ribulose monophosphate cycle [37, 64].

When the cellular formaldehyde concentration is increased and it can not be anymore metabolized efficiently by inclusion in the metabolic pathways, it becomes inhibitory.

In view of that, Battat et al. [5] showed that to avoid inhibition on the growth of *Pseudomonas* C on a mixture of methanol and formaldehyde, the formaldehyde concentration in a chemostat should be kept as low as 2 mg/l.

In batch growth of *Pseudomonas putida* at an initial formaldehyde concentration of 250 mg/l, exponential death was observed due to formaldehyde toxicity. However following this event, exponential growth was found to occur. Formaldehyde was transformed simultaneously in equimolar basis into methanol and formic acid. This transformation was attributed to a constitutively produced formaldehyde dismutase enzyme. Furthermore it was stated that the production of this enzyme was enhanced by the presence of formaldehyde [1].

Formaldehyde was also found inhibitory at 200 mg/l to fodder yeast when cultivated in chemostats. At this concentration the biomass yield decreased but the synthesis of protein and

RNA were stimulated [46]. It was found that some soil fungi can assimilate formaldehyde at formaldehyde concentrations as high as 1000 and 2000 mg/l [78]. The surprisingly high resistance of these fungi to such formaldehyde concentrations was attributed to very high formaldehyde dehydrogenase activities.

Even non-methylotrophic organisms were found to contain enzyme activity for formaldehyde oxidation. Addition of 2000 mg/l of formaldehyde inhibited growth of a non-methylotrophic yeast in a chemostat with glucose as carbon source. Yeast growth occurred only after the formaldehyde concentration had dropped below 30 to 40 mg/l. Formaldehyde was oxidized by formaldehyde dehydrogenase and formate dehydrogenase, a process indicated as detoxification by oxidation [45].

The resistance of some microorganisms to formaldehyde has been attributed to the presence of formaldehyde dehydrogenases. Formaldehyde dehydrogenase activity prevails irrespective of whether the bacteria were grown in the presence or absence of formaldehyde [4, 47, 78]. Formaldehyde dehydrogenase systems and mechanisms regulating their activity have been described for different microorganisms [2, 15, 30, 38, 92, 94]. A main role of such systems is to prevent the accumulation of formaldehyde within the cell. This role is extremely important in methylotrophic and methanotrophic bacteria since the formation of formaldehyde is an integral step in the growth of these organisms [3, 31]. Without having the ability to remove any free formaldehyde, cell death would readily occur.

The role of formaldehyde in methanogens. HS-CoM (coenzyme M) and formaldehyde react chemically in water to form $HOCH_2$-S-CoM (i.e. 2-(hydroxymethylthio) ethanosulfonic acid). When this compound was added to cell extracts of *Methanobacterium thermoautotrophicum*, methane was formed at rates comparable with the rate of CH_3-S-CoM reduction. The reduction of directly added formaldehyde to CH_4 required HS-CoM. Maximal rates of methanogenesis were observed at a ratio of formaldehyde to HS-CoM of 1. When formaldehyde was in excess to HS-CoM, formaldehyde became inhibitory to the system. Any free HS-CoM and formaldehyde were only observed in case one of these components was in excess over the other [77].

Formaldehyde can also react chemically with H_4MPT to form CH_2=H_4MPT, methylene-H_4MPT [16, 17]. In this way, cell suspensions of methanogens can catalyze the reduction of formaldehyde via methylene-H4MPT to CH_4. When methanogenesis was inhibited by 2-bromoethanesulfonate, formaldehyde was oxidized to CO_2 and $2H_2$ by cell suspensions of *Methanosarcina barkeri* [42].

The possible reactions of formaldehyde with components of the methanogenic route are presented in Figure 1.4.

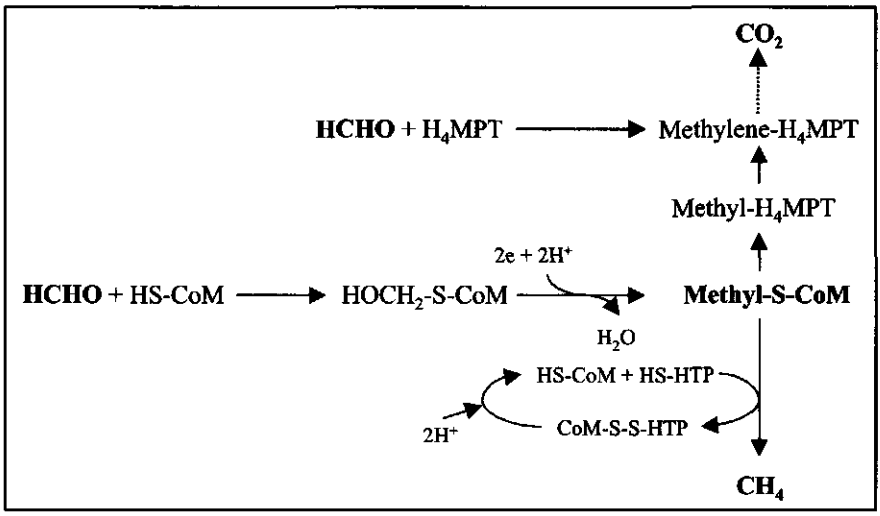

Figure 1.4 *Reactions of formaldehyde with components of the methanogenic route. $HOCH_2$-S-CoM 2-(hydroxymethylthio) ethanesulphonate. Other abbreviations used are defined in the legend of Figure 1.2. The dashed arrow indicates several reactions already described in Figure 1.2. Formaldehyde reactions with H_4MPT and HS-CoM are chemical reactions.*

Cell-free extracts of *Methanobacterium thermoautotrophicum* oxidize formaldehyde to generate reducing equivalents for the reduction of formaldehyde to CH_4 (disproportionation events). In these conditions a CH_4/HCHO ratio of 1/2.9 was observed. The oxidation of 2 mol of formaldehyde sufficed for the generation of the needed reducing power for the conversion of 1 mol of formaldehyde to CH_4. The proposed reactions are the following:

		$\Delta G^{o\prime}$ kJ/mol
	$2HCHO + 2H_2O \rightarrow 2HCOO^- + 2H_2 + 2H^+$	2(-23.4)
	$HCHO + H_2 \rightarrow CH_3OH$	-44.8
	$CH_3OH + H_2 \rightarrow CH_4 + H_2O$	-112.5
Total	$3HCHO + H_2O \rightarrow CH_4 + 2HCOO^- + 2H^+$	-204.1

Although the experimental results suggest the production of formate, free formate was not detected after completion of the reaction, indicating possibly a bound formyl moiety [16].

In similar experiments with cell-free extracts of the methanogens *Methanococcus voltae* and *Methanococcus jannaschii* a CH_4/HCHO of 1/2.01 and 1/2.05 was observed, respectively. In this case, the following reactions were proposed [16].

		$\Delta G^{o\prime}$ kJ/mol
	$HCHO + H_2O \rightarrow HCOO^- + H_2 + H^+$	-23.4
	$HCOO^- + H_2O \rightarrow HCO_3^- + H^+$	1.3
	$HCHO + H_2 \rightarrow CH_3OH$	-44.8
	$CH_3OH + H_2 \rightarrow CH_4 + H_2O$	-112.5
Total	$2HCHO + H_2O \rightarrow CH_4 + HCO_3^- + H^+$	-179.4

In the above enzymatic studies, formaldehyde has been used as a substrate (in bound form) to assess if it is capable of donating the C_1 unit to the methanogenic pathway. Excess formaldehyde and therefore free-formaldehyde was not used because under such conditions it may inhibit the enzymes involved.

With respect to the question whether formaldehyde can be used as carbon and energy source, Schink [79] reported that formaldehyde can be used as a growth substrate for homoacetogenic bacteria. However, extreme care has to be taken to avoid severe bacterial decay due to the toxic effect of formaldehyde [79]. In enrichment cultures with 30 mg/l of formaldehyde, methanogenic bacteria were selected together with *Acetobacterium*-like cells, and acetate and methane were formed as products. These cultures could never be maintained (Schink unpublished), indicating that the survival of a population growing on a rather toxic substance is difficult, if possible at all. These studies clearly demonstrate the challenge to apply anaerobic treatment for wastewaters from the chemical industries because the formaldehyde concentration can be considerably higher here than those used for metabolic studies.

Formaldehyde in waste streams and feasibility of anaerobic treatment

Process wastewaters derived from the production of urea-formaldehyde resins, dimethyl therephtalate (DMT), and the formaldehyde production itself may contain formaldehyde concentrations ranging from 1 to 10 g/l [59, 83, 104]. But even at concentrations as low as 5 mg/l, formaldehyde already is highly toxic to many organisms. Compared to phenol, another commonly used disinfectant, formaldehyde toxicity for most of the organisms tested is more severe [25, 97].

Since formaldehyde is a mutagenic and carcinogenic agent [28], direct discharge of chemical effluents containing formaldehyde threatens life in the receiving water, and imply the need for efficient treatment systems. Anaerobic treatment could represent an attractive option, however the implementation of anaerobic systems for the treatment of waste streams of the chemical industry has been hampered mainly by a lack of understanding of the response of anaerobic systems to this toxic compound. So far, there is no information about the mechanisms of formaldehyde toxicity, but in literature some information is available about formaldehyde toxicity in batch and continuous systems, which is summarized in Table 1.1 and Table 1.2, respectively.

The available information in literature is difficult to interpret and extrapolate, and yet insufficient for rational design purposes. Furthermore, most of the research has been performed with acetate enriched cultures so that the impact of formaldehyde conversion capacity of other bacterial trophic groups remains unclear.

It is obvious that there is an urgent need to improve the fundamental insight in this matter because otherwise the application of anaerobic wastewater treatment process to formaldehyde containing effluents remains extremely risky. Moreover also the design and mode of operation of anaerobic treatment systems would remain a matter of trial and error.

Apart from the toxicity aspects in the particular case of formaldehyde containing wastewaters, there also exists a more general need for a better understanding of the kinetics and metabolic characteristics of immobilized anaerobic consortia such as present in granular types of sludge.

Table 1.1 *Formaldehyde toxicity studies in anaerobic batch systems at 35°C unless stated otherwise.*

Inoculum	Main substrate	Tested HCHO mg/l	* IC_{50} mg/l	Additional information	Reference
anaerobic digested sludge		10-100		Recovery observed for all doses except for 100 mg/l	[35]
acetate enriched methanogenic sludge	acetate			≈ 0.3 gVSS/l Threshold dose <100 mg/l	[67]
domestic watewater		1-10000	200	22°C No acclimatization	[70]
granular sludge from a UASB treating petrochemical wastewater	sucrose		254	1 g VSS/l 30°C	[98]
methanogenic sludge treating water from seeafood processess	VFA	50-200	125	1-1.5 g VSS/l Temperature not indicated	[63]
acetate enriched methanogenic sludge	acetate	100		0.99 gVSS/l HCHO removed in 200 h	[72]
		60		0.36 gVSS/l HCHO removed in 100 h	
activated sludge from a plant treating wood processign industry wastewater, plus anaerobic digester sludge	glucose	2-400	300	90 % of HCHO removed, but at 3000 only 14%	[59]

* IC_{50} refers to 50% inhibition concentration

Table 1.2 *Formaldehyde toxicity studies in continuous systems at 35°C unless stated otherwise*

System	Biomass	Main substrate	SRT# d	Influent HCHO mg/l	% Rem.	Threshold Dose mg/l	Additional information	Reference
Continuous mixed with SP&	anaerobic digestor sludge	acetate methanol	>200	375	95-98		7 gVSS/l	[83]
Continuous mixed without SP&			10-18	125	85-88		2.2 gVSS/l	
Anaerobic filter		acetate		400			Maximum tolerable	[67]
Continuous mixed	acetate enriched	acetate	50	400			Maximum tolerable	[66]
Chemostat	acetate enriched	acetate	14	1110			HCHO degradation of 74 mg/l.d. No acclimatization observed.	[72]
Semi-continuous	acetate enriched	acetate	15	45		<50 as slug 0-50 as continuous		[68]
			25	200			Maximum tolerable values. 30°C Acclimatization is suggested.	
			50	>400		<50 as slug 186 as continuous		
Chemostat	acetate enriched	acetate	40	Slug 51			No effect	[6]
				128			System failed	
Semi-continuous mixed reactor	acetate enriched	acetate	50	Slug 100 500			Days to recover 15 35	[67]

refers to sludge retention time

& refers to support biomass particles

Reliable, plain and straightforward approaches should be used for the assessment of the kinetic properties and metabolic characteristics of the sludge. For this purpose, a novel approach was developed to study methanogenic systems (Chapter 6). This approach was applied in the research presented in this thesis.

If we consider that the complexity of anaerobic digestion still hampers a clear mathematical interpretation of the process, it becomes clear that a better understanding of the kinetic and metabolic properties of sludge is of eminent importance for rational design and performance monitoring of anaerobic reactors. The work presented in this thesis focuses on the kinetics and metabolic properties of anaerobic sludge under specific process conditions that may be found in the treatment of waste streams from the chemical industries, basically the lack of micronutrients and toxicity.

Scope of this thesis

Chapter 2 deals with studies concerning Ni and Co limitations to methanogenesis from methanol. Methanol was chosen because (i) it is one of the main components in several industrial waste streams, and (ii) its conversion is mediated by microorganisms which have special trace element requirements.

Chapter 3 addresses the impact of diffusion and mass transfer resistance on substrate utilization by granular sludge. Mass transfer limitations are particularly important at low substrate concentrations, which need to be maintained in well-mixed reactors in order to achieve very low effluent concentrations, such as in EGSB reactors. The EGSB reactor is currently preferred for the treatment of waste streams from the chemical industry.

Chapter 4 and 5 deals with toxicity studies concerning the effect of formaldehyde to methylotrophic and acetoclastic methanogenesis. Formaldehyde is found in several waste streams not only as wastewater constituent, but also as added contaminant when formaldehyde is used as process disinfectant.

Chapter 6 describes a novel approach for the study of methanogenic systems. The methodology described in that chapter was used throughout the research of this thesis. Case studies and further potentials of the methodology are presented.

The general discussion of the results with conclusions and recommendations is presented in chapter 7.

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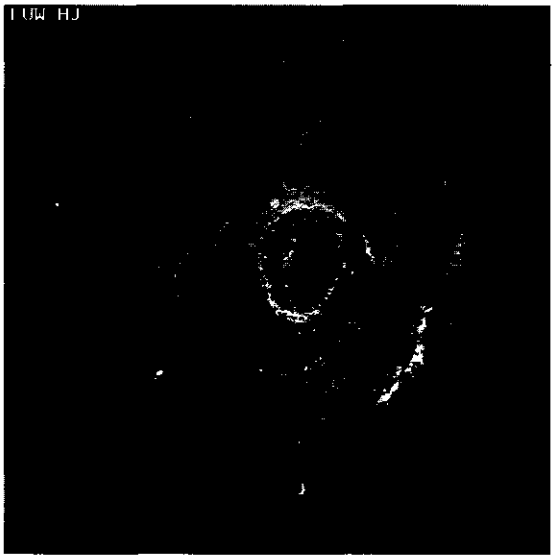

Top picture. Scanning electron image of a cross-sectioned granule. The darker zones indicate zones with higher contents of biomass.

Bottom picture. Energy disperse 'X' ray analysis (EDXA) of the same granule. The light zones indicate the distribution of iron precipitates.

Methanogenic biomass is highly dependent on trace elements like iron nickel and cobalt. In anaerobic environments due to the ubiquitous presence of sulfide, strong metal precipitation may occur. Precipitated metals are not directly available for biomass metabolism. Insights in the processes involved in the bioavailability of trace metals may help to optimize metal addition for anaerobic treatment.

2

Effects of Nickel and Cobalt on the Kinetics of Methanol Conversion by Methanogenic Sludge as Assessed by On-Line CH_4 Monitoring

When essential trace metals were added in a pulse mode to methylotrophic-methanogenic biomass, three methane production rate phases were recognized. Increased concentrations of Ni and Co accelerated the initial exponential and final arithmetic increase in the methane production rate and reduced the temporary decrease in the rate. When Ni and Co were added continuously, the temporary decrease phase was eliminated and the methane production rate increased exponentially. We hypothesize that the temporary decrease in the methane production rate and the final arithmetic increase in the methane production rate were due to micronutrients limitations and that the precipitation-dissolution kinetics of metal-sulfides may play a key role in the bioavailability of these compounds[✓].

[✓] A modified version of this chapter was published in *Appl. Environ. Microbiol.* (1999), **65:** 1789-1793.

Introduction

Previous research [6, 20, 26, 28] has shown that metal deficiencies can limit the performance of anaerobic digestion systems and that metal supplementation may substantially improve the treatment performance of such systems. In particular, the benefits of nutrient supplements during methanization of industrial wastewaters have been recognized [27, 28].

Methanogenesis from methanol may proceed by several pathways: (i) methanol can be directly converted to methane by methylotrophs, (ii) methanol can be converted with bicarbonate to acetate by acetogens, and the acetate can be metabolized by acetoclastic methanogens, and (iii) methanol can be converted to H_2 and CO_2, which can be used by hydrogenophilic methanogens and/or acetogens to form methane and acetate, respectively [6]. *Methanosarcina* type bacteria are responsible for direct conversion of methanol to methane, and Ni, Co, and Fe are components of the enzymes that catalyze many of the reactions of this methylotrophic pathway [4, 5, 11, 13, 29]. It has been reported that metal supplementation may substantially improve anaerobic treatment of different types of waste streams [8, 10, 21, 28]. However, the previously described metal additions that are optimal vary by several orders of magnitude [28]. Furthermore, previously published information concerning the relationship between kinetic data and "optimum" metal concentrations is rather unclear. This is illustrated by the fact that for similar batch systems, metal (Ni or Co) doses vary from 0 to 40 μM [17, 22-24].

Since methanol-consuming methanogens have specific trace metal requirements and since many industrial waste streams contain methanol as an important contaminant, in the present study we examined the kinetics of methanol consumption by methanogenic biomass in the presence of different Ni and Co concentrations.

Materials and Methods

Anaerobic granular sludge from a full-scale expanded granular sludge bed, a Biobed EGSB reactor (location, Caldic Europoort) developed by Biothane Systems (Delft, The Netherlands) was used. The sludge treated wastewater generated during the production of formaldehyde. The waste stream contained primarily methanol and formaldehyde as organic substrates [30]. To prevent mass transfer limitations, the granular structure was disrupted with a blender, and the sludge suspension obtained was used as inoculum. The initial concentration of the inoculum expressed as the amount of volatile suspended solids (VSS) in the reactors was 1.5 g/l. The standard medium used contained the following components: 15 mM NH_4Cl, 7 mM H_2PO_4, 4 mM $MgSO_4$, 5mM $CaCl_2$, and 35.74 mM $NaHCO_3$. This medium also contained

yeast extract (100 mg/liter) and trace elements, which were present in the following concentrations: H_3BO_3, 4.03 μM; $ZnCl_2$, 1.84 μM; $CuCl_2$, 1.11μM; $MnCl_2$, 12.63μM; $(NH_4)_6Mo_7O_{24}$, 0.2 μM; $AlCl_3$, 1.86 μM; Na_2SeO_3, 3.12 μM; and $FeCl_2$ 50, μM. Ni and Co were added as $NiCl_2$ and $CoCl_2$, respectively at the concentrations indicated below. Methanol was used as the carbon source at an initial concentration of 208 mM (10 g of chemical oxygen demand [COD] per liter). The pH in each reactor during the experimental period was 7 to 7.2. All chemicals were of analytical grade, and most chemicals were purchased from Merck (Darmstad, Germany); yeast extract was purchased from Oxoid Unipath Ltd. (Hampshire, England).

The reactors used were completely stirred plastic vessels. They were filled with 2.5 liters of mineral medium and flushed with a 70% N_2-30% CO_2 mixture for 20 min. Subsequently $Na_2S.8H_2O$ was added to obtain a final concentration of 0.54 mM; finally, Ni and/or Co was added. After a 24-h period, in which we assumed that chemical equilibrium was established, the inoculum and methanol were added. The reactors were operated in a batch mode in a temperature-controlled (30 ± 1 °C) room. The biogas produced was passed through an Erlenmeyer flask filled with a 20% NaOH solution and then through a tube filled with soda lime pellets with thymol blue indicator. Finally the gas passed through a Mariotte flask system containing water for quantification of the methane production. The methane produced was monitored continuously by measuring the weight of the displaced water with a pressure sensor (model QB 745; DS-Europe) connected to a programmable data logger system (model CR10; Campbell). The data were recorded every 10 s and were averaged over a 30-min interval. A personal computer programmed to function as a terminal emulator was used to communicate with the data logger. All assays were performed in duplicate.

To assess the main pathway of methanol conversion, methane production by the biomass during degradation of methanol was monitored in 250-ml serum bottles by adding specific inhibitors to 100 ml of the reaction medium in order to block different metabolic routes [7]. The inhibitors used were bromoethanesulfonic acid and vancomycin which were purchased from Sigma Chemical Co. (St. Louis, Mo.) and Janssen (Tilburg, The Netherlands), respectively. The biomass added (previously activated with methanol) contained 0.8 g VSS/l; methanol and inhibitors were added at concentrations of 31 and 50 mM, respectively. The methanol, methane, volatile fatty acids (VFA), and hydrogen in the head-space were measured during the experiment. H_2 and CH_4 contents were determined as previously described [7]. VFA and methanol were analyzed by the method of Kortekaas et al. [12], except that for methanol the temperature of the oven was 70° C. A spectrophotometric method [2] was used to routinely analyze for the presence of formaldehyde. VSS contents

were determined by standard methods[1]. Granule samples were prepared and analyzed for scanning electron microscopy as described previously [9].

The equilibrium concentrations of dissolved and precipitated species were calculated with a chemical equilibrium program written in Turbo Pascal. Phosphate, carbonate, and sulphide species, including metal sulphide complexes, were included. The input concentrations were the concentrations in the standard medium used. The mass balances and the reaction equations were solved iteratively by using the Newton-Raphson method. Most of the equilibrium constants were obtained from reference [25]; the metal sulfide complexes constants were obtained from reference [16].

Results

Scanning electron microscopy observations showed that the predominant bacteria in the sludge were *Methanosarcina* type archaea. This was confirmed by assessing the main metabolic route of methanol degradation. The results indicated that methanol was converted via direct methanogenesis and that methane formation through acetate or H_2/CO_2 did not play an important role.

The kinetics of methanogenesis from methanol in the sludge were characterized by the following three phases: (i) an exponentially increasing rate (phase I), (ii) a temporary decrease in the rate (phase II), and (iii) an additional arithmetic increase in the rate (phase III) (Figure 2.1). A possible explanation for the decrease in the rate was that the biomass was micronutrients limited. Hence, experiments with different Co and Ni additions were performed.

Either Ni or Co was added at concentrations of 0, 4, 40 and 400 μM while the other metal was added at a concentration of 40 μM. All the curves showed that there was a decrease in the methane production rate after approximately 40 h (Figure 2.2A, 2.2B). However, as the concentration of Co increased, the magnitude of the temporary decrease in the methane production rate decreased and the slope of the arithmetic increase (phase III) increased (Figure. 2.2A). Nickel concentrations of 4 and 40 μM increased the methane production rate during phase I (Figure 2.2B). Furthermore, it seemed that nickel affected phase I most, whereas cobalt affected phase III most. However in contrast to the cobalt experiments, addition of 400 μM nickel was inhibitory.

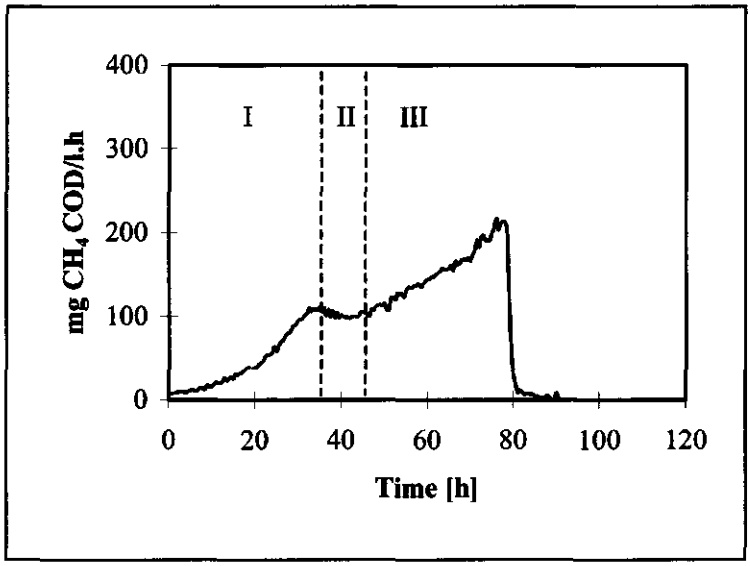

Figure 2.1 *Methane production rate curve from methanol degrading sludge. Three phases could be distinguished as indicated in the figure:* I) *Exponential methane production rate,* II) *Drop in the rate,* III) *Further arithmetic increase in the rate. Ni and Co were dosed at 1μM each.*

Additions containing Ni plus Co were also tested. These micronutrients were simultaneously added at concentrations of 0, 1, 4 and 40 μM each. As in the previous experiments, the metals were added 24 h prior to inoculation. The results (Figure 2.2C) clearly demonstrated that metal bioavailability was limited since as the amount of metal increased, the exponential growth during phase I increased, the temporary decrease in phase II was less pronounced, and the arithmetic rate of increase during phase III was faster. However, the observed limitations were not completely eliminated even with metals concentrations as high as 40 μM Ni and 40 μM Co. Therefore, continuous addition of metals was studied to see whether micronutrient limitations could be overcome.

Co and Ni were added continuously at rates of 0, 0.05, 0.2 and 2 μmol/h; hence, the concentrations in the reactor after 50 h of addition or 80-h experimental period (the first doses were added after 30 h) were 0, 1, 4 and 40 μM, respectively. The results of this test are shown in Figure 2.2D.

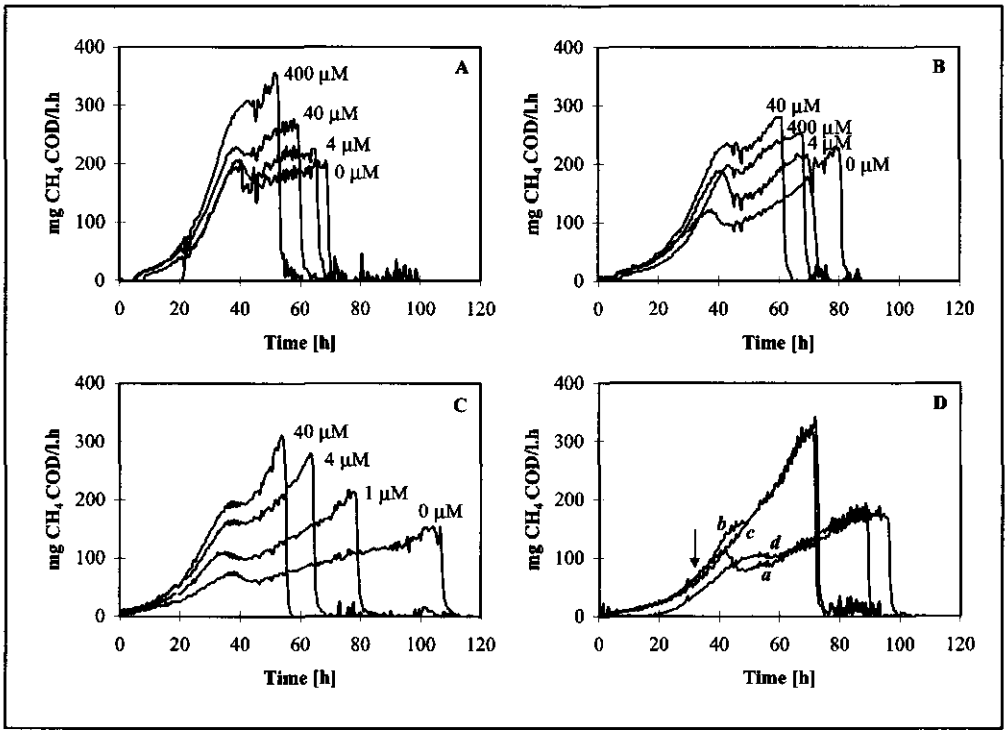

Figure 2.2 *Methane production rate curves from methanol:* **(A)** *At different initial cobalt concentrations. The nickel addition in all cases was 40µM.* **(B)** *At different initial nickel concentrations. The cobalt addition in all cases was 40µM.* **(C)** *At different Ni and Co concentrations dosed simultaneously.* **(D)** *Continuous dosing of Ni and Co during methanol degradation. Different rates of addition were tested. The arrow indicates the start of the addition of the metals. The dosing rates were in µmol/h : a 0, b 0.05, c 0.2, d 2.*

Doses of 0.05 and 0.2 µmol/h (1 and 4 µM respectively) seemed to eliminate limitations, and the previously observed decrease in the methane production rate during phase II was not observed in this experiments. Also, the period of exponential increases in the methane production rate was extended. However, a dose of 2 µmol/h (40 µM) appeared to be inhibitory.

Discussion

Rate curves having the shape shown in Figure 2.1 and 2.2A to C can reflect either (i) limitations due to substrate or nutrient depletion or (ii) production of a toxic compound. The temporary decrease in the rate could not be attributed to substrate limitations since in all cases the methanol concentration after 40 h was more than 3,000 mg of COD/l and the reported apparent substrate affinity coefficient (K_S) for methylotrophic methanogens was 12 mg of COD/l [7]. Possible limitations due to the production of formaldehyde, which is a toxic compound and an intermediate (at the oxidation level) during the oxidation of methanol [11], were not detected. Furthermore, the limitations could not be attributed to macronutrient limitations since in separate experiments performed with different amounts of macronutrients the methane production rate was not affected (data not shown).

Spiked addition of metals. The results of this work clearly show that the biomass was nickel and cobalt limited. Two main theoretical aspects should be considered in order to understand the limitations observed: (i) Assuming that *Methanosarcina barkeri* Fusaro cells grown on methanol contained 0.135 and 0.06 mg of Ni and Co per g of cells, respectively [19], and that the yield was 0.088 g of cells/g of methanol COD [22], then 2 μM Ni and 0.89 μM Co would be required for conversion of the 10 g of methanol COD liter/l added in the experiments (Table 2.1). According to this reasoning, addition of more than 2 μM metal should fulfill the nutrient requirements. However, as the spiked dose experiments showed, this was not the case, and the metals added (>2μM) seemed to be not available for the biomass since limitations were still observed. (ii) If it is assumed that there is chemical equilibrium, then the following trends can be expected: the calculated concentrations of free Ni and Co should increase sharply only when the initial dose of Ni and initial dose of Co are more than 100 μM, corresponding to the moment that the amount of sulfide added becomes limiting for precipitation reactions (Figure 2.3). Although metal sulfide complexes may be important soluble species at pH values greater than 7 [16], at metal concentrations below 100 μM the concentration of dissolved metals that occur as (i) free metals (M^{2+}), as well as and the concentrations of (ii) free metal plus sulfide complexes (MHS^{+}), should be less than 0.01 and 0.05 μM, respectively (Figure 2.3). If dissolution of the precipitated metals were negligible, these concentrations of dissolved metals would not fulfill the nutrient requirements, as indicated in Table 2.1. Consequently, we concluded that metal shortage must have been compensated by dissolution of precipitated metal sulfides.

Table 2.1 *Calculated Ni and Co requirements for the degradation of 10 g methanol-COD.*

Metal	Cell composition mg/g cell	Y µmol metal/g cell	Requirement µmol/g COD		Requirement phase I* µmol/3-6g COD	Requirement µmol/10g COD
Ni	0.135	2.3	0.2	*a*	0.6-1.2	2
Co	0.06	1.02	0.089	*a*	0.27-0.5	0.89
Ni			0.1059	*b*	0.3-0.6	1.059
Co			0.084	*b*	0.25-0.5	0.84

a Calculated considering the metals cell composition reported by Scherer et al. [19] and assuming a yield of 4.23 g cell/mol methanol (0.088 g cell/g COD) from Silveira et al. [22].

b Experimental values reported by Nishio et al. [18].

* In general during phase I, ± 3-6 g COD-methanol are consumed.

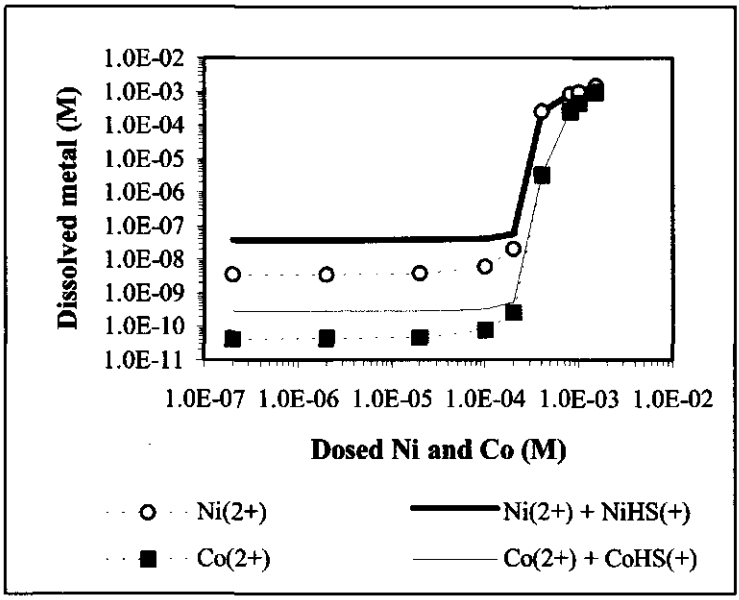

Figure 2.3 *Calculated equilibrium dissolved metals concentration at different initial metal doses of Ni and Co.*

Based on these considerations, the general trend in the methane production rate (Figure 1.1) can be explained as follows. During the first 30 to 40 hours of the process (phase I), readily

dissolved precipitates provide the required nutrients, which result in exponential increase in the methane production rate of the methylotrophic population. However, if the metal uptake rate is higher than the rate of dissolution, then metal-limited conditions for the bacteria prevail. This situation occurred during phase III, when the growth rate increased arithmetically; this increase may have been determined by the rate of dissolution of metal sulfides. The temporarily decrease in the methane production rate (phase II) may have been due to severe metal limitation that was even greater than that in phase III, suggesting that in addition to bacterial growth the specific methane conversion rate is affected by limiting concentrations of Co and/or Ni. It should be noted that different types of precipitates of the same metal can be present in a system (some compounds dissolve faster than others) and that the dissolution rate of a specific metal sulfide may not be constant since it depends on the total surface area.

Continuous dose of metals. Nutrient limitations can be overcome if the essential metals are added continuously at a proper rate so that their availabilities in solution can fulfill the requirement for biomass activity and growth. The results obtained in the continuous addition experiments clearly show this effect and thus indicate the importance of the dissolution rate kinetics of metal sulfide precipitates in anaerobic digestion processes.

When the metals were added continuously at rates of 0.05 to 0.2 μmol/h, the temporary decrease in the methane production rate was eliminated and the period of exponential methane production was extended (Figure 2.2D). These rates of addition corresponded to metal concentrations of 1 to 4 μM and agree well with the calculated metal requirements based on the biomass yield and Ni and Co contents of biomass (Table 2.1) for *Methanosarcina* spp. grown on methanol. Other studies have also shown that when essential nutrients are added continuously in fed batch systems, the period of exponential growth of *M. barkeri* is drastically extended compared to a system in which nutrients are added in one dose [18]. Thus, when the carbon-substrate concentration is not limiting, metal dissolution might become the rate-limiting step.

Practical implications. During operation of a continuous reactor, less metals may be required than the amount required in a batch system, because more biological ligands may be produced [3, 14, 15] and because continuous addition of nutrients ensures free metal availability for biomass uptake. In practice, in view of the clear evidence that supplying metal enhances the treatment of several effluents, there is a tendency to add nutrients in excessive amounts. This could lead to inhibitory effects for the biomass and/or to metal accumulation in the sludge. Hence, in order to determine a more rational way to add essential metals, studies to understand the process kinetics of precipitation and dissolution in anaerobic systems in

combination with kinetics of nutrients uptake by the microorganisms are necessary. In this paper we show that a rational supply of metals can be achieved, which in turn may open the possibility of either enhancing or diminishing the production of biomass depending on the treatment and remediation needs.

On-line CH_4 monitoring. With on-line measurements of the methane production very detailed kinetic information can be obtained. To our knowledge, there are no previous reports on kinetic data being analyzed by on-line measurement of the CH_4 production rate. Furthermore, the informative value of the results can be maximized when rate-time curves are plotted instead of cumulative curves of the CH_4 production.

Accurate rate data can be obtained only by on-line measurements. The observed decrease and subsequent further increase in the rate (Figure 2.1, phase II) would have been overlooked with cumulative plots or may have been treated as an analytical error.

Finally, although kinetic data are commonly assessed by assuming that only one substrate is limiting, discrepancies in previously reported kinetic constants could be due to nutrient limitations in batch experiments, as shown in this study. This suggests that straightforward comparison of previously published data of kinetic parameters is not possible.

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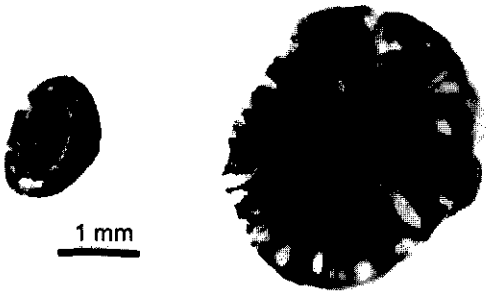

Top picture. Increase in the mean diameter of granular sludge over a one-year period at full-scale conditions.

Bottom picture. Biogas bubble inside a granule.

At high granule mean diameters the transport of substrate inside the biofilm could become limited, resulting in an overall limitation of reactor capacity.

So far it is not known under which conditions biogas bubbles are formed inside the biofilms. However, there exists the speculation that the out-flux of gas may create convective flow inside granular sludge.

3

Kinetics and Mass Transfer Phenomena in Methanogenic Granular Sludge

The kinetic properties of acetate degrading methanogenic granular sludge of different mean diameters were assessed at different up-flow velocities (V_{up}). Using this approach, the influence of internal and external mass transfer could be estimated. First, the apparent Monod constant (K_S) for each data set was calculated by means of a curve-fitting procedure. The experimental results revealed that variations in the V_{up} did not affect the apparent K_S-value, indicating that external mass transport resistance normally can be neglected. With regard to the granule size, a clear increase in K_S was found at increasing granule diameters. The experimental data were furthermore used to validate a dynamic mathematical biofilm model. The biofilm model was able to describe reaction-diffusion kinetics in anaerobic granules, using a single value for the effective diffusion coefficient in the granules. This suggests that biogas formation did not influence the diffusion-rates in the granular biomass[*].

[*] Submitted for publication.

Introduction

Anaerobic wastewater treatment is nowadays a well-accepted technology. This is mainly due to the development of high-rate reactors. High-rate reactors have the capacity to uncouple solid and liquid retention times resulting in high concentrations of biomass. The high biomass concentrations are accomplished either by means of bacterial growth on an inert carrier as in fluidized bed (FB) and fixed film (FF) reactors, or by self-immobilization of biomass in the form of granules in upflow anaerobic and expanded granular sludge bed reactors (UASB and EGSB, respectively).

Thick biofilms may give rise to mass transfer limitations, resulting in an overall limitation of reactor capacity. In these conditions, the in-flux of substrate and/or out-flux of products may become the rate determining step. Mass transfer limitations are particularly important at low substrate concentrations, which are normally maintained in well-mixed reactors with low effluent concentrations, such as EGSB-reactors.

Mass transport phenomena have hardly been studied in anaerobic systems. Contradicting results have been described regarding the impact of external and internal mass transport. Whereas some reports indicate that both internal and external diffusion-limitations influence the rate of substrate utilization [9, 30]; others indicate that mass transport limitations are not observed in anaerobic biofilms [5, 25] even at a film thickness of 2.6 mm [18]. In addition, it has been concluded that pH profiles inside anaerobic granules may affect more the conversion rate than mass transport limitations [5].

The impact of diffusion and mass transfer resistance on substrate utilization has been studied extensively in aerobic systems where clearly oxygen can be externally and internally mass transport limited due to its poor water solubility [12, 29]. However, in case of anaerobic systems still controversies exist with respect to: 1) Whether diffusion can be rate limiting in anaerobic biofilms, 2) whether increased liquid upflow velocities (V_{up}) enhance mass transport in granular sludge and 3) the relative importance of external and internal mass transport limitations in anaerobic granules.

Several biofilm models have been developed that comprise the effects of mass transfer in anaerobic films. They include steady-state simulations of substrate profiles for multi-trophic films with homogeneous [7] and heterogeneous biomass distribution [3, 13, 27]. Other models additionally include pH effects on kinetics [10, 11]. However detailed experimental data to validate the models is limited.

The objective of this study was to investigate under which conditions anaerobic granular sludge could be mass transport limited and if increased V_{up} could enhance mass transport. Furthermore, the relative importance of external and internal mass transport limitations in anaerobic granules was assessed.

Materials and Methods

Inoculum. Anaerobic granular sludge from a full scale expanded granular sludge bed (EGSB) reactor (Heineken, Zoeterwoude, The Netherlands) treating brewery wastewater was used. Both granular and dispersed granular sludge were used. Granular sludge of different particle sizes was obtained by sieving the sludge through metallic sieves under water. The dispersed sludge was obtained by disrupting the granular structure with a blender (Braun 4164).

Assessment of kinetic parameters of the inoculum. The maintenance coefficient (Ms) is defined as the quotient of the decay rate over the yield coefficient ($Ms = k_d / Y_{XS}$) as described by Leenen et al. [20]. By assessing the decay rate (and provide that the yield is known), the maintenance coefficient can be estimated. The decay rate of dispersed granular biomass was assessed by determining the loss of specific methanogenic activity in absence of substrate. 24 serum bottles of 120 ml were prepared by adding to each bottle, 2 g VSS/l and 50ml of medium. This medium contained the following components in g/l of demineralized water: $NaHCO_3$ 4, NH_4Cl 0.28, K_2HPO_4 0.25, $MgSO_4 \cdot 7H_2O$ 0.1, $CaCl_2 \cdot H_2O$ 0.01, yeast extract 0.1, and trace elements 1 ml/l with the composition described below. The bottles were flushed with N_2 and CO_2 (70:30 [vol/vol]) and incubated at 30°C without substrate in a shaker. At 0,3,7,14,21,35,70, and 180 days, three bottles were taken, and the headspace was flushed with the same gas mixture, and 1 g sodium acetate COD/l was added to each bottle. The bottles were incubated again, and the methane in the headspace was measured over time. The maximum specific activity (A^{max}) was obtained from the slope of the steepest part of the cumulative-methane *vs.* time curve. The decay coefficient was obtained from the slope of the $\ln (A^{max}(t)/(A^{max}(0))$ *vs.* time curve.

Maximum growth rate (μ^{max}). In a continuously stirred batch reactor operated at 30°C, dispersed granular sludge at a concentration of 0.5 g VSS/l, and 2.5 l of mineral medium of the composition described below were placed. The reactor was flushed with 100% N_2 during 15 min, then 2 g acetate-COD/l was added. During 11 days, substrate was added daily in order to keep the concentration in the reactor between 1.5 to 2 g acetate-COD/l, and the

methane production was measured on-line as described below. For zero order kinetics ($S >> K_S$), the Monod-based equation for product formation, in this case methane, can be integrated (neglecting maintenance and/or decay), and the following equation apply:

$$C_{M,R}(t) = C_{M,R}(0) - f_{S,M} \cdot (1 - Y_{XS}) \cdot \frac{C_{X,R}(0)}{Y_{XS}} \cdot (1 - e^{\mu^{max} \cdot t}) \quad (3.1)$$

Where, $f_{S,M}$ refers to the COD-based stoichiometry for the conversion of substrate into product (methane). By using the biomass yield on acetate reported by Haandel and Lettinga [14], and the on-line measurement of the methane in the reactor ($C_{M,R}$) as a function of time, the initial biomass concentration ($C_{X,R}(0)$), and μ^{max} were estimated with an optimization procedure included in a spreadsheet program. The optimization was based on minimizing the absolute error between measured and calculated values of methane.

Mineral medium. The medium used contained the following components in g/l of demineralized water: NH_4Cl 0.28, $MgSO_4.7H_2O$ 0.1, KH_2PO_4 6.79, $Na_2HPO_4.2H_2O$ 8.89, yeast extract 0.1, trace elements 1ml/l. The trace elements solution contained the following components in mg/l of demineralized water: $FeCl_2.4H_2O$ 2000, H_3BO_3 50, $ZnCl_2$ 50, $CuCl_2.2H_2O$ 38, $MnCl_2.4H_2O$ 500, $(NH_4)_6Mo_7O_{24}.4H_2O$ 50, $AlCl_3.6H_2O$ 90, $CoCl_2.6H_2O$ 2000, $NiCl_2.6H_2O$ 142, $Na_2SeO.5H_2O$ 164, EDTA 1000, Resazurine 200, HCl 36% 1 ml/l. All chemicals were of analytical grade and purched from Merck (Darmstadt, Germany); resazurine was from Fluka (Buchs, Switzerland) and yeast extract from Oxoid Unipath Ltd. (Hampshire, England).

Preliminary studies on the kinetics of dispersed sludge *vs.* granular sludge. The kinetics of acetate degradation by crushed and granular sludge were determined by on-line measurement of the methane production rate in continuously stirred batch reactors of 2.5 l. The reactors were filled with medium and inoculated with 2 g VSS/l of dispersed or granular biomass. Acetate was used as substrate at an initial concentration of 2 g COD/l. With the phosphate buffer added, the pH was kept at 6.8-7.2 and the reactors were operated in a temperature-controlled ($30 \pm 1^{o}C$) room.

Kinetics of granular sludge. The characteristics of acetate degradation by anaerobic granular sludge of different diameters and at different V_{up} values were determined by on-line measurement of the methane production rate in batch-recirculated systems. A schematic

representation of the experimental set-up is shown in Figure 3.1. The system consisted of a glass reactor of 0.16 l and substrate was recirculated over the reactor continuously from a batch plastic vessel of 5 l. The system was filled with mineral medium and the reactors were seeded with 42.5 g of VSS/l reactor, of granular sludge of the specific mean diameter to be tested.

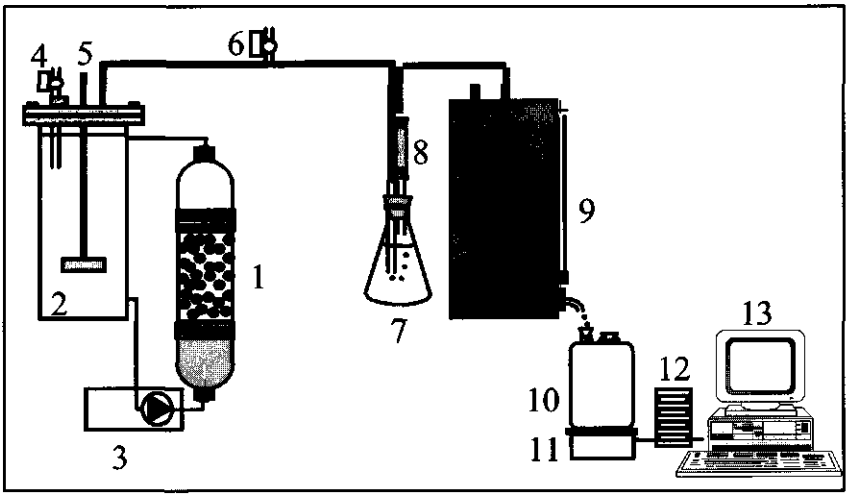

Figure 3.1 *Experimental set-upfor the assessment of the kinetics of granular sludge at different upflow velocities.* 1 *Glass reactor,* 2 *Recirculation vessel or buffer tank,* 3 *Recirculation pump,* 4 *Liquid sampling port,* 5 *Stirrer,* 6 *Gas sampling port,* 7 *NaOH washing bottle,* 8 *Lime soda pellets,* 9 *Mariotte flask,* 10 *Plastic container,* 11 *Balance,* 12 *Data logger,* 13 *Personal computer. The set-up was the same for the kinetics of crushed vs. granular sludge except that the glass reactor and the pump were not present; the reactor was the buffer tank.*

The granules were left to expand according to the V_{up} applied. To avoid biomass wash-out from the reactor, metallic sieves were placed at the bottom and top of the expanded sludge bed. The system was then flushed with 100% N_2 for about 15 min. Thereafter, subsequent feedings of 2 g acetate-COD/l were applied. After the second feed, substrate was added as acetic acid, which was neutralized by the bicarbonate produced in the previous feed. After each feed with acetic acid, the reactors were flushed with 100% N_2 for 15 min in order to remove the CO_2 formed by the chemical reaction. Every new feeding was added after the

former feeding had been depleted. A complete run consisted of 4 feedings. The phosphate buffer added kept the pH at 6.8-7.2. The reactors were operated at 30 ± 1°C.

On-line methane production monitoring. The biogas produced was passed through an Erlenmeyer flask filled with 20% NaOH solution and then through a tube filled with soda lime pellets with thymol blue indicator. Finally the gas passed through a Mariotte flask system filled with water for quantification of the methane produced. The methane production was monitored continuously by measuring the weight of the displaced water with a pressure sensor (model QB745, DS-Europe) connected to a programmable data logger system (model CR10, Campbell). The data were recorded every 10 s and averaged over a 15-min interval. To communicate with the data logger, a personal computer programmed to function as terminal emulator was used.

Analytical methods. The concentration of volatile fatty acids in the medium was determined with a gas chromatograph (Hewlett Packard 5890A). A glass column (2 m × 6 mm) packed with Supelcoport (100 to 120 mesh), coated with 10% Fluorad FC 431, was used. The temperatures of the column, injection port and flame ionization detector were 130, 200, and 280°C, respectively. Nitrogen gas saturated with 100% formic acid was used as a carrier gas at a flow rate of 40 ml/min. Methane was analyzed with a gas chromatograph (Hewlett Packard 438/S), equipped with a steel column (2m × 2mm), packed with Poropak Q (80 to 100 mesh). The temperatures of the column, injection port and flame ionization detector were 60, 200 and 220°C, respectively. All gas samples were taken with a gas-tight syringe (Dynatech, Baton Rouge, Lousiana). The concentration of volatile suspended solids (VSS) was determined by standard methods [1]. The mean diameter of the granular sludge was determined before and after every experimental run with an image analyzer. Sludge samples were placed in a 6 mm petri dish; pictures of the samples were made with a camera (Sony CCD) equipped with a 55 mm micro Nikkon lens. The pictures were digitalized and analyzed with a Magiscan 2 Image Analysis System (Applied Imaging, Tyne & Wear, UK) using the image-analysis software package GENIAS v. 4.6 (Applied Imaging Tyne & Wear, UK). By sieving the dispersed sludge through metallic sieves, it was determined that its size was smaller than 200 μm.

Mathematical model description

Two mathematical models were used to analyze the experimental results: A Monod-based model for calculation of the apparent K_S values of the granular sludge under different

operational conditions, and a biofilm model was developed to validate reaction-diffusion kinetics.

Monod model

Based on Monod kinetics, the rate of substrate consumption ($R_{S,R}$), biomass growth ($R_{X,R}$), and product formation ($R_{M,R}$) per unit of volume reactor in a batch reactor can be described by the following equations [4].

$$R_{S,R} = -\left(\frac{\mu^{max}}{Y_{XS}} + Ms\right)\cdot\left(\frac{C_{S,R}}{C_{S,R} + K_S}\right)\cdot C_{X,R} \tag{3.2}$$

$$R_{X,R} = \left\{\mu^{max}\cdot\frac{C_{S,R}}{C_{S,R} + K_S} - Ms\cdot Y_{XS}\cdot\left(1 - \frac{C_{S,R}}{C_{S,R} + K_S}\right)\right\}\cdot C_{X,R} \tag{3.3}$$

$$R_{M,R} = -R_{S,R} - \mu^{max}\frac{C_{S,R}}{C_{S,R} + K_S}\cdot C_{X,R} \tag{3.4}$$

Where μ^{max} is the maximum growth rate, K_S is the Monod half-saturation constant, Ms is the maintenance coefficient, Y_{XS} is the biomass yield, and $C_{S,R}$ and $C_{X,R}$ stand for the substrate, and biomass concentration in the reactor, respectively.

By combining these kinetic equations with mass balances for acetate conversion, the differential equations can be derived. These equations were integrated by using a fourth-order Runge-Kutta algorithm with adaptive step size control. The K_S-value, and the initial substrate ($C_{S,R}(0)$) and biomass concentrations ($C_{X,R}(0)$) were estimated by minimizing the absolute error between measured and calculated values of the methane production rate. The estimated values for $C_{S,R}(0)$ and $C_{X,R}(0)$ were later used as input for the reaction-diffusion model.

Biofilm model

General assumptions. The biofilm model here described is subject to the following basic assumptions: 1) The granules are spherical in shape and uniform in size (for each test run, granules were size selected), 2) the number of granules and the size of the granules are constant in time, 3) for each experimental run, the V_{up} is considered constant, 4) only radial diffusion transport is considered and is described by Fick's law, 5) the diffusion coefficient is constant, 6) there are no active biomass gradients in the granular biomass at time zero, 7) the biological reactions are described according to the model suggested by Beeftink et al. [4].

Model description. Discretization in time of the partial differential equation describing reaction-diffusion kinetics in a spherical particle (i.e. granule) results in the following equation:

$$\frac{d^2C_{S,P}(r)}{dr^2} = -\frac{2}{r}\cdot\frac{dC_{S,P}(r)}{dr} + \frac{R_{S,P}}{\alpha\cdot D_S} \tag{3.5}$$

Where $C_{S,P}$ is the substrate concentration in the particle, $R_{S,P}$ is the volumetric substrate conversion rate in the particle, α is the relative effective diffusivity, and D_S is the diffusion coefficient of substrate in water.

The two boundary conditions to solve Equation 3.5 are:

$$\left.\frac{dC_{S,P}}{dr}\right|_{r=0} = 0$$

$$C_{S,R} = C_{S,P}\Big|_{r=Rp} + \frac{\alpha\cdot D_S}{k_l}\cdot\left.\frac{dC_{S,P}}{dr}\right|_{r=Rp}$$

Equation 3.5 was solved numerically using a fourth-order Runge-Kutta algorithm with Brent's method to satisfy the boundary conditions [23], in a similar manner as proposed by de Gooijer et al. [12].

The change of the location dependent increase in biomass concentration in the particle ($C_{X,P}$) is obtained through discretization:

$$C_{X,P}(r,t+\Delta t) = C_{X,P}(r,t) + R_{X,P}(r,t)\cdot\Delta t \tag{3.6}$$

with the volumetric rate of biomass production in the particle ($R_{X,P}$) defined as:

$$R_{X,P}(r) = \left\{\mu^{max}\cdot\frac{C_{S,P}(r)}{C_{S,P}(r)+K_S} - Ms\cdot Y_{XS}\left(1-\frac{C_{S,P}(r)}{C_{S,P}(r)+K_S}\right)\right\}\cdot C_{X,P}(r) \tag{3.7}$$

The biomass concentration in the reactor ($C_{X,R}$) is calculated by correcting for the number of particles (PNo) in the reactor.

$$C_{X,R}(t+\Delta t) = \frac{\sum_{i=0}^{Rp} C_{X,P}(r,t+\Delta t)\cdot V_{PSeg}(r)}{V_{PT}}\cdot PNo \tag{3.8}$$

Where, $V_{PSeg}(r)$ stands for the location in the biofilm dependent volume of the particle segment, and V_{PT} refers to the total particle volume. The (constant) number of particles (PNo) in the reactor is calculated according to:

$$PNo = \left.\frac{C_{XT,R}\cdot V_{PT}}{C_{XT,P}\cdot V_R}\right|_{t=0} \tag{3.9}$$

Where, $C_{XT,P}$ and $C_{XT,R}$ are the total biomass concentration in the particle and reactor, respectively, and V_R is the reactor volume.

Integration in time of the substrate and product concentrations in the system is obtained by introduction of the efficiency (η_S) for substrate conversion according to:

$$\eta_S = \frac{3 \cdot \alpha \cdot D_S \cdot \left.\frac{dC_{S,P}(r)}{dr}\right|_{r=Rp}}{Rp \cdot R_{S,R}^{max}} \tag{3.10}$$

Where $R_{S,R}^{max}$ stands for the maximum substrate conversion rate in the reactor in the absence of diffusion limitations. Introduction of the η_S in the differential equations for description of substrate conversion and product formation in time, results in the following equations:

$$\frac{dC_{S,R}(t)}{dt} = \frac{Q}{V_R} \cdot \left(C_{S,B}(t) - C_{S,R}(t)\right) + R_{S,R} \cdot \eta_S \tag{3.11}$$

$$\frac{dC_{S,B}(t)}{dt} = \frac{Q}{V_B} \cdot \left(C_{S,R}(t) - C_{S,B}(t)\right) \tag{3.12}$$

$$\frac{dC_{M,R}(t)}{dt} = \frac{dC_{S,R}(t)}{dt} - R_{XT,R} \tag{3.13}$$

Where Q is the flow rate, $C_{S,B}$ is the substrate concentration in the buffer or recirculation tank, V_B is the volume of the buffer tank, and $R_{XT,R}$ was calculated using equations similar to Equations 3.7 and 3.8, without correction for biomass decay.

Computation procedure. The computation starts by introducing the parameter values, variables, and initial conditions described in Table 3.1 and proceeds as follows: 1) The substrate profile in the granule is calculated (Equation 3.5). 2) Based on this substrate profile, the biomass concentration in each segment of the particle (Equation 3.6), as well as the biomass concentration in the reactor after one time step (Equation 3.8) is calculated. 3) The efficiency factor for substrate conversion is calculated (Equation 3.10). 4) Then, the substrate and product concentrations in the reactor are integrated (Equations 3.11 to 3.13) by using the biomass calculated in step 2. This results in a new substrate concentration in the reactor. 5) The computation returns to step 1, and the loop stops until the substrate in the reactor is depleted.

Table 3.1 *Parameters, initial conditions, and variables in the reaction-diffusion model and the corresponding methods used for determination or literature source.*

	Units	Value	Source
Initial conditions			
$C_{S,R}(0)$	g S-COD/m^3		Estimated with the Monod model using the measured methane production rate data
$C_{S,B}(0)$	g S-COD/m^3		
$C_{X,R}(0)$	g X-COD/m^3		
$C_{XT,P}(0)$	g X-COD/m^3		Measured*
$C_{XT,R}(0)$	g X-COD/m^3		Measured ¤
$C_M(0)$	g CH_4-COD/m^3	0	
Parameters			
μ^{max}	d^{-1}	0.2	Measured
K_S	g S-COD/m^3	30	Measured
Ms	g S-COD/g X-COD.d	0.57	Measured
Y_{XS}	g X-COD/g S-COD	0.03	[14]
D_S	m^2/d	0.00013	[10]
V_R	m^3	0.00016	Measured
V_B	m^3	0.005	Measured
Variables			
Rp	m		Measured
V_{PT}	m^3		Calculated based on Rp
Q	m^3/d		Measured
k_l	m/d		Calculated based on an empirical relation valid for Reynolds number between 0 and $3 \cdot 10^5$ [16]
α	-		

*$C_{XT,P} = C^1_{XR} \cdot \rho_X \cdot \gamma_X$ where C^1_{XR} is the biomass concentration per unit of weight of wet sludge (g X-VSS/g sieved sludge), ρ_X is the density of sieved sludge ($1040 \cdot 10^3$ g sieved sludge/m^3 sieved sludge), and γ_X is the degree of reduction of biomass (1.36 g X-COD/g X-VSS).

¤$C_{XT,R} = \dfrac{MW_{XT} \cdot C^1_{XT} \cdot \gamma_X}{V_R}$ where MW_{XT} is the amount of sieved sludge (g) added to the reactor.

Results

Transport limitations in anaerobic granular sludge

The kinetics of acetate degradation by dispersed and granular methanogenic sludge were studied. The initial substrate ($C_{S,R}(0)$), and biomass concentration ($C_{X,R}(0)$), as well as the apparent K_S-value were estimated from the measured methane production rate as a function of time with Equations 3.1 to 3.3.

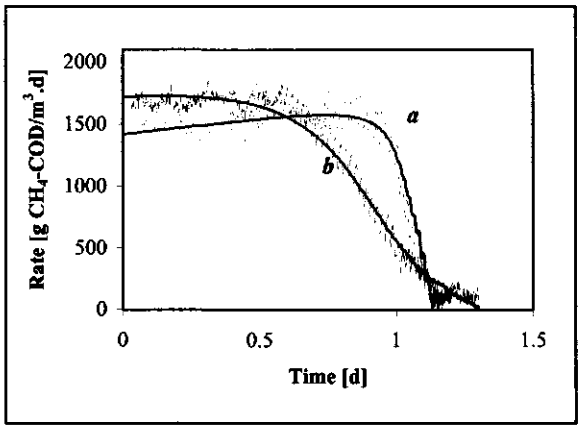

Figure 3.2 *Measured (thin lines) and calculated (thick lines) methane production rate of dispersed sludge (line **a**) and granular sludge (line **b**). The apparent K_S-values for the dispersed sludge and granules were 32 ± 10 and 208 ± 35 g COD/m^3, respectively.*

The apparent K_S-value represents the affinity for the substrate and strong changes on its value serve as an indication for substrate transport limitations.

The results from Figure 3.2 clearly show that the anaerobic intact granules were mass transport limited. The measured apparent K_S-values for dispersed and granular sludge were 32 ± 0.01 and 208 ± 0.035 g COD/m^3, respectively. The particles of the dispersed sludge were smaller than 200 μm, while the diameter of the granules was (3.5 ± 0.3) · 10^{-3} m.

Relative importance of internal and external mass transport

To determine the quantitative impact of respectively external and internal mass transfer, experiments were conducted at varying V_{up} and granule mean diameters. Experiments were conducted at variable V_{up} or variable granules mean diameter while keeping the other parameter constant. An example of the recorded methane production rate curves of such experiments is shown in Figure 3.3.

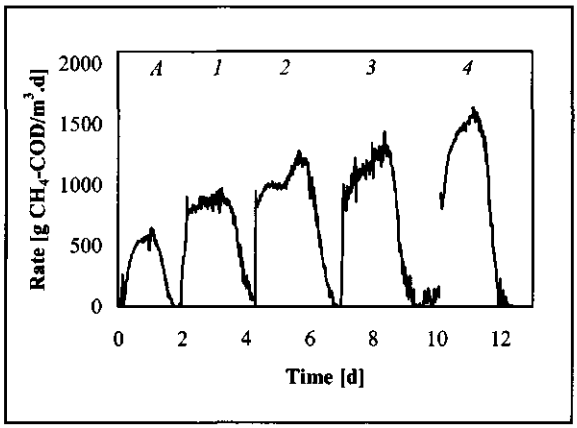

Figure 3.3 *Example of on-line measurement of the methane production rate. The run was performed with granules of 2.39±0.09 mm and liquid upflow velocity (V_{up}) of 3 m/h. During the tests, acetate was added at 2000 g S-COD/m^3 in four subsequent feedings (indicated by the numbers in the plot area). The letter A represents an acclimatization dose of ~500 g S-COD/m^3.*

From the methane production rate curves obtained, the apparent K_S-value was calculated by using the K_S as a fitting parameter. For this section, the K_S calculations were performed with the simple Monod model (i.e. without reaction-diffusion kinetics).

The effect of the liquid upflow velocity. The effect of different V_{up} on the apparent K_S-value of granules with a mean diameter of $(2.39 \pm 0.09) \cdot 10^{-3}$ m was studied. The results show that increased V_{up}-values did not have an impact on the K_S-value of anaerobic granular sludge, suggesting that the effect of external mass transfer is limited (Figure 3.4).

In subsequent feedings, a slight increase in the calculated K_S was observed. The maximum substrate consumption rate increases in time due to bacterial growth, which results in higher active biomass concentration in the granule. As a result of the increased substrate consumption rate, mass transfer limitations become more important, and consequently, the value of the apparent K_S -value in time (e.g. subsequent feedings) increases.

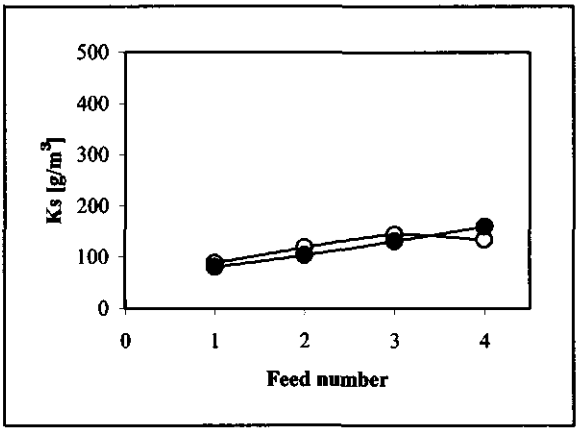

Figure 3.4 *Apparent* K_S*-values of granular sludge with a mean diameter of (2.39± 0.09) · 10^{-3} m, assessed at a* V_{up} *of 3 m/h (○), and 10 m/h (●).*

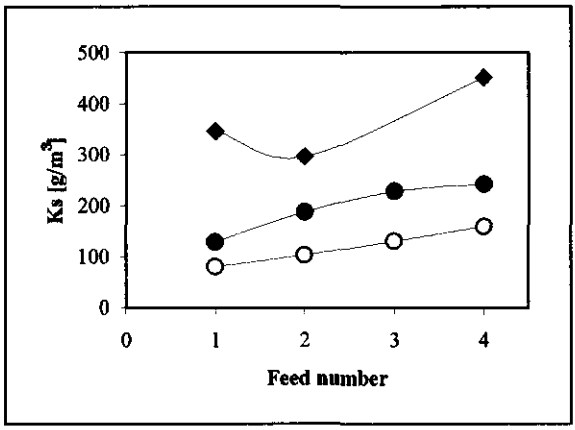

Figure 3.5 *Apparent* K_S*-values at* V_{up} *of 10 m/h for different biofilm sizes. The mean diameters of the granules were: (2.39 ± 0.09) · 10^{-3} m (○); (2.88 ± 0.26) · 10^{-3} m (●); and (3.52 ± 0.28) · 10^{-3} m (◆). A technical problem resulted in incomplete data during the third feed of one of the experiments,and the* K_S*-value could not be calculated.*

Effect of different granule diameter. The effect of the granule diameter on the apparent K_S was studied at a V_{up} of 10 m/h. The particle diameters tested were (2.39 ± 0.09) · 10^{-3} m, (2.88 ± 0.26) · 10^{-3} m, and (3.52 ± 0.28) · 10^{-3} m. At increasing granule diameter, the substrate consumption rate of the granules was decreased, as is reflected by an increase on the apparent K_S-value (Figure 3.5).

These results are clear indications for internal mass transport limitations in anaerobic granular sludge.

Biofilm model calculations

Using the data sets described above, we investigated if the biofilm theory could describe the experimental results (model validation). The procedure was the following: a) The measured methane production data were fitted by using the K_S-value as a fitting parameter (previous section). In this fitting procedure, also the initial biomass and substrate concentrations were estimated, and used as input for the biofilm model. b) Next, the same

data was fitted again, this time including the biofilm kinetic expressions in the model and setting the K_S constant at the value measured for dispersed sludge (30 g/m^3). Here, only the value of effective diffusion (α) was used as a fitting parameter.

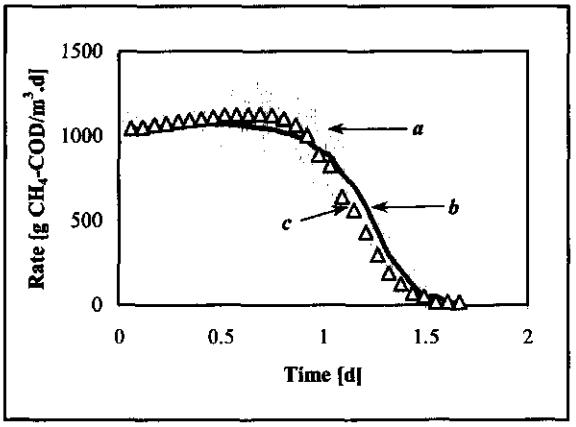

Figure 3.6 *Analysis of the methane production rate of granular sludge. The mean diameter of the granules was (2.88 ± 0.26) · 10^{-3} m, and the upflow velocity was 10 m/h.* ***a****) Measured data.* ***b****) Fitting by varying the* K_S*-value.* ***c****) Fitting with the biofilm kinetic theory and varying the effective diffusivity* α.

An example of the model running sequences is shown in Figure 3.6. The biofilm model was able to relate the reaction-diffusion dynamics inside the granules with the measured parameters of the system and the measured CH_4 production rate changes outside the biofilms. The α values for optimal description of the experimental data were similar for all the operational conditions tested (e.g. different granule diameter and different V_{up}). The values of α that resulted in an optimal description of the experimental results are shown in Table 3.2.

Table 3.2 *Values of the effective diffusivity* α *(-), that best described the methane production rate curves.*

Diameter (m)	**Upflow velocity (m/h)**		
	3	10	15
$2.39 \cdot 10^{-3}$	0.3 ± 0.1	0.3 ± 0.1	
$2.88 \cdot 10^{-3}$		0.4 ± 0.2	0.4 ± 0.2
$3.52 \cdot 10^{-3}$		0.3 ± 0.1	

Sensitivity analysis of the biofilm model

According to the film theory, the V_{up} would have a positive impact on the external mass transfer by reducing the stagnant liquid layer around the granule. This means that, at higher V_{up}, the external mass transfer coefficient (k_l) increases. On the other hand, the kinetic properties, and the size of the granule will be mainly related to the internal mass transport.

The relative importance of external and internal mass transfer limitations can be theoretically estimated. For this purpose, a sensitivity analysis of the biofilm model was made around the set point values indicated in Table 3.3.

Table 3.3 *Parameters used in the sensitivity analysis.*

Parameter	Set point value	Units
$C_{S,R}$	50	g S-COD/m^3
$C_{X,P}$	10000	g X-COD/m^3
K_S	30	g/m^3
r	0.001	m
k_l	1	m/d
α	0.3	-

Each parameter was varied in the range 0.1-2.0 times the set point value, keeping all other variables constant. The predicted efficiency for substrate conversion (η_S), or relative substrate uptake rate at steady state was used for comparison.

The simulations clearly show that C_S, R_P and $C_{X,P}$ have a high impact on the relative substrate uptake rate (Figure 3.7*a*), whereas K_S, k_l and α have a low impact (Figure 3.7*b*). These simulation outputs will be discussed in comparison with the experimental results in the next two sections.

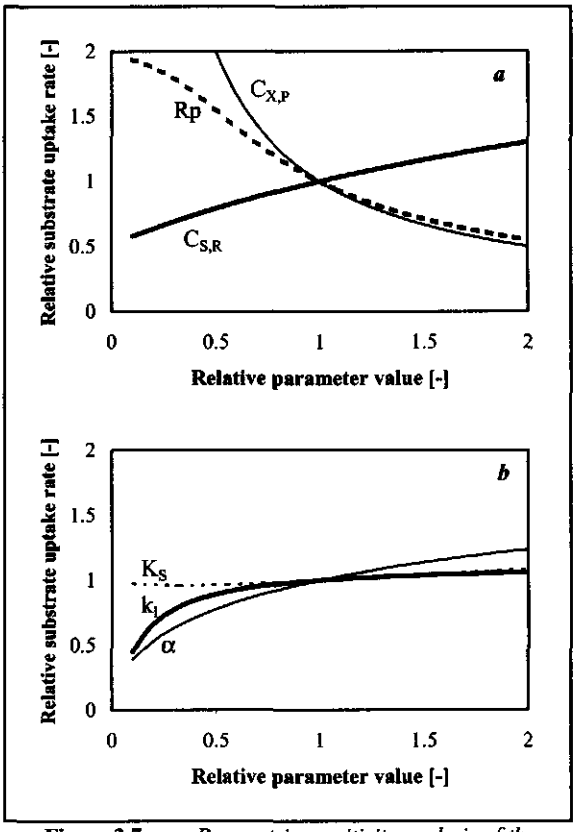

Figure 3.7 *Parametric sensitivity analysis of the model.* (*a*) *High sensitive parameters.* $C_{S,R}$ *is the substrate concentration in the reactor,* Rp *is the radius of the particle, and* $C_{X,P}$ *is the biomass concentration in the particle.* (*b*) *Low sensitive parameters.* K_S *is the Monod half-saturation constant,* k_l *is the external mass transfer coefficient, and* α *is the relative effective diffusivity.*

Discussion

External mass transfer limitations

The sensitivity analysis demonstrated that external mass transfer limitations may play an important role in anaerobic granular biomass at k_l values lower than 0.3 m/d (Figure 3.7*b*). This k_l value corresponds, according to an empirical relation valid for Reynolds from 0 to $3 \cdot 10^5$, to V_{up}-values lower than 1 m/h, suggesting that external mass transfer limitations are not important under the conditions normally encountered in anaerobic bioreactors.

There are several empirical relations to estimate k_l values, however, independent of the empirical relation used, the k_l values calculated for V_{up} of 1 m/h were in all cases higher than 0.3 m/d (Figure 3.8). We can conclude that the V_{up} would hardly influence the substrate conversion rate of anaerobic granules at values higher than 1 m/h. This corresponds well with our experimental results that showed that at increasing values of V_{up} the apparent K_S-values did not changed. This confirms that external mass transport resistance is normally not rate-limiting for anaerobic granular sludge.

According to literature, external mass transfer resistance can be neglected in denitrifying biofilters [8], and in acetate fed methanogenic fluidized bed [28], and packed bed reactors in aggregates up to 2 mm [21]. In other reports it has been suggested that liquid-film mass transfer resistance plays a strong role in substrate utilization in UASB reactor systems [6, 30]. However, the reduction of liquid-phase mass transfer resistance [31] and reduction on the K_S values at increasing V_{up}-values [17] in these studies may be attributed to enhanced mixing of the liquid and the sludge bed. The effect of the V_{up} may then be attributed to the reduction of preferential channeling of the influent wastewater and not to a direct effect on transport phenomena in the anaerobic biofilms. Furthermore, it is important to consider that the gas production will contribute more than the V_{up}, to the mixing in reactors. The gas production per unit surface area is directly related to the height of the reactor so that in tall and thin reactors the gas load (m^3/m^2) can be very high. This is mainly true in full-scale compared to lab-scale systems.

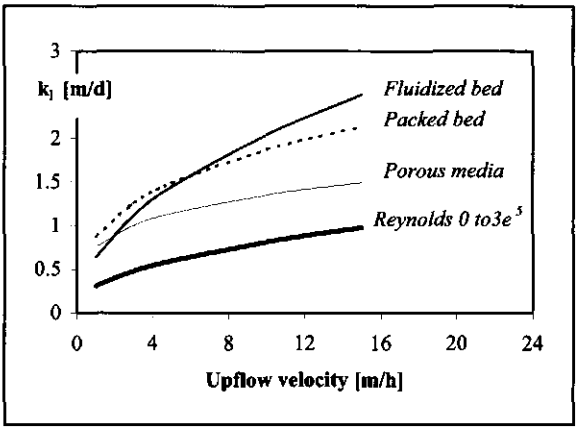

Figure 3.8 *Calculated external mass transfer coefficient-values* (k_l)*, as a function of the upflow velocity* (V_{up})*. For the calculations several empirical relations were used. In all cases the diameter of the biofilm was 3.5 · 10^{-3} m. The empirical relations to calculate the curves for fluidized and packed bed systems were taken from Kissel* [19]*. The curve for porous media was computed as described by Rittmann et.al.* [24]*, while the empirical relations to calculate the curve valid for Reynolds from 0 to 3 · 10^5 were taken from Hooijmans et. al.* [16].

Internal mass transport

The simulations of the sensitivity analysis show that the substrate concentration in the reactor (i.e. bulk), the radius of the granule, and the biomass concentration have a high impact on the relative substrate uptake rate (Figure 3.7*a*). The experimental results also showed that, in

contrast to the V_{up}, the diameter of the granules had a high impact on the apparent K_S. With increasing particle size, the K_S-value increased. This clearly indicates internal mass transport limitations of the anaerobic sludge.

Diffusion limitations have been reported for granular sludge from UASB systems [9, 30]. On the contrary, Alphenaar [2] found no relationship between the measured K_S and the granule diameter. However, in this study, a limited amount of biomass was active in the outer layer and the cores of the granules are reported to be empty. Schmidt and Arhing [25] did not observe differences on acetoclastic methanogenesis of disintegrated and intact granules. Likely, the small size of the granules in their study ($0.44 \cdot 10^{-3}$ m) played a role in the results.

Although it was clearly shown that anaerobic biofilms could be mass transport limited, if we considered, (i) the substrate concentrations commonly found in wastewaters, (ii) that the biofilm thickness experienced in most anaerobic systems is of mean diameters smaller than $1 \cdot 10^{-3}$ m [22], and (ii) the not very high specific methanogenic activity on acetate ranging from 0.4 to 0.6 g CH_4-COD/g VSS · d, [26] of most of granular sludge treating different waste streams, this suggests that in practice, diffusion limitations might not be of extreme importance. Hence the conversion of the rate limiting substrate (i.e. acetate) will be mainly controlled by its biological kinetics.

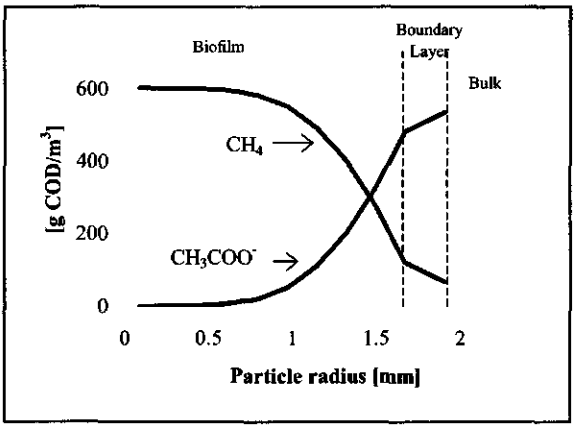

Figure 3.9 *Acetate* (CH_3COO^-), *and methane* (CH_4) *profiles in a granule. The methane concentration at saturation was calculated considering 70% of* CH_4 *in the headspace. The acetate profile that led to the estimation of the methane profile was obtained from the experimental data set at a* V_{up} *of 10 m/h, with granules of* $3.5 \cdot 10^{-3}$ *m, during a 2nd feed run, and from a specific kinetic time.*

Another aspect that should be considered in the biofilm conversion process is the formation of gaseous products. By assuming that (i) only molecular diffusion applies, (ii) that the diffusion coefficient for acetate is equal to that of CH_4, and (iii) that complete conversion of substrate into CH_4 occurs, then, it is possible to get an approximate estimation of the CH_4 profiles inside the granules. This estimation is shown in Figure 3.9.

The substrate profile that

consecutively led to the CH_4 profile was obtained as computed from one of the data sets already described. It can be seen from Figure 3.9 that at high granule diameters with a high active biomass concentration, steep substrate profiles, and consequently steep CH_4 profiles may develop. The high CH_4 concentration difference between the inner biofilm and the bulk liquid will create a high out-flux of CH_4. A gas with low aqueous solubility like CH_4 may reach supersaturated concentrations inside the anaerobic granules and under supersaturated conditions CH_4 gas bubbles may be formed.

It has been questioned how the formation and out-flux of the formed gas bubbles affects the activity of biofilms. In addition it has become speculative if microcurrent movements can be generated due to bubble movements and whether those currents in turn may cause substrate transport by convection [15]. Such situation would result in insignificant diffusional resistance.

To assess if transport other than diffusion (e.g. convection) would play a role in the kinetics of biofilms, the parameter α can be used as a tool. The value of α is expected to be rather constant for the same type of sludge, as long as it depends on the structure of the biofilm. The parameter α is defined as the ratio between the diffusion coefficient of substrate in the biofilm and in pure water. If the values of α that best describe the experimental results were different for different operational conditions (i.e. V_{up} or granule diameter), it would indicate that diffusion in the biofilm is being affected by those operational conditions. Furthermore, α values higher than 1 may also indicate that other types of mass transport (e.g. convection) may play a role in the conversion process.

In this study there was no evidence of convective flow inside the anaerobic biofilms. Internal diffusion resistance was evident and was not eliminated by the gas produced during the tests or by increased V_{up}. The α value that explained the experimental results were similar for all the experimental conditions tested, and no indications of enhanced substrate transport could be found. The substrate uptake could be satisfactorily explained only by diffusion in the biofilm. This experimental result confirms the generally accepted assumption that diffusion is the predominant transport process that takes place in anaerobic biofilms [21].

Nomenclature

α	relative effective diffusive permeability	-
μ^{max}	maximum growth rate	1/d
η_S	relative substrate conversion efficiency	-
γ_X	degree of reduction of biomass	g X-COD/g X-VSS
ρ_X	density of sieved sludge	g sieved sludge /m^3 sieved sludge
A^{max}	maximum specific methanogenic activity	g CH_4-COD/g VSS · d
$C_{M,R}$	methane concentration in the reactor	g CH_4-COD/m^3
COD	chemical oxygen demand	
$C_{S,B}$	substrate concentration in the buffer tank	g S-COD/m^3
$C_{S,P}$	substrate concentration in the particle	g S-COD/m^3
$C_{S,R}$	substrate concentration in the reactor	g S-COD/m^3
$C_{X,P}$	active biomass concentration in the particle	g X-COD/m^3
$C_{X,R}$	active biomass concentration in the reactor	g X-COD/m^3
$C_{XT,P}$	total biomass concentration in the particle	g X-COD/m^3
$C_{XT,R}$	total biomass concentration in the reactor	g X-COD/m^3
C^1_{XR}	biomass concentration per unit of weight of wet sludge	g X-VSS/g sieved sludge
D_S	diffusion coefficient of substrate in water	m^2/d
$f_{S,M}$	COD-based stoichiometry for the conversion of substrate into product (methane)	
k_d	decay coefficient	1/d
k_l	external mass transfer coefficient	m/d
K_S	Monod half-saturation constant	g S-COD/m^3
Ms	maintenance coefficient	g S-COD/g X-COD · d
PNo	number of particles in the reactor	
Q	recirculation flow rate	m^3/d

r	radius	m
Rp	radius of the particle	m
$R_{S,R}^{max}$	volumetric maximum substrate conversion rate in the absence of diffusion limitations	g S-COD/$m^3 \cdot d$
$R_{M,R}$	volumetric methane production rate in the reactor	g CH_4-COD/$m^3 \cdot d$
$R_{S,P}$	volumetric substrate conversion rate in the particle	g S-COD/$m^3 \cdot d$
$R_{S,R}$	volumetric substrate conversion rate in the reactor	g S-COD/$m^3 \cdot d$
$R_{X,P}$	volumetric biomass growth rate in the particle	g X-COD/$m^3 \cdot d$
$R_{X,R}$	volumetric biomass growth rate in the reactor	g X-COD/$m^3 \cdot d$
V_B	volume of the buffer tank	m^3
V_{PSeg}	volume of the particle segment	m^3
V_{PT}	total volume of the particle	m^3
V_R	volume of the reactor	m^3
VSS	volatile suspended solids	
V_{up}	liquid upflow velocity	m/h
Y_{XS}	yield coefficient	g VSS-COD/g COD

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4

Toxicity Effects of Formaldehyde on Methanol Degrading Sludge and its Anaerobic Conversion in Biobed EGSB reactors

Methanogenesis from formaldehyde mainly occurred via intermediates as confirmed by the increased concentrations of methanol and hydrogen in the liquid and gas phases, respectively during formaldehyde conversion. While formaldehyde was readily transformed, the methane production rate was immediately and strongly inhibited. Formaldehyde toxicity was in part reversible since the methane production rate recovered after formaldehyde depletion. This recovery can not be explained by biomass growth. The toxicity of formaldehyde was also in part irreversible, since the degree of recovery was not complete. This loss in the methane production rate likely can be attributed to biomass decay as suggested by its linear relation with the amount of formaldehyde dosed, as well as by scanning electron microscopy observations. The addition of the same amount of formaldehyde either in a slug or in a continuous mode caused the same loss in the methane production rate. Thus for the treatment of formaldehyde-containing streams, a balance between formaldehyde-related decay and bacterial growth should be attained. By combining good biomass retention and internal dilution of the wastewater, industrial streams containing formaldehyde can still be treated anaerobically[✓].

[✓] A modified version of this chapter was published in *Wat. Sci. Tech.* (1999), **40(8)**: 195-202.

Introduction

The limited knowledge with respect to the susceptibility of methanogenic sludge to toxic compounds still hampers the applicability of anaerobic treatment for industrial wastewaters. This is particularly important whenever the substrate consists of a mixture of toxic and non-toxic compounds like in the case of methanol and formaldehyde mixtures. Formaldehyde is frequently encountered in industrial waste-streams; its concentration in wastewaters from chemical industries can be as high as 10 g/l. Due to its mutagenic and carcinogenic effects [5], direct discharges of those streams threaten life in the receiving water. Data concerning formaldehyde toxicity from previous research is difficult to extrapolate since there is no understanding of the mechanisms of toxicity as well as its biodegradation. Furthermore it is not clear from literature whether formaldehyde was directly converted to methane, or its dismutation products (i. e. methanol, formic acid) are the actual substrate for methane formation. In previous studies except those from Qu and Battacharya [11], there are no measurements of formaldehyde, hence its fate is not clear. Controversies exist with respect to the non-toxic concentrations as well as whether formaldehyde toxicity is avoided by continuous dosing instead of slug dosing. Hickey et al. [6] showed that in batch systems, formaldehyde was toxic from 10-100 mg/l; recovery of the methanogenic activity was observed for all doses except for 100 mg/l. Lu and Hegemann [8] reported a 50% inhibition at 300 and 150 mg/l with glucose and glue wastewater. In continuous systems, Sharma et al. [13] reported that biomass support particles can resist formaldehyde toxicity up to 375 mg/l (influent concentration) with sludge retention time (SRT) higher than 200 d. Parkin et al. [9, 10] stated that continuous addition of 400 mg/l of formaldehyde to a chemostat did not cause failure of the system at a SRT of 50 d, but a slug dose of 150 mg/l caused immediate acetate accumulation. Furthermore, it is reported that a chemostat with a SRT of 14 d and a VS-concentration of 444 mg/l can tolerate influent formaldehyde concentrations of 1110 mg/l with a 99% formaldehyde removal efficiency [11].

The objective of this study was to investigate the fate of formaldehyde in anaerobic bioreactors. Three main aspects were considered. The first one was to investigate whether formaldehyde or its dismutation products were the actual substrates for methanogenesis. Secondly, whether formaldehyde toxicity was reversible or irreversible. And third, if formaldehyde toxicity was load or concentration dependent.

Materials and methods

Inoculum. Anaerobic granular sludge from a full scale expanded granular sludge bed reactor, developed by Biothane Systems (Biobed® EGSB) was used. The sludge treated the wastewater generated in a chemical factory that produces formaldehyde out of methanol (Caldic Europoort, The Netherlands). The waste stream contains primarily methanol and formaldehyde as organic substrates. The mean diameter of the sludge was determined by an image analysis technique [7].

Formaldehyde conversion. The conversion of formaldehyde was followed in 250-ml serum bottles according to the method described by Florencio et al. [3]. The biomass concentration used (previously activated with methanol) was 0.8 g VSS/l. Formaldehyde was added as carbon source from a solution containing 37% formaldehyde. This solution contained also 10% of methanol. Formaldehyde, methanol, as well as methane and hydrogen in the headspace were measured over time.

Formaldehyde toxicity studies at low biomass concentration (increasing rate, i.e. growth). These studies were done in 2.5 l continuously stirred batch reactors. The reactors were inoculated with a sludge suspension to a final concentration of 1.4 g VSS/l. Methanol was added as carbon source at an initial concentration of 10 g COD/l. The mineral medium used contained the following components in mg/l demineralized water: NH_4Cl 802, KH_2PO_4 952, $MgSO_4.6H_2O$ 912, $CaCl_2$ 556, $NaHCO_3$ 3000, $Na_2S.8H_2O$ 120, H_3BO_3 0.25, $ZnCl_2$ 0.25, $CuCl_2.2H_2O$ 0.076, $MnCl_2.4H_2O$ 2.5, $(NH_4)_6Mo_7O_{24}.4H_2O$ 0.2, $AlCl_3.6H_2O$ 0.45, $Na_2SeO_3.5H_2O$ 0.82, $FeCl_2.4H_2O$ 7.96, $CoCl_2.6H_2O$ 9.52, $NiCl_2.6H2O$ 9.52 and yeast extract 100. Since higher metal additions can be required during exponential increase of the CH_4 production rate by methylotrophic-methanogens, the medium used here was based on a previous study [4]. Formaldehyde was dosed in a slug mode. Its addition was done after approximately 2 g of methanol COD/l was consumed. At this moment, all the reactors had the comparable methane production rate.

Toxicity studies at high biomass concentration (initial constant maximum rate, i.e. activity). These studies were performed in a 4 l EGSB-reactor inoculated with granular sludge at a final concentration of 23 g VSS/l. The reactor was continuously operated at a liquid upflow velocity of 6 m/h with a volumetric load of 10 g of methanol COD/l.d. For each toxicity test, the reactor was operated in a batch-recirculated mode. Methanol was added as carbon source at an initial concentration of 5 g COD/l. The mineral medium used contained the following components in mg/l: NH_4Cl 1392, KH_2PO_4 226, $(NH_4)_2SO_4$ 226, $MgCl_2.6H_2O$ 200, KCl 360, $NaHCO_3$ 3000, H_3BO_3 0.0064, $ZnCl_2$ 0.0064, $CuCl_2.2H2O$ 0.0049, $MnCl_2.4H_2O$ 0.064, $(NH_4)_6Mo_7O_{24}.4H_2O$ 0.0064, $AlCl_3.6H_2O$ 0.0115, $Na_2SeO_3.5H_2O$ 0.02,

$FeCl_2.4H_2O$ 0.256, $CoCl_2.6H_2O$ 0.256, $NiCl_2.6H_2O$ 0.018 and yeast extract 26. Formaldehyde was added either in a slug or in a continuous mode. The toxicant was dosed when a constant methane production rate was achieved. At this moment, less than 3 g of methanol-COD/l had been consumed.

Analytical methods. The methanol and formaldehyde contents, hydrogen in the headspace and on-line monitoring of the methane production rate were assessed as indicated elsewhere[4]. Intact and cross sectioned (cross sectioned with a scalpel) granules were prepared and analyzed for scanning electron microscopy as described previously [7]. All assays were performed in duplicate.

Results and Discussion

Formaldehyde conversion

In this experiment, formaldehyde conversion was followed in serum bottles. Formaldehyde was added as the only carbon source (plus methanol, as indicted in the materials and method section) at different initial concentrations. It was observed that part of the formaldehyde dosed was readily transformed into methanol (Figure 4.1*b* and 4.1*c*).

At initial formaldehyde doses of 200 and 600 mg COD/l, all substrate-COD could be recovered as methane COD (Figure 4.1*a* and 4.1*b*). At an initial dose of 1400 mg COD/l, formaldehyde was highly toxic and no methane production was observed. However, the conversion of formaldehyde into methanol was not inhibited (Figure 4.1*c*). During formaldehyde conversion, the hydrogen concentration in the headspace increased. The maximum hydrogen peak measured was directly related to the amount of formaldehyde dosed; 50, 28 $\times 10^3$, 43 $\times 10^3$, and 150 $\times 10^3$ ppm when formaldehyde was added at 0, 200, 400 and 600 mg/l, respectively.

Likely formaldehyde was first oxidized to formate and then reduced to methanol, with intermediate accumulation of hydrogen. Considering that all formaldehyde is converted into methanol and formate the following reactions are proposed:

Oxidation $HCHO + H_2O \rightarrow HCOOH + H_2$

Reduction $HCHO + H_2 \rightarrow CH_3OH$

Total $2HCHO + H_2O \rightarrow CH_3OH + HCOOH$

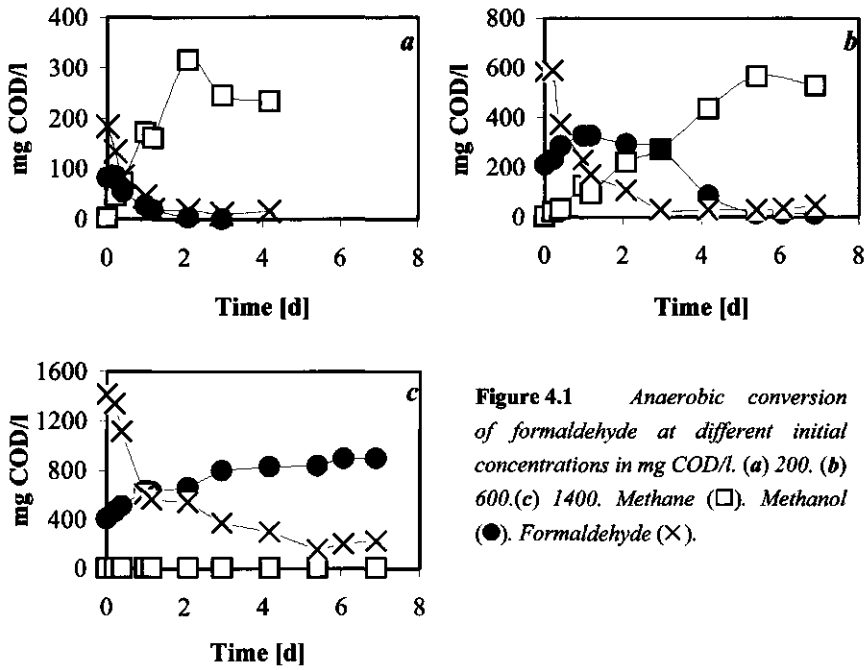

Figure 4.1 *Anaerobic conversion of formaldehyde at different initial concentrations in mg COD/l.* (*a*) *200.* (*b*) *600.*(*c*) *1400. Methane* (□). *Methanol* (●). *Formaldehyde* (×).

According to the above stoichiometry, experimentally, only ≈ 55-75 % of the expected methanol was formed (Figure 4.1*c*). Since the balance is not completely closed, it is likely that some of the formaldehyde reacted chemically with cell components. Furthermore, it is also possible that some of the formaldehyde was adsorbed on the biomass [1]. A similar oxidation-reduction mechanism has been reported in which the oxidation of formaldehyde by cell-free extracts of *Methanobacterium thermoautotrophicum* can provide the reducing power for the reduction of formaldehyde to methane [2]. At a formaldehyde dose of 1400 mg-COD/l, the reaction rate of formaldehyde conversion decreased in time (Figure 4.1*c*). This suggests that some enzymes (e.g. dehydrogenase) and/or cofactors can become inactivated due to the excess of formaldehyde. The limitation of coenzymes can be particularly important at high formaldehyde doses as suggested by Romesser and Wolfe [12]. On the other hand, Qu and Bhattacharya [11] concluded that formaldehyde degradation followed the Monod pattern. However, this was only true at initial formaldehyde concentrations not higher than 30 mg/l.

Formaldehyde toxicity at low biomass concentration

The effect of slug doses of formaldehyde on the methane production rate by methanol degrading sludge was studied in batch systems. Due to the low initial amount of biomass an exponential increase of the methane production rate was observed (i.e. growth was not negligible). The results are shown in Figure 4.2. At a formaldehyde dose of 10 mg/l, there was no effect on the rate curves (data not shown). However, formaldehyde concentrations of 50 mg/l and higher caused an immediate and sharp decrease of the methane production rate.

The time required to reach the maximum methane production rate after formaldehyde dosage was related to the concentration of the toxicant dosed. From the curves depicted in Figure 4.2, three phases can be described. 1) Exponential increase of the methane production rate. 2) Dosage of formaldehyde resulting in a sharp drop of the methane production rates likely due to biomass deactivation and/or biomass decay and 3) Recovery of the methane production rate.

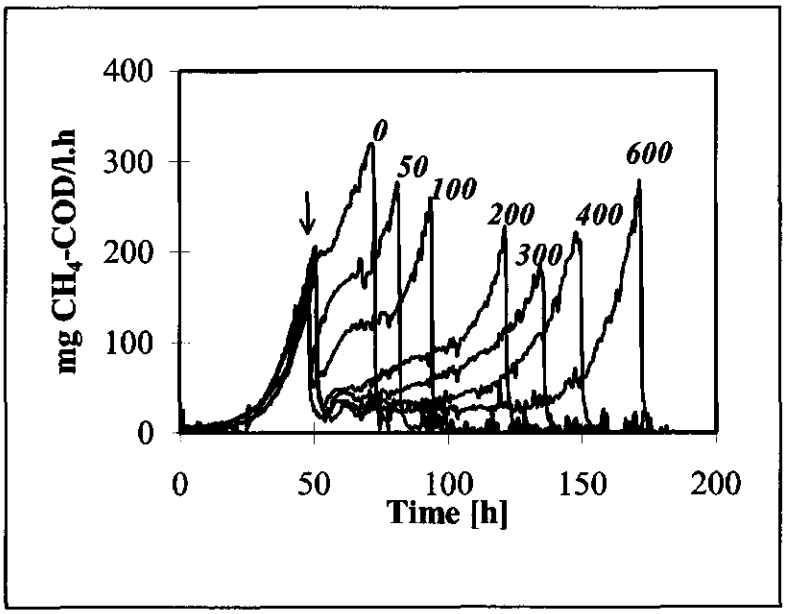

Figure 4.2 *Effect of a slug dose of formaldehyde on the methane production rate at low initial biomass concentration. The numbers on top of each curve indicate in mg/l the concentration of formaldehyde dosed and the arrow indicates the time of addition.*

Even at formaldehyde doses of 600 mg/l, the methane production rate did not cease completely. Apparently a small fraction of the activity remained since methanol was consumed during the flat part of the recovery period (data not shown). However, although formaldehyde was immediately converted, growth was observed only after 100 h. The physiological meaning of this observation remains to be elucidated.

During the experiment, formaldehyde and hydrogen were measured in the liquid and gas phases, respectively. These results are shown in Figure 4.3. It was observed that although the methanogenesis was strongly inhibited (Figure 4.2), formaldehyde was immediately transformed without any lag (Figure 4.3*a*). Immediately after the addition of formaldehyde, the hydrogen concentration in the headspace increased (Figure 4.3*b*).

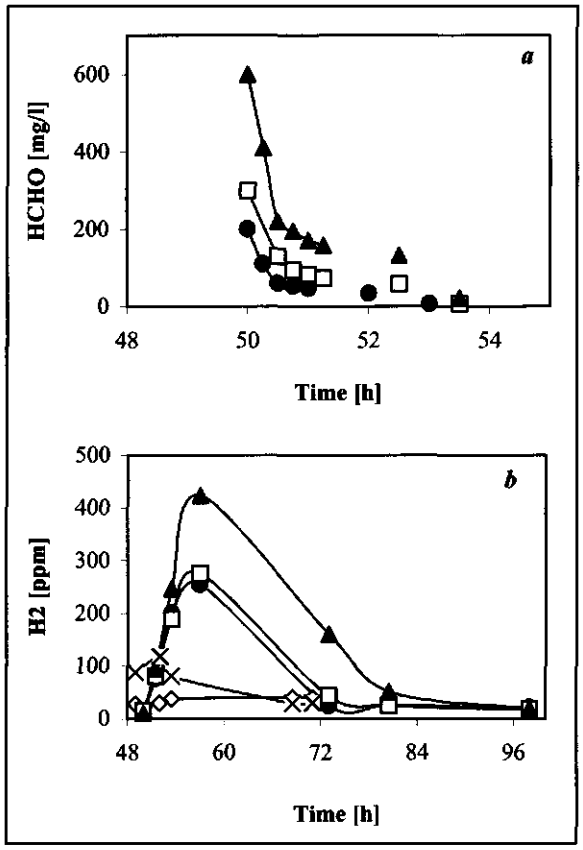

Figure 4.3 (*a*) *Formaldehyde depletion and* (*b*) *hydrogen production during slug doses of formaldehyde to methanol consuming sludge. Note that the panels have different time scales. Slug dose in mg/l: 600* (▲); *300* (□); *200* (●); *100* (×); *0* (◊).

However, the maximum hydrogen concentration did not correspond to the maximum rate of formaldehyde conversion suggesting mass transfer limitation from the liquid to the gas-phase. Nonetheless, the maximum concentration of hydrogen measured in the gas phase was dependent on the amount of formaldehyde dosed, but the hydrogen concentration on COD based balances was negligible.

Low concentrations of methanogenic biomass were able to recover even at formaldehyde additions as high as 600 mg/l. Assuming exponential growth, the calculated specific growth rate (μ)

for all the curves before formaldehyde dosing was 0.092 h^{-1}. After dosing and during the exponential increase of the methane production rate, the calculated μ-values ranged from 0.03 to 0.07 h^{-1}. In these experiments, the observed recovery can likely be attributed mainly to bacterial growth. The long period (≈ 100 h) between formaldehyde depletion and the start of the recovery at a formaldehyde addition of 600 mg/l can physiologically not be explained yet.

Formaldehyde toxicity at high biomass concentration

Slug addition. The effect of slug doses of formaldehyde on the methane production rate by methanol degrading sludge was studied in an EGSB reactor operated in batch mode. The initial amount of biomass was high, and consequently bacterial growth could be neglected during the tests. The toxic compound was dosed when a maximum and constant methanol conversion rate was achieved as reflected by a constant methane production rate. After formaldehyde addition, the methane production rate was immediately inhibited, but formaldehyde conversion proceeded without a lag-period as has been shown previously (Figure 4.1*c*). For all the concentrations tested, formaldehyde was removed within two hours after dosage (data not shown). Like in the experiments with low biomass concentration, the hydrogen concentration in the biogas increased, but its amount on COD basis was negligible. After a slug addition of formaldehyde, 4 phases in the methane production rate curve could be observed as indicated in Figure 4.4*a*. The results of the slug doses of formaldehyde showed strong inhibition at all the concentrations tested. The methane production rate dropped immediately and sharply after the addition of formaldehyde. The slope of the inhibition (phase II) was similar for all tests. However, the slopes of the recovery part (phase III) as well as the maximum rate obtained after the addition of the toxicant (phase IV) were dependent on the amount of the formaldehyde dosed. The loss in the maximum rate of methane production between phase I (Ro) and phase IV (Re), equal to Ro-Re, was directly related to the concentration of formaldehyde dosed as shown in Figure 4.4*b*.

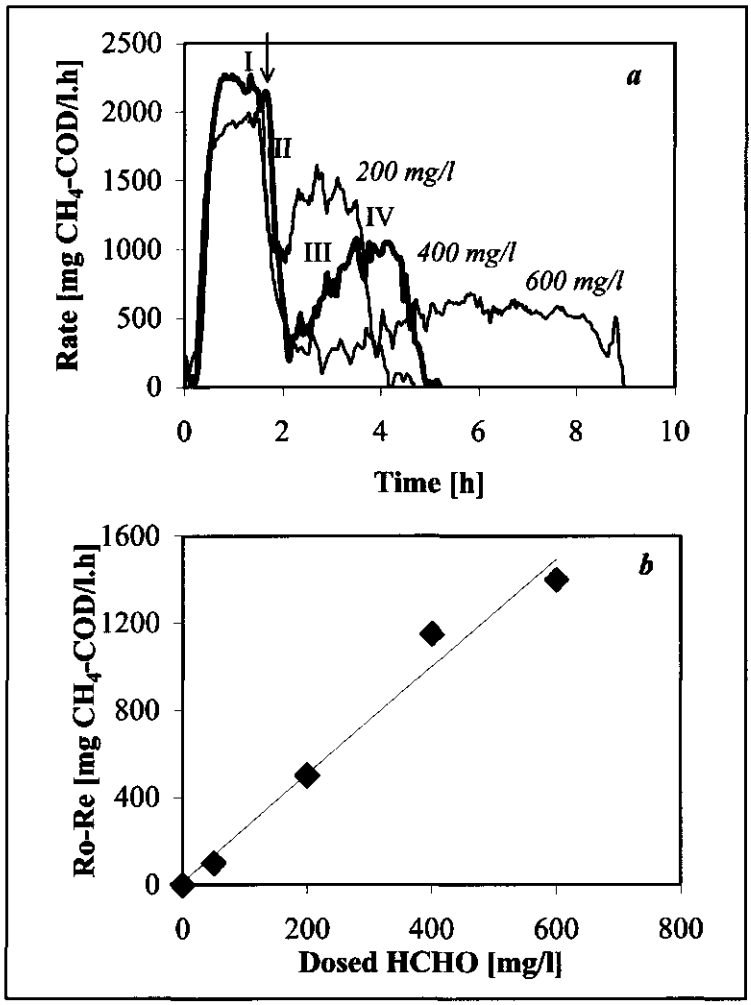

Figure 4.4 (*a*) *Effect of slug doses of formaldehyde on the methane production rate of methanol degrading sludge. The arrow indicates the time of addition. Several phases are observed as indicated for the 400 mg/l curve:* (I) *Maximum methane production rate before dosing.* (II) *Inhibition.* (III) *Recovery.* (IV) *Maximum methane production rate after dosing.* (*b*) *Loss in the maximum rate of methane production between phase* I (Ro) *and phase* IV (Re), *equal to* Ro-Re, *at different formaldehyde doses.*

These results clearly show that formaldehyde toxicity was in part reversible (phase III Figure 4.4*a*) since the methane production rate recovered after formaldehyde conversion. This temporary de-activation was concentration dependent. Contrary with the low biomass experiments, here the observed recovery can not be attributed to bacterial growth as shown in Table 4.1. Assuming exponential growth with a μ of 0.092 h^{-1} (as observed in the previous section), the elapsed time from phase III to phase IV would have lasted longer than what was observed in Figure 4.4*a*. Formaldehyde toxicity was also in part irreversible since the degree of recovery was not complete (phase I compared to phase IV). This irreversible toxicity seemed to have a linear relation with respect to the dosed formaldehyde. The irreversibility reflected by the loss (Ro-Re) in the methane production rate likely can be attributed to biomass decay.

Table 4.1 *Comparison between the observed elapsed time and the calculated elapsed time assuming growth for the recovery phase in Figure 4.4a. A specific growth rate of 0.092 h^{-1} was assumed.*

Dosed HCHO (mg/l)	Average rate start phase III (mg CH_4-COD/l.h)	Average rate at phase IV (mg CH_4-COD/l.h)	Elapsed time from phase III to phase IV assuming growth (h)	Elapsed time observed (h)
200	900	1400	4.8	0.5
400	400	1000	9.9	1.5
600	250	600	9.5	3

Continuous addition. The effect of continuous dosage of formaldehyde was studied to assess whether the irreversible toxicity of formaldehyde was dependent on its concentration in the bulk or on the total amount of formaldehyde dosed (i.e. load). Formaldehyde was dosed continuously during one hour at rates of 200 and 400 mg/l.h. Its dosing started after a constant and maximum methane production rate was attained. The results are shown in Figure 4.5. It was observed that although the formaldehyde concentration in the liquid phase was low (below 7 and 50 mg/l for the test at 200 and 400 mg/l.h, respectively), the methane production rate was gradually decreasing. The final decrease of the rate in the continuous experiments was similar to that of the slug tests (Figure 4.5). This implicates that the addition of the same amount of formaldehyde either in a slug or in a continuous mode caused the same loss in the methane production rate.

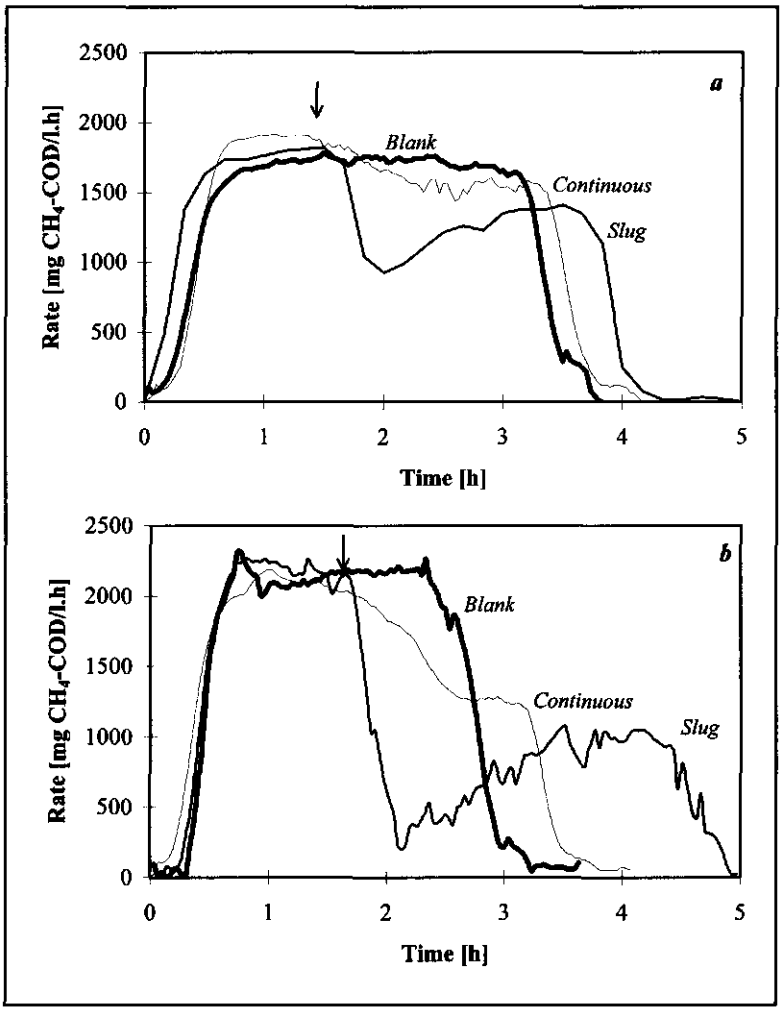

Figure 4.5 *Effect of continuous dosing of formaldehyde on the methane production rate by methanol consuming sludge. For comparison, the curves obtained with slug doses as well as blank curves are plotted. The arrow indicates the dosing of the toxicant either in continuos or slug mode. The continuous doses lasted 1h.* (***a***) *Continuous dosing of 200 mg/l.h and slug dose of 200mg/l.* (***b***) *Continuous dosing of 400 mg/l.h and slug dose of 400 mg/l.*

This loss in the rate during the continuous dosing suggests an irreversible toxicity, likely due to biomass decay. SEM of the sludge showed that the outer part of the granules was mainly composed of *Methanosarcina*-type bacteria (Figure 4.6*a*). Observations on the surface of the

granules showed noticeable amounts of lyzed bacteria (Figure 4.6*b*) suggesting enhanced bacterial decay due to formaldehyde.

The results of the continuous dosing clearly show that independent of the type of dose mode (i.e. slug or continuous), a certain amount of formaldehyde reduces the methane production rate to a certain extent. Thus for the treatment of formaldehyde-containing streams, a balance between formaldehyde-related decay and bacterial growth should be attained.

Since the irreversible toxicity was found to be dependent on the load, this suggests that it would also be independent of the reactor configuration and/or mode of operation. However, it is possible that a kind of irreversible toxicity-concentration threshold exists.

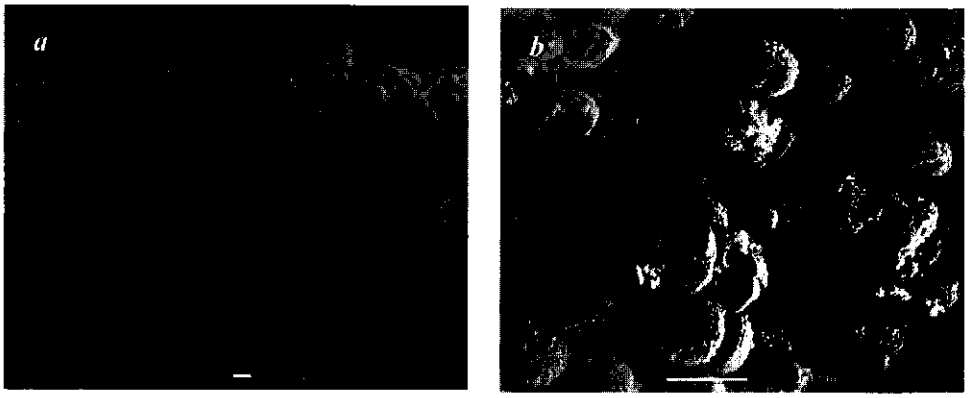

Figure 4.6 *(a) Outer layer of a cross-sectioned granule showing mainly Mthanosarcina-type bacteria. (b) Surface of the granule showing lyzed Methanosarcina type cells.Bars indicate 1 μm.*

Full scale treatment of methanol-formaldehyde wastewater

From the previous results, it is clear that the ratio formaldehyde/methanol is important since it will determine the net growth of the methanogenic population. The formaldehyde-related decay should be compensated by growth. Thus industrial streams containing formaldehyde can still be treated anaerobically by providing good biomass retention. Biothane has successfully implemented the treatment of wastewater containing a mixture of methanol and formaldehyde in their EGSB Biobed® reactor. The reactor tolerates very high liquid upflow velocities without biomass wash-out (the sludge settling velocity amounts approximately 50

m/h). The performance of this reactor has been described previously [14]. A general characterization of the sludge that developed in such a system showed that the mean diameter of the granules was 2.2 ± 1 mm and that they were mainly composed by *Methanosarcina*-type bacteria as shown in Figure 4.6*a*. If formaldehyde is mainly transformed in the outer layers of the biofilm, and there exists a threshold concentration for the irreversible toxicity, then the granular structure of EGSB sludge may provide protection to the inner biomass, conferring more stability to the system compared with dispersed biomass ones. Interestingly lized *Methanosarcina*-type bacteria were only observed on the surface of the granules.

Conclusions

Formaldehyde conversion proceeded via intermediate formation of methanol and hydrogen (and probably formate) in the liquid and gas phase, respectively.

At low concentrations of methanogenic biomass, the methane production rate can be recovered even at formaldehyde additions as high as 600 mg/l. This recovery after formaldehyde depletion can probably be attributed to bacterial growth.

At high biomass concentrations, formaldehyde toxicity was in part reversible since the methane production rate recovered after formaldehyde conversion. This observed recovery can not be explained by bacterial growth. Since the degree of recovery was not complete, we conclude that formaldehyde toxicity was also in part irreversible. This loss in the methane production rate likely can be attributed to biomass decay as suggested by its linear relation with respect to the amount of formaldehyde dosed as well as by SEM observations.

Independent of the type of dose mode (i.e. slug or continuous), a certain amount of formaldehyde reduces the methane production rate to a certain extent. Thus for the treatment of formaldehyde-containing streams, a balance between formaldehyde-related decay and bacterial growth should be attained.

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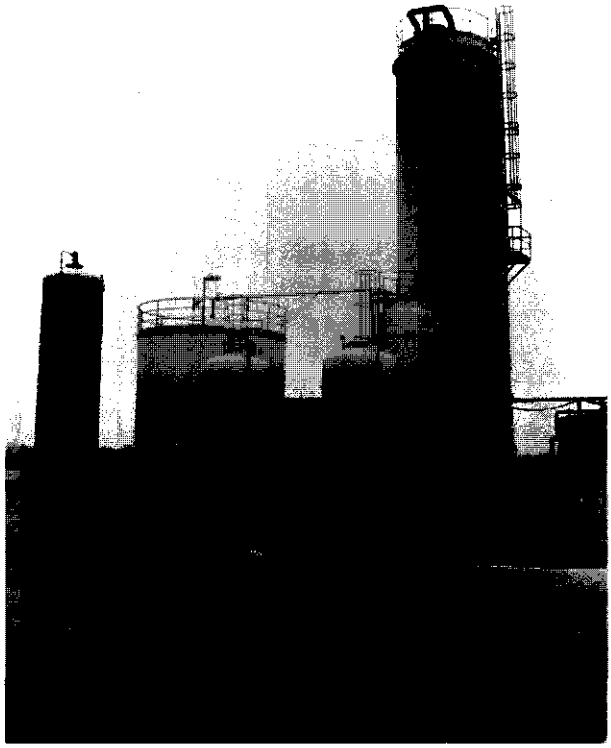

Picture. Full scale expanded granular sludge bed (EGSB) reactor treating a chemical wastewater containing as main components methanol and formaldehyde.

Successful implementation of anaerobic treatment to toxic biodegradable waste streams can be achieved through the understanding of the mechanisms of toxicity and biodegradability of the target compounds.

5

Conversion and Toxicity Characteristics of Formaldehyde in a Acetoclastic Methanogenic Consortium

An unadapted mixed methanogenic sludge transformed formaldehyde into formic acid and methanol. The $CH_3OH/HCHO$ ratio obtained were 0.7/2 to 0.9/2. Formaldehyde conversion proceeded without any lag phase suggesting the constitutive character of the formaldehyde conversion enzymes involved. Since the rate of formaldehyde conversion declined at increased formaldehyde additons, we hypothesize that some enzymes and/or cofactors might become denatured due to the excess of formaldehyde. Furthermore, formaldehyde was toxic to acetoclastic methanogenesis in a dual character. Formaldehyde toxicity was in part reversible since once the formaldehyde concentration was extremely low or virtually removed from the system, the methane production rate was partially recovered. Since the degree of this recovery was not complete, we conclude that formaldehyde toxicity was also irreversible. The irreversible toxicity likely can be attributed to biomass formaldehyde-related decay. Independent of the mode of formaldehyde addition (i.e. slug or continuous), the irreversible toxicity was dependent on the total amount of formaldehyde added to the system. This finding suggest that in order to treat formaldehyde containing waste streams a balance between formaldehyde-related decay and biomass growth should be attained[□].

[□] To be submitted for publication.

Introduction

Because of its relatively low costs and chemical reactivity, formaldehyde has become one of the world's most important industrial and research chemicals.

The main use of formaldehyde is in the production of amino and phenolic resins. About 80% of the slow-release fertilizer market is based on urea-formaldehyde containing products. Formaldehyde is also used in the manufacture of acetal resins and permanent-press finishes of cellulosic fabrics. It is furthermore used as an industrial raw material for the production of diverse derivatives [5].

Formaldehyde can enter the environment through exhaust from gasoline and diesel combustion and via discharge of waste streams from diverse chemical industries. The formaldehyde concentrations of industrial waste streams can range from 0.2 to as high as 10 g/l [11, 17]. Since it was shown that formaldehyde is a mutagenic and carcinogenic agent [7], direct discharge of those chemical effluents is prohibited in view of the fact that they threaten life in the receiving water.

Anaerobic technology can be an attractive option for the treatment of formaldehyde containing waste streams, although so far high-rate anaerobic reactors mainly have been successfully applied for the treatment of medium to high-strength non-complex wastewaters [10]. The implementation of anaerobic technology to waste streams from the chemical industry is still scarce mainly due to the lack of understanding of the kinetics and mechanisms of toxic compounds to anaerobic systems. Proper insights in these aspects are a prerequisite for rational design, and reliable monitoring and control of the process.

Concerning data on the toxicity of formaldehyde, controversies exist and available information is insufficient for non-empirical design and control purposes. It is not clear from literature whether formaldehyde is converted directly to methane or if products of its chemical dismutation are the actual substrates for methanogenesis. Furthermore it is not clear under which conditions toxicity can be avoided, Hickey et al. [8] reported that formaldehyde is already toxic at 10 mg/l, but recovery occurred except when doses of 100 mg/l or more were used. Other researchers report 50% inhibition values of 125-300 mg/l [11, 12]. In the case of continuous reactor systems there is no consensus in the available literature dada. Continuous reactors with immobilized biomass were found to resist toxicity of formaldehyde up to influent concentrations of 375 mg/l with sludge retention time (SRT) higher than 200 d [16]. On the other hand, chemostats and anaerobic filters could tolerate 400 mg/l at a SRT of 50 d [3, 13]. Qu and Battacharya [14]. found that a chemostat could treat influent

formaldehyde concentrations as high as 1110 mg/l at a SRT of 14 d. None of the studies conducted so far explain the mechanisms of formaldehyde toxicity and/or biodegradation.

In the present study, we assessed the fate of formaldehyde in anaerobic systems. We furthermore, investigated whether formaldehyde toxicity was reversible or irreversible and whether the toxicity was load or concentration dependent. Based on the results obtained, the practical implications for the treatment of formaldehyde containing wastewaters are presented.

Materials and methods

Inoculum. Anaerobic granular sludge from a full scale expanded granular sludge bed (EGSB) reactor (Heineken, Zoeterwoude, The Netherlands) treating brewery wastewater was used. Dispersed granular sludge was used in all experiments except otherwise stated. The granular structure was disrupted with a blender (Braun 4164).

Formaldehyde conversion. The conversion of formaldehyde as sole carbon source was followed in 300 ml serum bottles with a liquid volume of 150 ml. The mineral medium used contained the following components in g/l of demineralized water: $NaHCO_3$ 4.0, NH_4Cl 0.28, K_2HPO_4 0.25, $MgSO_4.7H_2O$ 0.1, $CaCl_2.2H_2O$ 0.01, yeast extract 0.1, trace elements 1ml/l. The trace elements solution contained the following components in mg/l of demineralized water: $FeCl_2.4H_2O$ 2000, H_3BO_3 50, $ZnCl_2$ 50, $CuCl_2.2H_2O$ 38, $MnCl_2.4H_2O$ 500, $(NH_4)_6Mo_7O_{24}.4H_2O$ 50, $AlCl_3.6H_2O$ 90, $CoCl_2.6H_2O$ 2000, $NiCl_2.6H_2O$ 142, $Na_2SeO.5H_2O$ 164, EDTA 1000, Resazurine 200, HCl 36% 1 ml/l. Medium and dispersed sludge (3.5 g VSS/l) were added to the serum bottles. The bottles were sealed with butyl rubber septa and aluminum screw caps and the headspace was flushed with a mixture of N_2 and CO_2 (70:30 [vol/vol]). To activate the sludge, 1 g acetate COD/l was added and the bottles were incubated at 30°C in a shaker. The methane production was measured over time. Once all the acetate was consumed as indicated by the methane production data, the serum bottles were flushed again with the N_2/CO_2 mixture. To perform abiotic tests, a number of bottles containing sludge were autoclaved (121°C at 1.5 bar) during 1 h. Possible reactions with medium components was checked in batches with medium without of sludge addition.

The formaldehyde conversion experiments were started by addition of the desired amount of formaldehyde. Formaldehyde was added as a sole carbon source from a stock solution prepared by adding 0.5 g of paraformaldehyde in 5 ml demineralized water. This solution was heated at 100°C during 12 h. The resulting formaldehyde concentration in the stock solution

was ≈ 90 g/l (3 M). Formaldehyde, formate, and methanol in the liquid phase, as well as methane and hydrogen in the headspace were measured in time.

Formaldehyde toxicity studies. These studies were conducted in 2.5 l continuously stirred batch reactors. The reactors were inoculated with a sludge suspension to a final concentration of 1.5 to 3 g VSS/l. The medium used contained the following components in g/l of demineralized water: NH_4Cl 0.28, $MgSO_4.7H_2O$ 0.1, KH_2PO_4 6.79, $Na_2HPO_4.2H_2O$ 8.89, yeast extract 0.1, trace elements 1ml/l (composition described above). The phosphate buffer used maintained the pH between 6.8 and 7.2. The reactors were then flushed with 100% N_2 for about 15 min.

At the start of an experimental run, 3000 mg acetate COD/l was added and the methane production rate recorded. When the methane production rate reached a maximum stable value, formaldehyde (from a 37% solution) was added at the desired concentration. Formaldehyde additions were done either in a slug or in a continuous mode. The concentrations of formaldehyde, formate, and methanol in the liquid phase, as well as methane and hydrogen in the headspace were measured over time.

Methane production monitoring. The biogas produced by the reactors was led through an erlenmeyer flask filled with a 20% NaOH solution followed by a tube filled with soda lime pellets with thymol blue indicator. Finally, the gas was collected in a Mariotte flask system containing water for quantification of the methane production. Displaced water was collected in plastic containers that were placed on pressure sensors (DS-Europe, QB 745) for continuous monitoring of the CH_4 production. The sensors were connected to a programmable datalogger system (Campbell CR10). The data were recorded every 10 seconds and averaged over a 20-min. interval. To communicate with the datalogger, a PC programmed to function as terminal emulator was used. All assays were performed in duplicate.

Analytical methods. For methanol analysis, samples were centrifuged (5 min at 10000 rpm), diluted with formic acid (3% [vol/vol]) and analyzed with a gas chromatograph (Hewlett Packard 5890A). A glass column (2 m by 6 mm) packed with Supelcoport (100 to 120 mesh), coated with 10% Fluorad FC 431, was used. The temperatures of the column, injection port and flame ionization detector were 70, 200 and 280°C, respectively. Nitrogen gas saturated with 100% formic acid was used as a carrier gas at a flow rate of 40 ml/min. The concentration of volatile fatty acids was determined by the same procedure as for methanol except that the temperature of the column was 130°C. Formaldehyde and formate were analyzed by high-pressure liquid chromatography (HPLC). Volumes of 10 µl of centrifuged samples (10000 rpm) were injected by using an autosampler (Applied biosystems 878 A).

Separation of the organic compounds was obtained by using an Ion 300 organic acid column (300 by 7.8 mm). The temperature of the column was 20°C. H_2SO_4 at 1.25 mM was used as a carrier phase at a flow rate of 0.5 ml/min. The separated components were detected by a refractive index (RI) detector (Showa Denko, Japan, Mod. RI-71). Chromatograms were stored and integrated using the software package Minichrom.

Hydrogen was analyzed with a chromatograph (Hewlett Packard mod. 5890A), equipped with a steel column (1.5m by 2mm) packed with molecular sieve 25H (60 to 80 mesh). The temperatures of the column, injection port and thermal conductivity detector were 40, 110 and 125°C, respectively. Argon was used as the carrier gas at a flow of 45 ml/min. Methane was analyzed with a gas chromatograph (Hewlett Packard 438/S), equipped with a steel column (2m × 2mm), packed with Poropak Q (80 to 100 mesh). The temperatures of the column, injection port and flame ionization detector were 60, 200 and 220°C, respectively. All gas samples were taken with a gas-tight syringe (Dynatech, Baton Rouge, Lousiana).

Volatile suspended solids (VSS) were analyzed according to standard methods [1].

Chemicals. All chemicals were of analytical grade, and most were supplied by Merck (Darmstadt, Germany); methanol was purchased from Labscan (Dublin, Ireland) and yeast extract from Oxoid Unipath Ltd.(Hampshire, England).

Results

Formaldehyde conversion

The conversion of formaldehyde as a sole carbon source was studied in serum bottles, and abiotic bottles were used to assess the degree of sorption to biomass.

Figure 5.1 shows the anaerobic conversion of formaldehyde added at an initial concentration of 33.3 mM.

Formaldehyde conversion for all the concentrations tested (i.e. 3-33.3 mM) proceeded without any lag period, and was concomitant with the formation of methanol and formate. The results show that methanol accumulated at a final molar ratio $CH_3OH/HCHO$ of 0.9/2. Formate did not completely accumulate because once the formaldehyde concentration had decreased below 4mM, formate was readily transformed into methane. Methanogenesis from methanol occurred after 50 h (Figure 5.1 bottom graph) suggesting that the methanol-consuming bacteria were present at low numbers. The results of the tests with autoclaved sludge show that formaldehyde removal due to adsorption and/or chemical reaction with biomass amounted to 10-15%. In the abiotic tests, neither formate nor methanol formation

was detected. Furthermore, no physical or chemical formaldehyde removal occurred in the presence of only mineral medium (Figure 5.1).

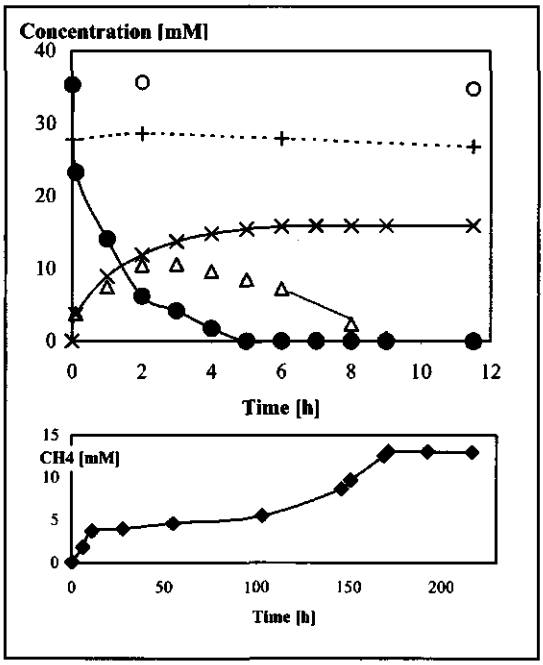

Figure 5.1 *Top graph: Anaerobic conversion of formaldehyde as sole carbon source. The inoculum was 3.5 g VSS/l of crushed anaerobic granular sludge. Formaldehyde (●), methanol (×), formate (Δ). Formaldehyde from the abiotic test containing autoclaved sludge (+), and from the test with only mineral medium (○). Bottom graph: Methane produced (◆) from the formate (first 10 h) and the methanol that resulted from the formaldehyde conversion as shown in the top graph.*

With respect to the formaldehyde conversion, we observed that the rate decreased at increased initial formaldehyde additions (Table 5.1).

Table 5.1 *Calculated first order rate constants at different initial formaldehyde concentrations. 3.5 g VSS/l were used.*

Initial formaldehyde [mM]	First order rate constant k [h^{-1}]
3.33	2
16.66	1.2
33.33	0.7

Fate of formaldehyde

We studied the fate of formaldehyde during acetoclastic methanogenesis. In these experiments, at time zero, acetate was added at a concentration of 47 mM, and the methane production rate was recorded on-line. Once the recorded methane production rate reached a stable maximum value, formaldehyde was added at a concentration of 33.3 mM (this chemical also contained 10% of methanol as an impurity). The results of the experiment are shown in Figure 5.2. During the tests, different phases in the methane production rate could be distinguished.

Immediately after the addition of formaldehyde, an inhibition phase (phase II) was observed which was characterized by a sharp drop in the methane production rate. Although during this phase, the methane production was strongly inhibited, formaldehyde conversion proceeded

without any lag phase. Formaldehyde was transformed immediately into formate/hydrogen and methanol (Figure 5.3). During the inhibition phase, acetate was virtually not consumed (Figure 5.3, bottom panel).

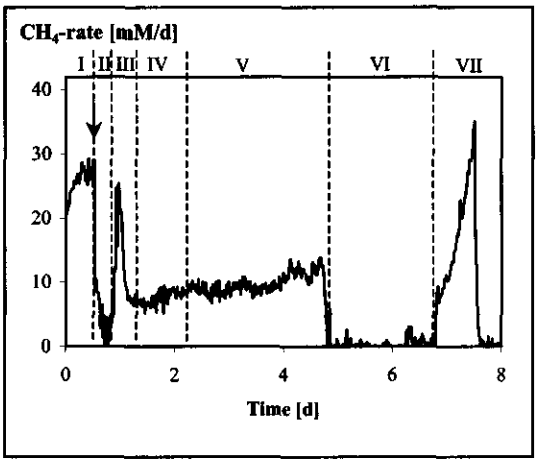

Figure 5.2 *Methane production rate. At time zero acetate was added at 47 mM, and at the time indicated by the arrow formaldehyde was added at 33.3 mM. The inoculum was 3 g VSS/l of crushed anaerobic granular sludge. Several phases can be observed:* (I) *Maximum acetoclastic CH_4-rate before formaldehyde addition.* (II) *Inhibition phase.* (III) *Methane production from formate and/or hydrogen.* (IV) *Maximum recovered acetoclastic CH_4-rate.*(V) *Increase of the acetoclastic CH_4-rate likely due to biomass growth.* (VI) *Depleted acetate substrate.* (VII) *Methanogenesis from methanol.*

After the inhibition, and when formaldehyde was virtually removed (formaldehyde concentration ≈ 4 mM), a conspicuous peak in the methane production rate occurred, phase III (Figure 5.2 , and Figure 5.3 top panel). This peak was likely due to methanogenesis from formate and/or hydrogen (Figure 5.3). Following this peak, methanogenesis from acetate consumption was recovered (phase IV). The maximum activity obtained in this period demonstrates that the recovery was not complete as can be seen by comparing with the maximum methane production rate before formaldehyde addition (phase I, Figure 5.2).

Acetate was totally consumed on day 5 and thus the methane production rate at that moment ceased due to acetate depletion (phase VI) (Figure 5.2 and Figure 5.3). Two days after this event, the degradation of methanol started, as indicated by a fast exponential increase of the methane production rate (phase VII). The relation between the methane production rate phases and the corresponding substrate being consumed at the end of the experiment (days 2 to 8) can be seen in Figure 5.3.

COD balances for all the experiments performed showed that formaldehyde was mineralized, for example, in the test shown in Figure 5.2, a total of 4471 mg COD/l were added (i.e. acetate 3000, plus 1066 formaldehyde, plus 405 methanol), and the recovered CH_4 at the end of the experiment was 4400 mg COD/l. Although in all cases the hydrogen concentration in the head-space increased as a result of formaldehyde conversion, its amount on COD basis

was negligible. The amount of hydrogen was dependent on the amount of formaldehyde added. The maximum measured hydrogen content in the head-space were 1.7 $\times 10^3$, 6.6 $\times 10^3$, 10 $\times 10^3$, and 17 $\times 10^3$ ppm when formaldehyde was added at 6.6, 13, 20 and 33.3 mM, respectively.

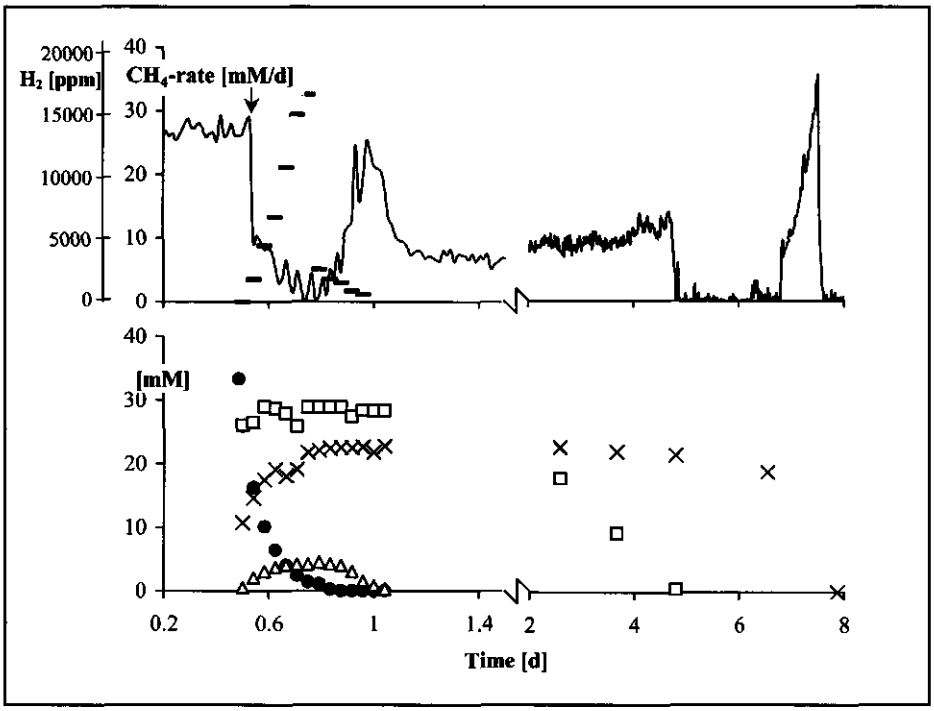

Figure 5.3 *Details of experiment sown in Figure 5.2. Methane production rate (continuous line) of acetate-consuming sludge during a toxicity test. At time zero acetate was added at 47 mM. The arrow indicates the time of formaldehyde addition at 33.3 mM. Symbols: Formaldehyde (●), methanol (×), formate (Δ), acetate (□), hydrogen (–).*

Characteristics of formaldehyde toxicity

The characteristics of formaldehyde toxicity were investigated in batch systems with either slug or continuous additions of the toxic compound.

Slug addition. Formaldehyde was added in a slug mode at concentrations in the range 3.3 to 33.3 mM. All the concentrations tested resulted in a strong inhibition of the methane production rate (Figure 5.4*a*). As described in the previous section, several phases in the

methane production rate can be distinguished. The results show that the slope of the inhibition phase was similar independent of the concentration of formaldehyde added. However, the acceleration of the recovery phase decreased as the concentration of formaldehyde increased.

Furthermore the results show that at all the concentrations tested, the methane production rate partially recovered. This recovery occurred when formaldehyde was either completely transformed or its concentration had decreased to very low values (below ≈1.5 mM). The recovery however, was clearly not complete since the maximum recovered rate after formaldehyde addition (Re) was, in all cases lower than the rate before formaldehyde addition (Ro). As follows from the results depicted in Figure 5.4*b*, the decrease in the methane production appeared to be linearly related to the amount of formaldehyde added.

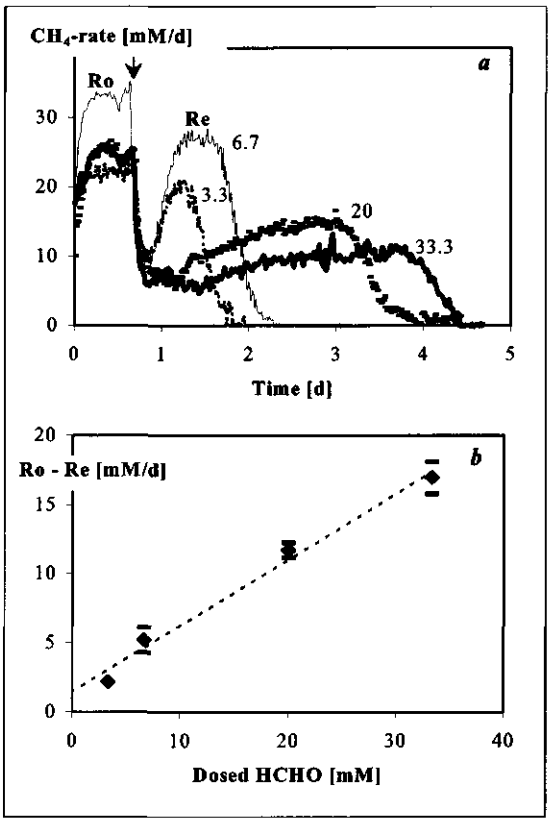

Figure 5.4 (*a*) *Effect of slug additions of formaldehyde on the methane production rate. The numbers beside each curve indicate in mM the concentration of formaldehyde added. The arrow indicates the time of addition.* Ro *and* Re *indicate the maximum methane production rate before formaldehyde addition, and after recovery, respectively as indicated in the case of the 6.7mM curve.* (*b*) *Decrease in the maximum methane production rate, equal* Ro-Re, *at the different additions of formaldehyde.*

To evaluate the effect of the initial active biomass concentration on the toxicity pattern of formaldehyde, runs were performed with different initial amounts of sludge while dosing the

same slug concentration of formaldehyde. The results of these experiments are shown in Figure 5.5. It can be seen from the results that independent of the initial methane production rate (i.e. initial active biomass), the absolute decrease on the methane production rate between phase I and IV (i.e. Ro-Re) was comparable at the same addition of formaldehyde. This is summarized in Table 5.2.

Table 5.2 *Decrease on the methane production rate due to formaldehyde addition at different initial biomass concentrations.*

Type of sludge	Dosed HCHO [mM]	Biomass [g VSS/l]	Loss in activity Ro-Re [mM/d]
Crushed	16.7	1.5	7
		3	7.5
Granules	20	1.5	12.5
		2	13.2
Granules	33.3	1.5	18.8
		2	21.1

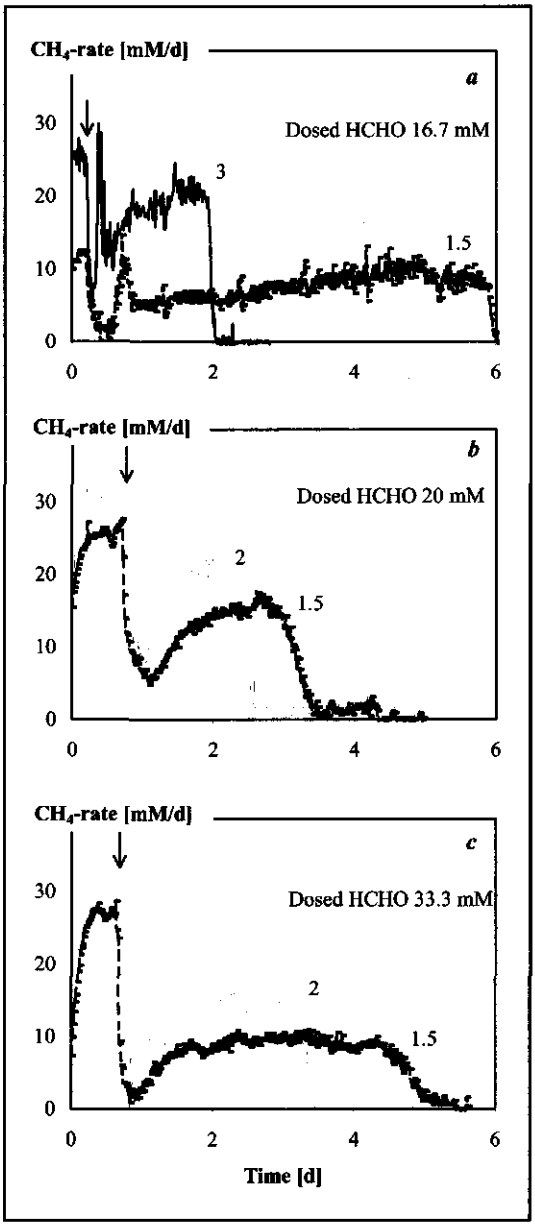

Figure 5.5 *Effect of a slug addition of formaldehyde to different initial active biomass concentrations as indicated by the different initial methane production rates before formaldehyde addition. The arrows indicate the time of addition, and the numbers beside the rate curves indicate the added biomass concentration in the batch reactor in g VSS/l. Dispersed sludge was used in the tests of panel (**a**). In panels (**b**) and (**c**) another type of sludge was used, and in this case, the tests were conducted with granular biomass.*

Continuous addition. Experiments with continuous addition of formaldehyde were performed to elucidate whether the loss in the methanogenic activity depended on the concentration or on the total amount of formaldehyde added (load). As was the case in the slug test, formaldehyde addition started once a maximum and stable methane production rate had been established. The results show that the maximum methane production rate recovered in phase IV was similar for the same amount of formaldehyde added, either in a continuous or in a slug mode (Figure 5.6*a-d*).

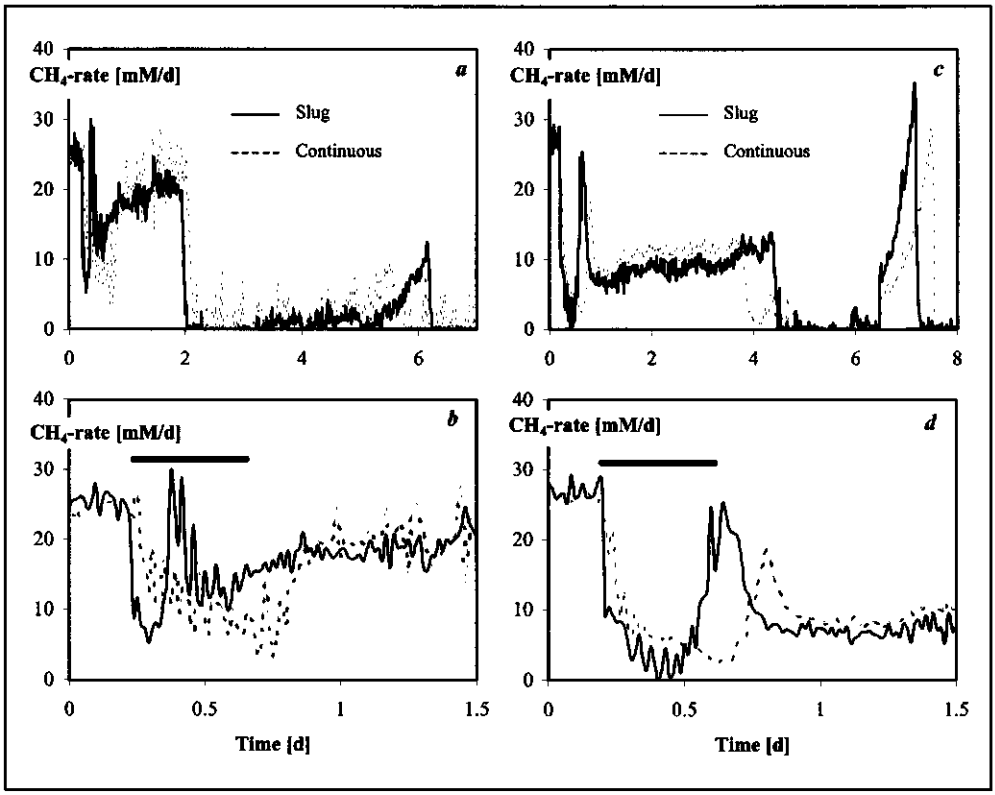

Figure 5.6 *Effect of continuous vs slug addition of formaldehyde on the methane production rate. Dash and solid lines in all cases refer to continuous and slug tests, respectively. The continuous additions were performed during 10 h as indicated by the bars.* (***a***) *Continuous and slug addition of 1.6 mM/h and 16.7 mM, respectively. A zoom-time image of this figure is depicted in panel* (***b***)*. Panel* (***c***)*, continuous and slug addition of 3.3 mM/h and 33.3 mM, respectively. A zoom-time image on this figure is depicted in panel* (***d***)*.*

It was observed however, that when formaldehyde was added continuously the decline of the methanogenic activity in phase II was more protracted (Figure 5.6*b* and Figure 5.6*d*). When formaldehyde was added in a slug mode the methane production peak due to formate consumption (phase III) was very clear. While in the experiments where formaldehyde was added continuously, the occurrence of this peak was either not prominent (Figure 5.6*b*), or it was delayed (Figure 5.6*d*).

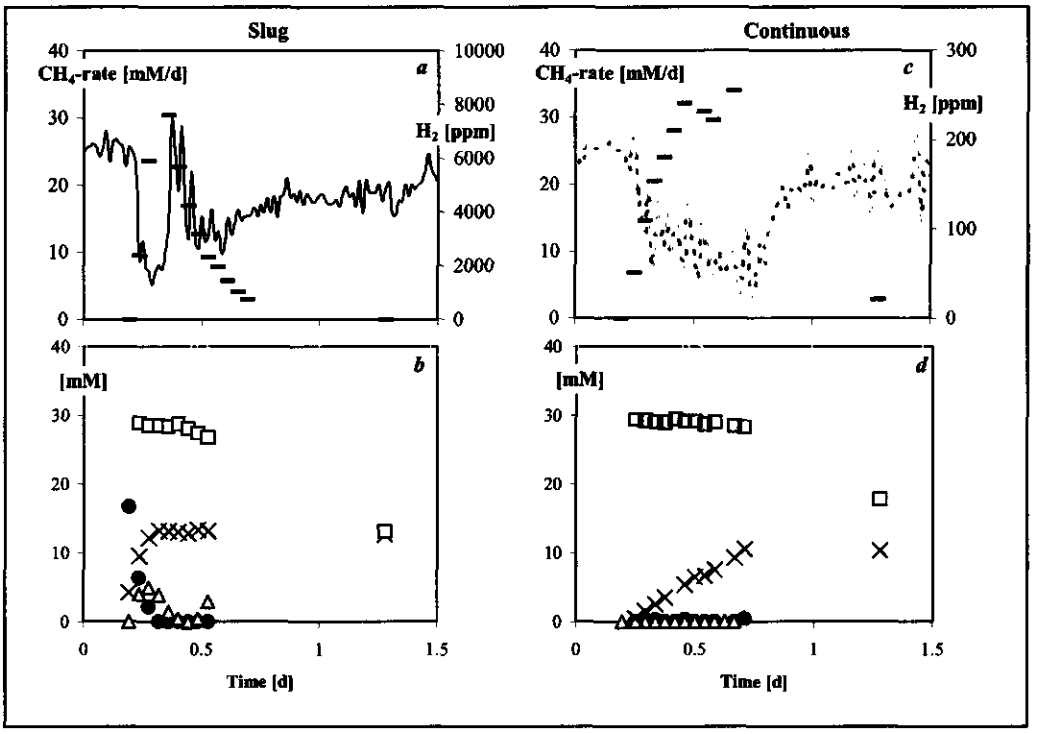

Figure 5.7 *Comparison of formaldehyde and its intermediate products during slug vs continuous tests. The continuous and slug additions were 1.6 mM/h during 10 h, and 16.7 mM, respectively. (**a**) and (**b**) show the components evolution for the slug test. (**c**) and (**d**) show the components evolution for the continuous test. Symbols: Formaldehyde (●), methanol (×), formate (Δ), acetate (□), hydrogen (–).*

For the case of the continuous-dose experiments conducted at 1.6 mM/h and 3.33 mM/h, the measured formaldehyde concentration in the bulk during the time of the continuous addition was below 0.4 mM (Figure 5.7*d*) and 2.6 mM, respectively. The methanol concentration in the continuous tests built-up gradually over time, while very little formate accumulation occurred. As an example, these trends are shown for the continuous test at 1.7 mM/h in Figure 5.7.

Discussion

Formaldehyde conversion and final fate

The results of formaldehyde conversion as sole carbon source clearly show that anaerobic sludge transformed formaldehyde into formic acid and methanol. Based on the $CH_3OH/HCHO$ ratios of 0.7/2 to 0.9/2 measured in this study, we suggest the following reactions for formaldehyde conversion:

Oxidation $HCHO + H_2O \rightarrow HCOOH + H_2$

Reduction $HCHO + H_2 \rightarrow CH_3OH$

Total $2HCHO + H_2O \rightarrow CH_3OH + HCOOH$

This mechanism has been also proposed for the conversion of formaldehyde by methanol degrading anaerobic sludge [6]. A similar oxidation-reduction mechanism has been suggested by Escalante-Semerena and Wolfe [4], for the oxidation of formaldehyde by cell-free extracts of *Methanobacterium thermoautotrophicum*.

The results of our study furthermore show that although the sludge used had not been previously exposed to formaldehyde, formaldehyde conversion proceeded immediately without any lag phase. This observation suggests the constitutive character of the formaldehyde converting enzymes in the biomass within the mixed culture. Formaldehyde conversion by acetate enriched methanogenic biomass also occurred without a lag phase [14]. In the case of some fungi and eubacteria, formaldehyde can be transformed by formaldehyde dehydrogenase enzymes which are present in those organisms, irrespective whether they were grown in the presence or absence of formaldehyde [2, 9, 15].

In our case, it is not possible to indicate which microorganisms are involved in the conversion of formaldehyde. Experiments performed with vancomycin and bromoethanesulfonic acid to inhibit the metabolism of eubacteria and methanogenic archaea, respectively showed that independent of the specific inhibitor used, formaldehyde conversion did not stop (data not shown). The use of these inhibitors do not discard the occurrence of a merely enzymatic reaction with formaldehyde either with eubacterial or with methanogenic dehydrogenases/hydrogenases enzymes.

However, it was observed that methanogenesis from formate apparently was less inhibited by formaldehyde (Figure 5.3). This observation only applies for the reversible toxicity (i.e. temporary deactivation). Compared to methanogenesis from formate, acetoclastic methanogenesis recovered once formaldehyde was almost depleted or not detected in the system.

With respect to the rate of formaldehyde conversion, we observed that the rate decreases at increasing initial formaldehyde concentrations. We hypothesize that this kinetic behavior might indicate that some enzymes (e.g. dehydrogenases) and/or cofactors could become denatured due to the excess of formaldehyde. In support of our hypothesis previous research showed that formaldehyde conversion stopped at high formaldehyde concentrations of 13 to 20 mM in anaerobic digester sludge [11], and at formaldehyde concentrations of 2 mM in acetate enriched biomass [14]. Furthermore, the formaldehyde conversion was largely independent of the initial presence (Figure 5.3, bottom panel) or absence of acetate (Figure 5.1). For comparison purposes, normalized first order rate constants for formaldehyde conversion were calculated for the 33.3 mM tests. The values obtained were 0.18 and 0.2 $h^{-1}.g^{-1}$VSS/l in the presence and absence of acetate, respectively.

Characteristics of formaldehyde toxicity

The toxicity of formaldehyde to acetoclastic methanogenesis exerted a dual character. Formaldehyde toxicity was in part reversible since the methane production rate was partially recovered. The temporary deactivation of the methane production rate was dependent on the formaldehyde concentration. Once the formaldehyde concentration reached a very low value, methanogenesis recovered. This recovery can not be attributed to bacterial growth since assuming a growth rate (μ) of *Methanosaeta* sp. of 0.2 d^{-1} for acetoclastic methanogenesis by anaerobic sludge, the elapsed time between the start and end of the recovery period would be much longer than that observed in Figure 5.2. Since the degree of recovery was not complete, we conclude that formaldehyde toxicity was also in part irreversible. This irreversible toxicity

is linearly related with the dosed formaldehyde. The same relation was found for methanol consuming sludge in which enhanced cell lysis was observed due to formaldehyde exposure [6]. The irreversible toxicity, as indicated by the loss on the methane production rate after formaldehyde addition, therefore can be likely attributed to formaldehyde-related decay.

Formaldehyde addition either in a slug or in a continuous mode caused similar irreversible toxicity. We suggest that a certain amount of formaldehyde irrevocably kills a certain amount of biomass, and thus reduces the methane production rate to a certain extent independent of the mode of formaldehyde addition. Other researchers suggest that compared to slug additions, higher concentrations of formaldehyde can be tolerated by acetate enriched biomass grown in chemostats when added continuously [3], or with repeated additions at low concentrations [14]. However, in such experiments the elapsed time between the repeated additions ranged from 20 h to 10 d. Considering a doubling time for an acetate enrichment culture of $\approx 0.2\ d^{-1}$, it is clear that biomass growth might occurred during the time between the additions that compensated the killing effect of formaldehyde.

Practical implications

The results presented here show formaldehyde toxicity characteristics that have important implications in the anaerobic treatment of formaldehyde-containing waste streams. Since formaldehyde is completely mineralized by anaerobic sludge via formate and methanol, anaerobic technology in principle can well be applied. Formaldehyde toxicity is in part reversible, and in part irreversible. Only after formaldehyde depletion, acetoclastic methanogenesis can be reverted.

The irreversible character of the toxicity implicates that to avoid failure of the anaerobic process, the formaldehyde-enhanced decay must be compensated by net biomass growth. Hence, the ratio formaldehyde/acetate (and/or other non-toxic substrate) is an important factor since it will determine the net growth of the microbial population.

Based on these reasoning it is clear that formaldehyde-containing wastewaters can be treated anaerobically provided that (i) good biomass retention, and (ii) a net growth of the methanogenic population are attained.

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6

A Novel Approach for Assessing Metabolic Properties and Kinetic Parameters of Methanogenic Sludge by On-Line CH_4 Production Rate Measurements

This paper presents a new approach to study the metabolic and kinetic properties of anaerobic sludge from single batch experiments. The two main features of the method are that the methane production is measured on-line with a relatively cheap system, and that the methane production data can be plotted as rate *vs.* time curves. The case studies of specific methanogenic activity, biodegradability and toxicity tests here presented, show that very accurate kinetic data can be obtained. The method is specifically useful in experiments where strong changes in the methane production rate occur, and it is proposed as a powerful tool to study methanogenic systems. Furthermore, the method is simple and could be implemented by industry to be used as a routine characterization method for the sludge[*].

[*] Submitted for publication.

Introduction

Anaerobic digestion is widely applied for the treatment of a great variety of wastewaters [11]. The monitoring and/or assessment of the metabolic properties of biomass grown in anaerobic reactors are crucial from both a scientific and practical point of view. Bioassays to assess some metabolic properties of sludge include (i) the maximum specific methanogenic activity either from a single or mixed substrates, (ii) biodegradability, and (iii) toxicity tests.

Methanogenic activity tests are frequently used as a sludge-monitoring procedure for operating reactors. Furthermore, to achieve a more rational reactor start-up procedure, it is necessary to characterize the seed sludge with respect to its metabolic capacity to transform anaerobically the main substrates in the wastewater. Biodegradability and/or toxicity tests are particularly important when complex and more toxic wastewaters need to be treated, such as those from the chemical industries. These tests can give some indications about the feasibility of the treatment process.

Several methods have been described to assess the metabolic and kinetic properties of anaerobic sludge. Methane as the final end product, is the priority compound to be measured during the bioassays, and therefore, the majority of the methods developed so far rely on the measurement of the methane produced. In general, these methods have been developed for batch-experiments with serum bottles in which quantification of the methane concentration is obtained by measuring (i) pressure increase, (ii) gas composition in the headspace, and (iii) liquid displacement systems.

Pressure-based methods like the modified Warburg respirometer described by James et al. [8] have the disadvantage that the gas composition has to be measured which is laborious and time consuming. Only for methanogenic substrates the gas-composition can be calculated from the pressure increase, the medium composition, and the ratio of headspace to liquid volume. An on-line technique consisting of an electronic pressure transducer, and based on the methodology described by Shelton and Tiedje [17] for data analysis, has been reported [3, 4]. However, when using a non-methanogenic substrate, the gas composition has to be measured and then the system becomes again an off-line system loosing the potential value of an on-line method. Nevertheless, an important advantage of the pressure-based method is that, with high precision sensors, very small gas productions can be measured.

The headspace method [5] is based on methane analysis by gas chromatography. Gas sampling is performed with a pressure-lock syringe, which allows quantification independent of the pressure inside the test vials. The method can be time consuming, and the liquid

sampling size can be also limited, but one of its advantages is that only small amounts of sludge are required.

Liquid displacement systems [18] are reasonably simple to implement and by using relatively high amounts of sludge, high accuracy can be obtained. In some cases the amount of sludge required might be a drawback. An advantage is that on-line measurements of the biogas can be relatively easy implemented by weighing the displaced liquid using computer-linked pressure sensors.

Recently, a sophisticated method using membrane mass spectrometry (MS) has been reported for on-line monitoring of dissolved gases. The technique has been used for the assessment of methanogenic activities [2] and for fast kinetic studies [12]. Due to the sensitivity of the MS, small concentration changes of dissolved gases like H_2, CO_2 and CH_4 can be measured. The methodology is however complex and expensive.

If we consider that the complexity of anaerobic digestion still hampers a clear mathematical interpretation of the process, it becomes clear that a better understanding of the kinetic and metabolic properties of sludge is necessary for rational design and performance monitoring of anaerobic reactors. Reliable, plain and straightforward approaches should be used for this purpose, which are accepted by a large number of researchers and beneficiaries of the system. With most of the approaches used so far valuable information may be overlooked or even lost, particularly during gradual changes on the methane production rate.

In the current paper we present a new approach to study the metabolic and kinetic properties of anaerobic sludge. The two important features of the method are that the methane production is measured on-line with a relatively cheap system, and that the methane production data can be plotted as rate *vs.* time curves. Examples are presented to show the potentials of this new methodology.

Materials and methods

Inoculum. Anaerobic granular sludge from a full scale expanded granular sludge bed (EGSB) reactor was used. The reactor treats brewery wastewater (Heineken, Zoeterwoude, The Netherlands), and was developed by Biothane Systems International (Delft, The Netherlands). Both intact and dispersed granular sludge were used. Granular sludge of a specific particle size was obtained by sieving the sludge through metallic sieves under water. The dispersed sludge was obtained by disrupting the granular structure with a blender (Braun 4164). The dispersed granular sludge here described was used, unless stated otherwise.

Mineral medium. The medium used contained the following components in g/l of demineralized water: NH_4Cl 0.28, $MgSO_4.7H_2O$ 0.1, KH_2PO_4 6.79, $Na_2HPO_4.2H_2O$ 8.89, yeast extract 0.1, trace elements 1ml/l. The trace elements solution contained the following components in mg/l of demineralized water: $FeCl_2.4H_2O$ 2000, H_3BO_3 50, $ZnCl_2$ 50, $CuCl_2.2H_2O$ 38, $MnCl_2.4H_2O$ 500, $(NH_4)_6Mo_7O_{24}.4H_2O$ 50, $AlCl_3.6H_2O$ 90, $CoCl_2.6H_2O$ 2000, $NiCl_2.6H_2O$ 142, $Na_2SeO.5H_2O$ 164, EDTA 1000, Resazurine 200, HCl 36% 1 ml/l. All chemicals were of analytical grade and purchased from Merck (Darmstadt, Germany); resazurine was from Fluka (Buchs, Switzerland) and yeast extract from Oxoid Unipath Ltd. (Hampshire, England).

On-line methane production monitoring. The experiments were conducted in continuously stirred plastic batch reactors. The reactors were filled up to 2.5 liter with mineral medium and inoculated with 2 g VSS/l of dispersed or granular biomass. Acetate or a mixture of fatty acids was added as primary substrate from concentrated stock solutions. Hereafter, the reactors were flushed with 100% N_2 for 20 min. and operated in a 30 (±1)°C temperature controlled room. With the phosphate buffer dosed, the pH was maintained at 6.8-7.2. The biogas produced was led through an Erlenmeyer flask filled with a 20% NaOH solution followed by a tube filled with soda lime pellets with thymol blue indicator. Finally the gas passed through a Mariotte flask system containing water for quantification of the methane production. Displaced water was collected in plastic containers that were placed on pressure sensors (model QB 745; DS-Europe) for continuous monitoring of the methane production. The sensors were connected to a programmable datalogger system (model CR10; Campbell). The data were recorded every 10 seconds and averaged over a 20-min. interval. To communicate with the datalogger, a PC programmed to function as terminal emulator was used. The set-up is schematically shown in Figure 6.1. All assays were performed in duplicate.

Analytical methods. The concentration of volatile fatty acids in the medium was determined with a gas chromatograph (Hewlett Packard 5890A). A glass column (2 m by 6 mm) packed with Supelcoport (100 to 120 mesh), coated with 10% Fluorad FC 431, was used. The temperatures of the column, injection port and flame ionization detector were 130, 200, and 280°C, respectively. Nitrogen gas saturated with 100% formic acid was used as a carrier gas at a flow rate of 40 ml/l. The volatile suspended solids (VSS) contents were determined by standard methods [1].

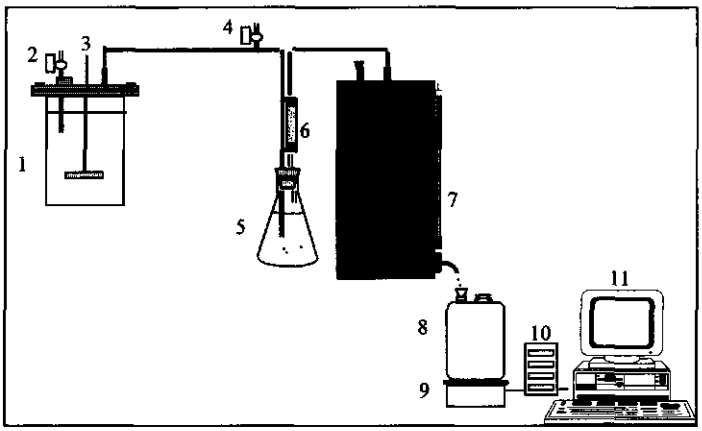

Figure 6.1 *Experimental set-up for on-line methane production monitoring.* 1 *Batch reactor,* 2 *Liquid sampling port,* 3 *Stirrer,* 4 *Gas sampling port,* 5 *CO_2 washing solution (20% NaOH),* 6 *Soda lime pellets,* 7 *Mariotte flask,* 8 *Plastic container,* 9 *Pressure sensor,* 10 *Datalogger,* 11 *Personal computer.*

Kinetic analysis. To analyze the methane production measurements during degradation of the volatile fatty acid (VFA) mixture, a mathematical model based on Monod kinetics was used. The volumetric rates of substrate consumption (R_S), biomass growth (R_X), and product formation (R_P) in a batch reactor can be described by the following equations.

$$R_S = -\frac{\mu^{max} \cdot S}{K_S + S} \cdot \frac{X}{Y_{XS}} \tag{6.1}$$

$$R_X = -R_S \cdot Y_{XS} \tag{6.2}$$

$$R_P = -f_{sp} \cdot (1 - Y_{XS}) \cdot R_S \tag{6.3}$$

where μ^{max} (d^{-1}) and K_S (mg COD/l) represent the maximum growth rate and the affinity constant, respectively. f_{sp} refers to the COD-based stoichiometry for conversion of substrate

S (mg S-COD/l) into product P (mg P-COD/l). The COD-based yield, Y_{XS} (g X-COD/g S-COD), represents the biomass yield for growth of biomass X (mg X-COD/l) on substrate (S). Combining these equations and mass balances based on the chemical reactions (A to C), the next differential equations can be described.

$$CH_3COO^- + H_2O \rightarrow HCO_3^- + CH_4 \quad \text{(A)}$$

$$CH_3CH_2COO^- + 0.75H_2O \rightarrow CH_3COO^- + 0.25HCO_3^- + 0.25H^+ + 0.75CH_4 \quad \text{(B)}$$

$$CH_3CH_2CH_2COO^- + 0.5H_2O + 0.5HCO_3^- \rightarrow 2CH_3COO^- + 0.5H^+ + 0.5CH_4 \quad \text{(C)}$$

$$\frac{dS_{C2}}{dt} = R_{C2} - [f_{C3-C2} \cdot (1 - Y_{XC3}) \cdot R_{C3}] - [f_{C4-C2} \cdot (1 - Y_{XC4}) \cdot R_{C4}] \quad (6.4)$$

$$\frac{dS_{C3}}{dt} = R_{C3} \quad (6.5)$$

$$\frac{dS_{C4}}{dt} = R_{C4} \quad (6.6)$$

$$\frac{dCH_4}{dt} = -f_{C2-CH4} \cdot (1 - Y_{XC2}) \cdot R_{C2} - f_{C3-CH4} \cdot (1 - Y_{XC3}) \cdot R_{C3} - f_{C4-CH4} \cdot (1 - Y_{XC4}) \cdot R_{C4} \quad (6.7)$$

$$\frac{dX_{C2}}{dt} = -R_{C2} \cdot Y_{XC2} \quad (6.8)$$

$$\frac{dX_{C3}}{dt} = -R_{C3} \cdot Y_{XC3} \tag{6.9}$$

$$\frac{dX_{C4}}{dt} = -R_{C4} \cdot Y_{XC4} \tag{6.10}$$

Equation 6.7 was derived assuming a lumped production of methane together with the conversion of propionate (C3) and butyrate (C4) according to reactions B and C, respectively. No product inhibition terms for the conversion of C3 and C4 were included since no substantial accumulation of acetate (C2) and hydrogen occurred during the experiments. The equations were integrated using the Euler method [14] implemented in Microsoft Visual Basic.

The parameter values to run the model were assessed as follows: From a batch experiment with only acetate and high initial biomass concentration, the apparent affinity constant (K_S) for acetate was calculated by solving Equations 6.4, 6.7 and 6.8 (for Equations 6.4 and 6.7 only the acetate terms were used). The K_S and the initial biomass concentration were estimated with an optimization procedure included in the spreadsheet program. The optimization was based on minimizing the absolute error between measured and calculated values of the methane production rate. From a batch experiments with low initial biomass concentrations, the μ^{max} of acetate, propionate, and butyrate consuming bacteria were calculated from the exponential part of the substrate depletion curves. For zero order kinetics ($S \gg K_S$), the Monod equation for substrate consumption can be integrated resulting in the following equation.

$$S_{(t)} = S_{(0)} + \frac{X_{(0)}}{Y_{XS}} \cdot \left(1 - e^{\mu^{max} \cdot t}\right) \tag{6.11}$$

By using calculated biomass yields (Y_{XS}, mg X-COD/mg S-COD) as described by Heijnen et al. [7], and measured substrate concentrations (S, mg COD/l) as a function of time, the initial biomass ($X_{(0)}$, mg X-COD/l) and μ^{max} (d^{-1}) were estimated with an optimization

procedure included in a spreadsheet program. The optimization was based on minimizing the absolute error between measured and calculated values of S.

Results and discussion

The potential advantages of the application of on-line measurements of the methane production rate will be presented based on three examples: (i) determination of affinity constant, (ii) metabolic characterization and kinetic parameter estimation for methanogenic sludge, and (iii) assessment of different properties of toxicants.

Assessment of kinetic parameters

In "conventional" batch systems, the K_S parameter is especially difficult to estimate mainly due to insufficient data at the low substrate concentrations. This experimental difficulty may be overcome by the on-line measurement of the methane production rate. In a batch system, the maximum methanogenic activity and the apparent K_S constant can be assessed at the same time. This is illustrated in Figure 6.2 for an experiment during acetate conversion by granular sludge, and crushed granular sludge.

The data in Figure 6.2*b* show that the maximum rate for both types of sludges is similar. However, the calculated apparent K_S for the granular and crushed sludge differed significantly with values of 208 and 32 mg COD/l, respectively. Clear differences in apparent K_S-values can be observed between dispersed and granular sludge when comparing the rate *vs.* time curves (Figure 6.2*b*). Such differences are less obvious when cumulative curves are plotted (Figure 6.2*a*).

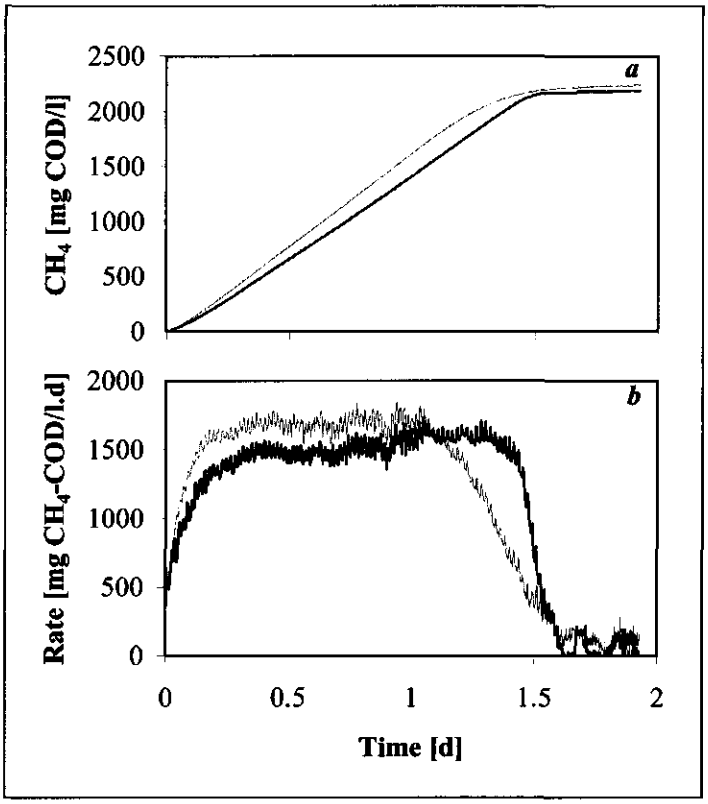

Figure 6.2 *Methanogenesis from acetate of crushed (thick lines) and granular (thin lines) sludge. The initial substrate concentration amounted ≈2000 mg COD/l. (**a**) Cumulative vs. time plot. (**b**) Rate vs. time plot.*

Assessment of Metabolic characteristics of the sludge

A metabolic characterization of anaerobic sludge can be performed when specific non-methanogenic substrates are used and their conversion is followed over time. Frequently this characterization is time consuming since not only the gas phase needs to be analyzed but the liquid phase as well. Our new methodology simplifies the characterization, and provides insight in the capacity of anaerobic sludge to degrade non-methanogenic substrates. As an example, Figure 6.3*b* shows the methane production rate from the conversion of a mixed substrate composed of acetate, propionate, and butyrate. Each fatty acid was added at an initial concentration of ≈1000 mg COD/l. The shape of the methane production rate changed during the duration of the experiment. These changes proceeded according to the type of

substrate that was being converted, and to the amount of initial active biomass degrading that substrate (Figure 6.3). At high amounts of initial active biomass, growth can be neglected and the methane production rate will stay more or less constant (i.e. activity curve in phase I from Figure 6.3*b*). On the contrary, when the initial amount of biomass that utilizes a certain compound is low, the methane production rate will increase constantly (i.e. growth rate curve in phase III from Figure 6.3*b*).

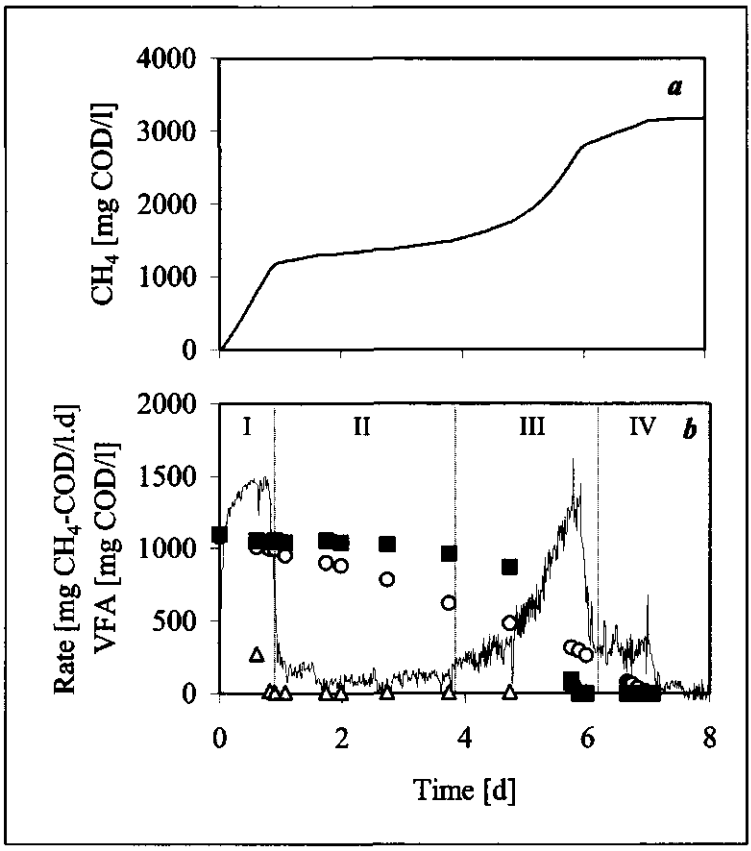

Figure 6.3 (*a*) *Cumulative methane production curve from batch conversion of a substrate mixture consisting of acetate, propionate, and butyrate.* (*b*) *Methane production rate curve (continuous line) and concentration of fatty acids. Acetate* (Δ), *propionate* (○) *and butyrate* (■). *The following phases on the methane production rate curve were observed:* I *Constant rate due to the conversion of acetate.* II *Rate due to the start of the conversion of propionate.* III *Exponential rate due mainly to the conversion of butyrate.* IV *Rate due to the end of the conversion of propionate.*

It is clear that rate *vs.* time plots (Figure 6.3*b*) provide significant more information than the cumulative methane plots (Figure 6.3*a*).

In order to validate the model described in the materials and methods section, experiments were conducted with sludge samples from different origin. Sludge Brewery 1 was the EGSB sludge already described. Sludge Paper mill and Brewery 2 were from full-scale UASB reactors treating paper mill and brewery wastewater, respectively. By (i) measuring the methane production rate on-line, (ii) the measurement of a few liquid samples, (iii) yield estimates, and (iii) with the aid of a simple mathematical model, the initial concentrations and the specific growth rate of VFA-degrading biomass could be estimated. The parameter values to run the mathematical model are shown in Table 6.1. The μ^{max} values of propionate and butyrate consuming biomass were calculated from the exponential part of the substrate depletion curves shown in Figure 6.3*b*. The μ^{max} of acetoclastic methanogens was obtained by the same approach in a separate experiment (data not shown). The apparent K_S value of acetate was measured (Figure 6.2*b*), while those of propionate and butyrate were taken, respectively, from Kaspar and Wuhrmann [9], and Labib et al. [10].

Table 6.1 *Parameter values used as input for the mathematical model. All parameters were experimentally measured or calculated, except stated otherwise.*

Parameter	Units	C2	C3	C4
μ^{max}	d^{-1}	0.2	0.3	1.1
K_S	mg COD/l	32	10 [a]	0.8 [b]
Y_{XS}	mg X-COD/mg S-COD	0.032	0.022	0.045
f_{sp} (acetate)		1	0.57	0.80
f_{sp} (methane)			0.43	0.20

a Average value reported by Kaspar and Wuhrmann, 1978.

b Value reported by Labib et al., 1993.

The methane production rate curves for the three different sludges were calculated with the model by using only the initial biomass as a fitting parameter. The results of the measured and calculated methane production rate curves are shown in Figure 6.4. The results of the calculated initial active biomass for the different trophic groups and for the different sludges are shown in Table 6.2.

Table 6.2 *Estimated initial active biomass of different trophic groups and for different sludge samples. Acetoclastic methanogens, propionate and butyrate consuming biomass are represented respectively by* X_{C2}, X_{C3}, *and* X_{C4}.

Sludge	X_{C2}	X_{C3}	X_{C4}
		mg X-COD/l	
Brewery 1	227	3.67	0.076
Paper mill	170	1.5	0.02
Brewery 2	17	0.018	0.00012

The metabolic capacities of the three sludges tested were significantly different. Sludge samples Brewery 1 (Figure 6.4*a*) and Paper mill (Figure 6.4*b*) had a high capacity for acetate conversion, but a low capacity for propionate and butyrate. Since the initial amount of propionate and butyrate degraders was low these biomass trophic groups grew during the test. Conversely, sludge sample Brewery 2 had a low capacity for all the added volatile fatty acids (Figure 6.4*c*).

The biomass composition will determine the removal efficiency of the intermediates during anaerobic digestion. Frequently the bacterial content of the sludge is measured by the solids method, however this does not give an indication of the active biomass concentrations or its capacity to degrade the main intermediates of the anaerobic digestion process. In fact, although the same amount of VSS was used in all the sludges tested, their metabolic properties were quite different. Reported methods for the quantification of methanogenic biomass include the traditional enumeration methods, and recently, fluorescent-based methods [16]. However these methods are time consuming, and they do not reflect the metabolic capacity of the sludge. Furthermore, direct correlation of fluorescent F_{420} content and methanogenic activity remains doubtful since the amount of this coenzyme varies for different species and for different environmental conditions [5, 13].

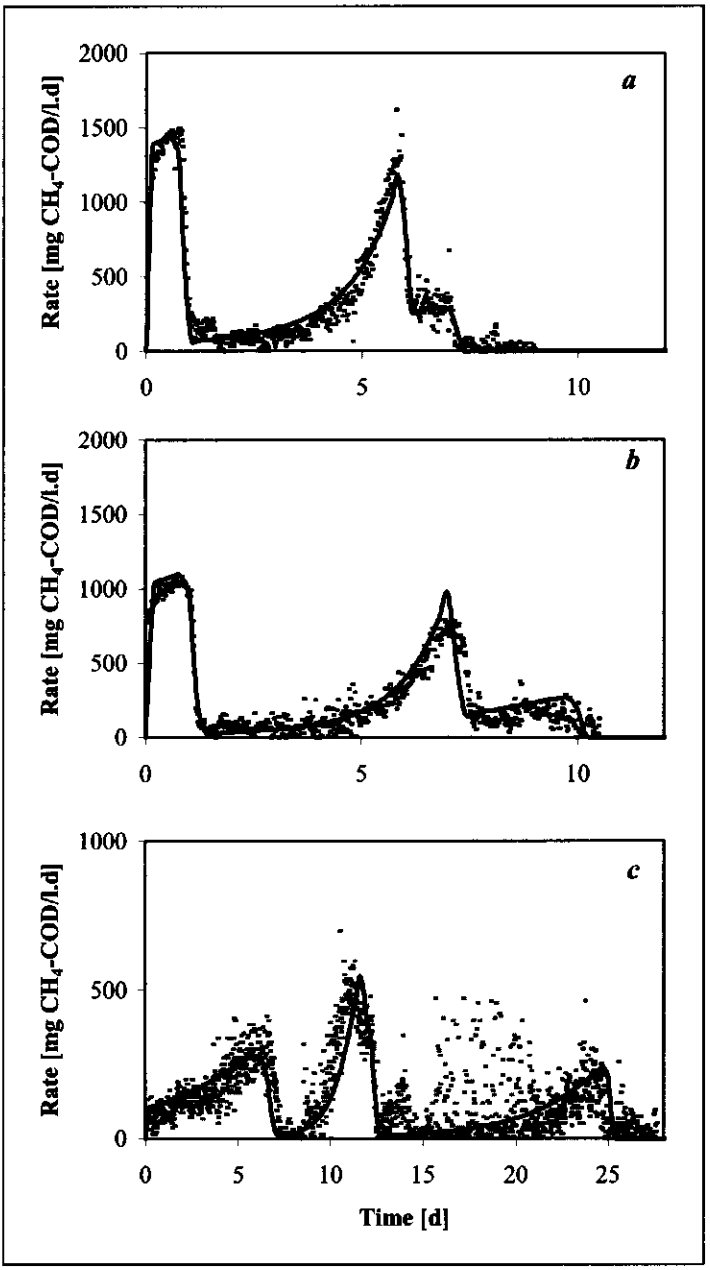

Figure 6.4 *Methane production rate from the conversion of a mixed substrate containing acetate, propionate and butyrate of three different sludge samples.(**a**) Brewery 1 sludge, (**b**) Paper mill sludge, and (**c**) Brewery 2 sludge. Markers correspond to measured data and the continuous lines represent the output of the model.*

The model presented here captures the anaerobic conversion of VFA. With the aid of this model and the on-line methane production rate measurements estimations of the concentration of active biomasses may be achieved.

Toxicity studies

Another important application of our methodology deals with toxicity studies of industrial chemicals. When treating industrial wastewater, possible toxic compounds may lead to a severe drop in the methanogenic activity. Batch toxicity test may contribute to the resolution of the question if (i) methanogenesis can recover during or after exposure and (ii) whether or not the toxic compound can be mineralized.

In the experiment described below the characteristics of formaldehyde toxicity to acetoclastic methanogenesis are shown as an example (Figure 6.5). At time zero, acetate was added and the methane production rate recorded. When the methane production rate reached a stable maximum value, the test compound was added. From Figure 6.5*b* it can be seen that the methane production rate drops immediately after the addition of the formaldehyde (phase II).

The rate, however, does not drop to zero and recovers (phase III). This recovery indicates the reversible character of the toxicity. This reversibility is attributed to formaldehyde conversion during phase II as shown by Gonzalez-Gil et al. [6]. The toxicity of the tested compound is also partly irreversible because the specific activity of the sludge was decreased. This decrease in activity is very clearly indicated by the difference between the maximum rate before the toxic addition (phase I) and the maximum rate recorded after the recovery (phase IV).

Gradual changes in the methane production rate can readily be seen from rate *vs.* time plots. For example, the recovery phase (phase III) is virtually "invisible" in the cumulative curve.

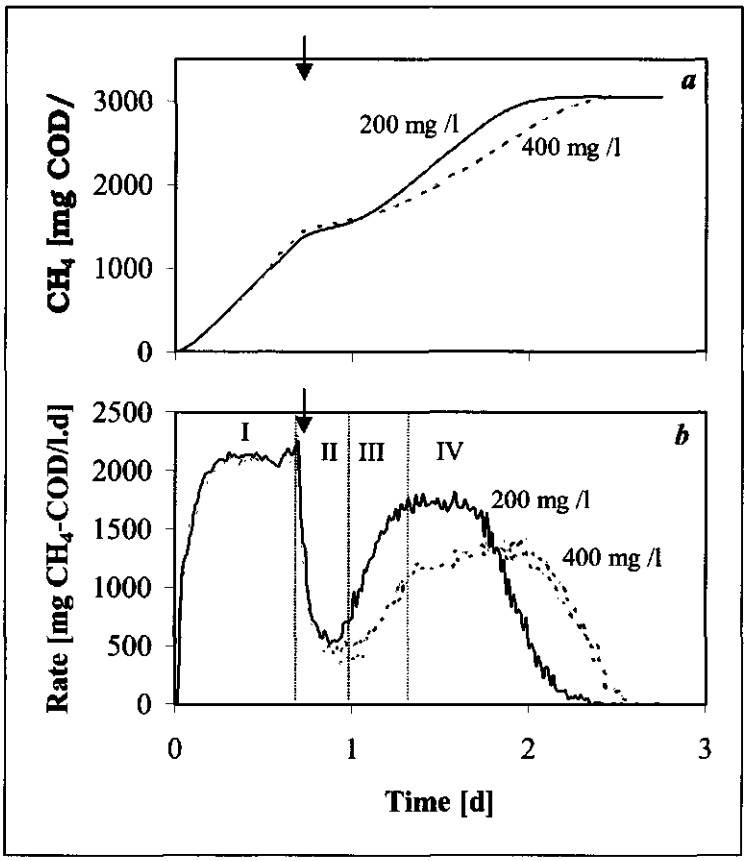

Figure 6.5 *Toxicity effect of formaldehyde to methanogenesis from acetate. Intact granular sludge was used in this test. Acetate was added at time zero at 3000 mg COD/l. Formaldehyde was dosed at the time indicated by the arrow and at the concentrations indicated in the plot area.* (***a***) *Cumulative methane production curve.* (***b***) *Rate curve.* I *indicates the maximum rate before the addition of the toxic compound,* II *indicates the inhibition period,* III *represents the recovery phase, and* IV *indicates the maximum rate after recovery.*

Not only detailed information about the reversible or irreversible character of the toxicity caused by a specific compound can be obtained, but also information concerning the fate of intermediates formed. This is illustrated by a toxicity test in which 1000 mg/l of formaldehyde was added during a batch conversion of 3000 mg acetate COD/l (Figure 6.6).

Immediately after the addition of formaldehyde the methane production rate dropped dramatically (phase II). However, at the end of phase II, a sharp peak in the methane production rate was observed (Figure 6.6*b*, phase III), followed by a lower and constantly increasing methane production rate phase (Figure 6.6*b*, phase IV). Since these two methane production rate phases had different shapes one may postulate that two different substrates were utilized. The later can be likely attributed to the conversion of acetate already present in the batch since its area corresponds with most of the COD added. However, the former indicates that methanogenesis was occurring either directly from the toxic compound (in this case formaldehyde) or from an intermediate product. This intermediate product has been detected as formate as indicated by specific experiments in our laboratory dealing with formaldehyde conversion in which formaldehyde is transformed into formate and methanol.

About two days after the depletion of acetate, another methane production rate phase appeared consisting of an exponential increase in the rate (Figure 6.6*b* phase V). The total amount of methane COD produced during this phase was ≈800 mg/l. This phase indicates the utilization of the ≈405 mg methanol COD/l added with the formaldehyde solution, plus the methanol formed during the conversion of formaldehyde.

The exponential increase in the methane production rate of this last phase also suggests that the methanol-consuming bacteria were present at low numbers.

In the above described batch test, 3000 mg COD/l of acetate were added plus 1066 mg COD/l of formaldehyde, and 405 mg COD/l of methanol. This methanol was present in the formaldehyde solution. Thus, in total 4471 mg COD/l were added. The total COD recovered as CH_4 as observed in the cumulative methane *vs.* time curve (Figure 6.6*a*) was ≈ 4400 mg COD/l. This COD balance indicates that the toxic compound was mineralized.

In general literature data is presented as cumulative production of gas [2, 3, 15]. Cumulative *vs.* time plots, in some cases, and particularly for toxicity assays, may be an inefficient way to analyze results because important information can be overlooked as was clearly shown here.

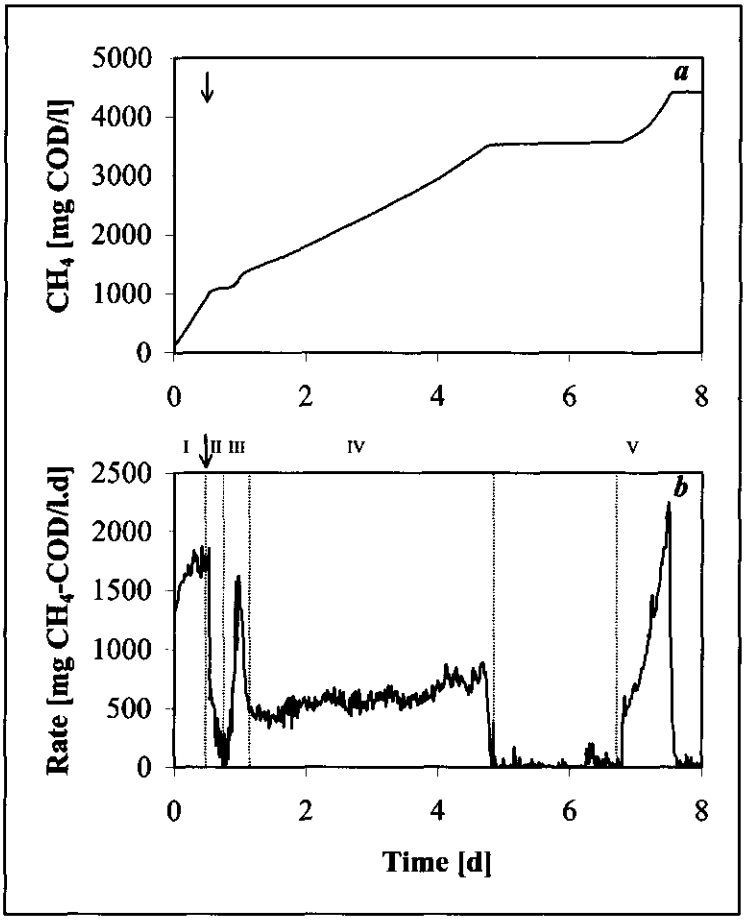

Figure 6.6 *Toxicity effect of formaldehyde to methanogenesis from acetate. Acetate was added at time zero at 3000 mg COD/l. Formaldehyde was dosed at the time indicated by the arrow at 1000 mg/l.* (***a***) *Cumulative methane production curve.* (***b***) *Rate curve. The roman numbers are explained in the text.*

Conclusions

The case studies presented here demonstrate the added value of the newly developed method for on-line measurement of the methane production rate.

The specific advantages of the method described here for conducting methanogenic activity, biodegradability and toxicity measurements are the following: Very accurate rate data can be obtained as shown by the different experiments presented. The method is simple and could be implemented by industry to be used as a routine analysis for the sludge. The system is specifically useful in experiments where strong changes in the methane production rate occur. For example with cumulative data and particularly with non on-line measurements, stages of low gas production or small changes of the gas production can be considered as analytical noise or even can be virtually "invisible".

In addition, our system can be used for farther other studies than the ones presented here. Studies dealing with continuous dosing of toxic compounds can well be performed. Also the system can be operated as a continuous reactor, and moreover, static substrate level tests can be implemented.

Some limitations of our system are that sludge with very low activity can give noisy recordings. Gas composition changes and air-pressure differences can have also an impact on the accuracy.

In summary, our methodology shows a straightforward way to evaluate kinetic data from single batch experiments. It can be proposed as a rational procedure to monitor the metabolic capacity of anaerobic sludge and also as a powerful tool to study methanogenic systems. A major advantage of the methodology deals with time flexibility concomitant with an on-line system. Furthermore, for metabolic studies, the number of liquid samples can be drastically reduced and can be done only at strategic times according to the development of the methane production rate curve.

Nomenclature

COD	chemical oxygen demand	
VSS	volatile suspended solids	
VFA	volatile fatty acids	
R_S	volumetric substrate consumption rate	mg S-COD/l.d
R_X	volumetric biomass growth rate	mg X-COD/l.d
R_P	volumetric product formation rate	mg P-COD/l.d
S	substrate concentration	mg S-COD/l
X	biomass concentration	mg X-COD/l
P	product concentration	mg P-COD/l
μ^{max}	maximum specific growth rate	d^{-1}
K_S	apparent affinity constant	mg COD/l
Y_{XS}	stoichiometric biomass yield on substrate	mg X-COD/mg S-COD
f_{sp}	COD-based stoichiometry for conversion of S into P	-
C2	acetate	
C3	propionate	
C4	butyrate	
CH_4	methane	
t	time	

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7

Summary and Concluding Remarks

Introduction

The phenomenon of granule formation and maintenance in UASB reactors has been exploited to extend further the application of anaerobic wastewater treatment. By introducing effluent recirculation and applying an improved gas-solid separator [6], the UASB system has been modified resulting in the development of the ultra-high rate EGSB reactor. Since EGSB systems can accept high liquid upflow velocities, high recirculation rates can be applied. This provides dilution of the influent wastewater, which makes the system very attractive for the treatment of industrial streams with biodegradable toxic compounds. In fact, the EGSB concept is increasingly being used for the treatment of waste streams from the chemical industry [1].

Frequently, wastes originating from chemical industries are unbalanced with respect to nutrients and/or micronutrients. Furthermore, in general these industrial effluents contain toxic compounds and EGSB systems, as mentioned above, may represent an attractive option for their treatment. However, since high recycle ratios are applied to reduce toxicity, this at the same time, may adversely affect substrate conversion rates due to mass transport limitations.

The main objectives of the work described in this thesis were to assess the kinetic impact of (i) nickel and cobalt limitations on the methanogenic degradation of methanol, (ii) the relative importance of mass transport phenomena in methanogenic granular sludge, and (iii) to characterize the toxicity and biodegradation of formaldehyde in the anaerobic conversion of methanotrophic substrates. Based on the obtained results, the practical implications of these factors for anaerobic treatment are discussed in this chapter.

Metal limitations

Since several methanogenic reactions are catalyzed by metallo-enzymes, anaerobic treatment is a process highly dependent on the availability of metals like nickel and cobalt. Previous research indicates that metal deficiencies can limit the performance of anaerobic systems and particularly the benefits of metal supplements for anaerobic treatment of industrial effluents have been recognized. So far, however, most of the information about metal deficiencies and enhancements in anaerobic systems reported in literature is fortuitous [11], since metal deficiencies were not the main subject of research. Reported data about "optimum" metal concentrations for anaerobic treatment varies by several orders of magnitude [14]. In order to establish optimum metal concentrations for anaerobic treatment it is necessary to understand several processes involved in the metal uptake by biomass. These processes include the

bioavailability of metals, the nature of metal transport systems and their kinetics. These process characteristics could be established for specific trophic groups so they could be extrapolated to the anaerobic consortia (i.e. granular sludge). Based on the waste composition and its anaerobic conversion metabolism, it could be possible to estimate the trophic composition of the granules and thus the metal requirements.

Trace metals like iron nickel and cobalt are main components of several enzymes involved in the metabolism of methanogenic archaea. Particularly in the case of anaerobic systems, the ubiquitous presence of sulfides resulting from sulfate reduction and organic matter mineralization will lead to strong metal precipitation as metal sulfides. These precipitated metals are not directly available for the biomass. Based on the results obtained in Chapter 2, we suggest that precipitation-dissolution kinetics of metal sulfides may play a key role in the bioavailability of nickel and cobalt during methylotrophic methanogenesis.

In batch systems, the available concentrations of nickel and cobalt were limiting during methylotrophic methanogenesis even at slug doses of these metals as high as 40 μM Ni and 40 μM Co. Based on the bacterial content of Ni and Co, and on biomass yield, only approximately 2 μM Ni and 0.9 μM Co would be required for conversion of the 10 g of methanol COD/l added in our experiments. However, spiked-dose experiments of metals showed that despite the high additions of metals (40 μM Ni and 40 μM Co), they seemed to be not available for the biomass. If chemical equilibrium was assumed, then the estimated dissolved metal concentrations should be less than 0.01 μM to 0.05 μM, which would not fulfill the nutrient requirements as indicated above. Therefore we conclude that metal shortage must have been compensated by dissolution of precipitated metal sulfides.

Results obtained in batch experiments in which Ni and Co were added continuously showed that nutrient limitations can be overcome if the essential metals are added continuously at a proper rate so that their availability in solution can fulfill the requirement for biomass activity and growth. Dosing rates of 0.05 to 0.2 μmol/h eliminated the observed limitations in the spiked-dose experiments. These rates of addition corresponded to metal to methanol ratios of 0.1-0.4 (μmol/g methanol-COD) and these values agree well with the calculated metal requirements based on the biomass yield [10], and Ni and Co content of methylotrophic methanogens (*Methanosarcina* sp.) grown on methanol [9]. While in the slug-addition experiments the methane production rate was limited by the dissolution rate kinetics of the metal sulfide precipitates, continuous addition of metals ensured free metal availability for biomass uptake.

In practice, in view of evidence that supplying metals enhances the treatment of several effluents [12, 13], there is a tendency to add nutrients/and or metals in excessive amounts. This could lead to inhibitory effects to the biomass or to metal accumulation in the sludge. We have shown that a rational supply of metals can be achieved, which in turn may open the possibility to either enhance or diminish the production of biomass depending on the treatment and remediation needs.

Important to mention is that another aspect hardly investigated is the role of precipitated metals in anaerobic systems as structural components of granular sludge [4, 15]. Thus the influence of metals may go far beyond their role as essential nutrients. In systems like EGSB-reactors, granular biomass is necessary for its operation, hence optimum metal dosage may also include that amount required to maintain physically the granular structure. Based on this reasoning metal dosage could also be used to enhance granule disruption in case that the diameter of the granules becomes so high that mass transport limitations and/or gas entrapment problems may occur. By reducing iron addition it was possible to reduce the mean diameter of granular sludge from a full-scale EGSB reactor (Zoutberg G., personal communication).

Mass transport

External mass transfer limitations may play an important role in anaerobic granular sludge at liquid upflow velocities lower than 1 m/h. It was found that at liquid upflow velocities exceeding 1 m/h liquid-film (external) mass transfer limitations normally can be neglected for acetate degrading methanogenic granular sludge. The reported reduction of apparent K_S-values at upflow velocities exceeding 1 m/h in previous research [5, 16], may be attributed to the reduction of preferential channeling of the wastewater and not to a direct effect on transport phenomena in anaerobic biofilms.

On the contrary, a clear increase in apparent K_S-value was found at increasing mean granule diameters. Herewith we have clearly shown that anaerobic biofilms can be internally transport limited. In addition we also demonstrated that substrate transport in the biofilm can be described by diffusion, i.e. there was no evidence of convective flow due to biogas production in the anaerobic granules.

Important to consider is that it is still necessary to improve the understanding on hydrodynamic characterization of EGSB systems in order to further optimize their performance. Depending on the operational conditions, the hydrodynamics could eventually be manipulated. Conditions favoring complete mix of the liquid will be desired in case of

substrate inhibited reactions or in case of presence of toxic compounds in the influent stream, while conditions favoring plug flow will be appropriate in most other cases.

Formaldehyde toxicity

A wider application of anaerobic digestion would involve the treatment of effluents containing toxic compounds. In this thesis, specifically, we assessed the characteristics of toxicity of formaldehyde. Formaldehyde is found in several waste streams not only as wastewater constituent, but also as added contaminant when formaldehyde is used as process disinfectant.

A sound knowledge of the mechanisms of toxicity of a certain compound allows the development and/or establishment of a rational design, as well as monitoring and control strategies. Here, the knowledge obtained in Chapters 4 and 5 is summarized. This information together with additional results about formaldehyde toxicity is used to intent a mathematical description of the anaerobic conversion and toxicity of formaldehyde by mixed methanogenic biomass.

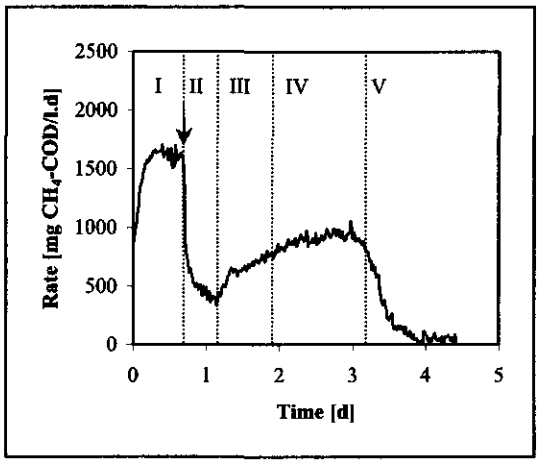

Figure 7.1 *Methane production rate during a toxicity test. The arrow indicates the time of formaldehyde addition at 600 mg/l.* (I) *Maximum rate before addition.* (II) *Inhibition* (III) *Recovery.* (IV) *Maximum rate after addition.*(V) *Depletion of acetate substrate.*

Reversible/Irreversible character of formaldehyde toxicity

Toxicity tests were performed by adding a methanotrophic substrate to anaerobic sludge, and the methane production rate was measured on-line. Once the methane production rate was at a maximum and constant value, formaldehyde was added at different concentrations.

An example of the on-line measurement of the methane production rate of a toxicity test is shown in Figure 7.1. This figure shows a general overview of the different phases observed during a toxicity experiment. The maximum rate before the addition of the toxic compound is indicated as phase I. The

drop on the methane production rate observed just after the formaldehyde addition is called the inhibition phase or phase II. The recovery phase or phase III comprises the moment that the methane production rate starts to increase until it reaches a new constant and maximum value. The start of the recovery phase can readily be identified. The end of the recovery phase was defined here as the point at which the slope (i.e acceleration) of the methane production rate changed noticeably. The new constant and maximum value after formaldehyde addition is represented as phase IV. The observed slight increases on the methane production rate in phase IV can be attributed mainly to biomass growth. Important to notice is that depending on the sludge used, the recovery phase (phase III) can show a slightly different 'shape' (see Figure 5.2) as will be shown later. Phase V indicates the depletion of substrate (in this example, acetate).

The characteristics of formaldehyde toxicity were independent of the methanotrophic substrate used. Slug addition of formaldehyde to formate, methanol, and acetate consuming sludges showed similar trends independent of the type of sludge used (Figure 7.2). Despite the high inhibition exerted by formaldehyde, the methane production rate partially recovered at all the concentrations tested. This recovery occurred when the formaldehyde concentration in the liquid was very low or virtually removed from the system (<50 mg/l). Formaldehyde in the system was transformed into formate and methanol (Chapter 5). Although the methane production rate recovered, its recovery was never complete. This can be clearly observed from the obtained difference between the maximum rate before addition (phase I) and the maximum rate after the recovery (phase IV). This decrease in the maximum methane production rate between phase I (Ro) and phase IV (Re), or irreversible toxicity, was directly related to the dosed amount of formaldehyde (Figures 7.2*b* and 7.2*d*).

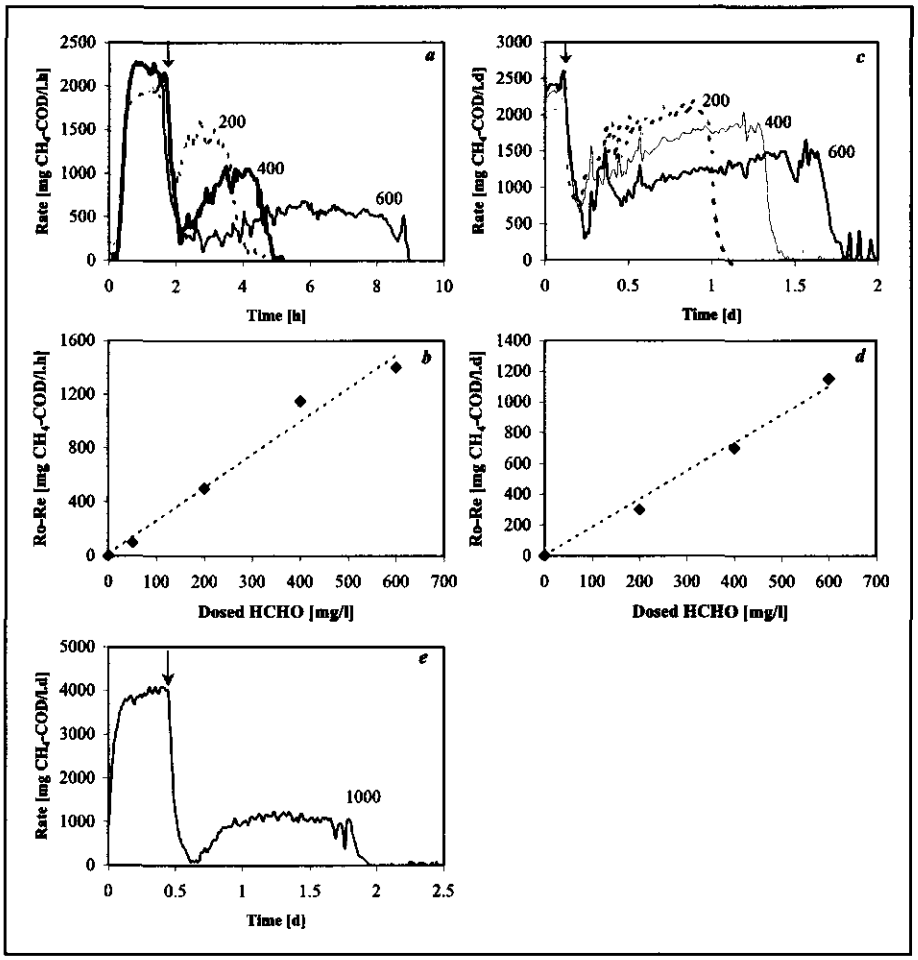

Figure 7.2 *Effect of slug additions of formaldehyde on the methane production rate of (a) methanol consuming sludge and (c) acetate consuming sludge. Decrease in the methane production rate between phase* I (Ro) *and phase* IV (Re), *equal to* Ro-Re, *at different formaldehyde doses for the (b) methanol consuming, and (d) acetate consuming sludge. Panel (e) shows the effect of a slug addition of formaldehyde during methanogenesis from formate. In all cases, the arrow indicates the time of addition, and the amount of formaldehyde added in mg/l is indicated beside each methane production rate line.*

Mode of formaldehyde addition (continuous *vs* slug)

Independent of the mode of dosing, either in continuous or in slug mode, the final decrease in the methane production rate always was the same. The formaldehyde concentration during the continuous dosing remained below 50 mg/l and 12 mg/l for the tests with methanol and acetate, respectively. However, despite the low formaldehyde concentrations in the bulk, apparently a certain amount of formaldehyde added to the system irrevocably reduces the methane production rate to a certain extent (Figure 7.3).

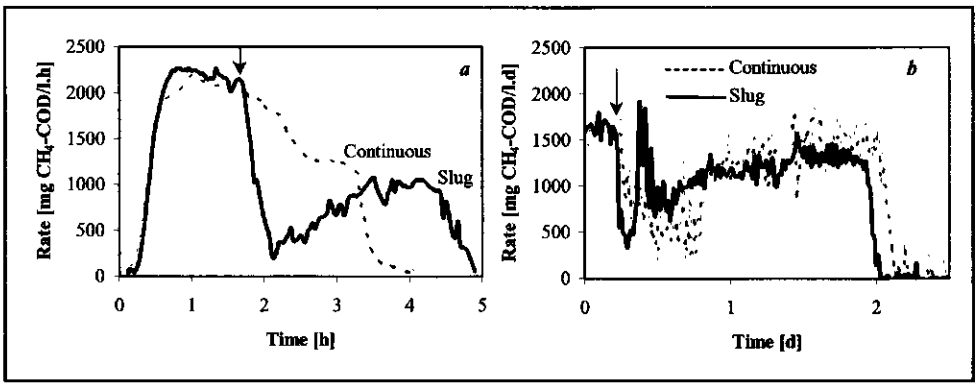

Figure 7.3 *Comparison of continuous vs. slug addition of formaldehyde. (**a**) Methanol consuming biomass. Dosing rate at 400 mg/l.h during 1 h and slug dose at 400 mg/l. (**b**) Acetate consuming biomass. Dosing rate at 50 mg/l.h during 10 h and slug dose at 500 mg/l. The arrow indicates the time of addition.*

This finding suggests that in order to avoid complete failure of the system, a balance between formaldehyde-related decay and biomass growth should be attained. Others have indicated that much higher concentrations of formaldehyde can be tolerated when added continuously [2, 8] and even that acclimatization of the sludge can occur [2, 7]. However, it should be considered that in those studies, likely growth of biomass may have compensated the formaldehyde-related decay and that possible growth of other biomass with a higher capacity of formaldehyde conversion may have occurred as well. The enrichment of biomass with specific organisms with the capacity to degrade toxic compounds frequently is referred as "acclimatization".

Effects of active biomass concentration and biomass diversity on toxicity response

In order to assess the relationship between formaldehyde toxicity and the amount of active biomass, two sets of experiments were conducted. In the first set, the same type of sludge was

used at two different initial sludge concentrations of 2 and 4 g VSS/l. The sludge for this test was sampled from the full-scale reactor in 1998 (see methods section Chapter 5). In the second set of experiments, different types of sludge were used. These sludges were sampled from the reactor in 1997, and in 1998. In all cases, acetate was used as main substrate at 3000 mg COD/l and formaldehyde was added in a slug mode at 1000 mg/l.

The results of experiments with the same type of sludge (i.e. samples 1998) showed that the degree of irreversible toxicity was proportionally related to the initial sludge concentration (Figure 7.4*a*). This can readily be observed from the differences between the rates before (phases I) and the rates after recovery (phases IV). Furthermore, the rate of formaldehyde conversion was directly related with the amount of initial biomass as can be observed in Figure 7.4*b*.

The results of experiments where different types of sludge (i.e samples 1997 *vs.* 1998) showed that the degree of irreversible toxicity was independent of the initial specific acetoclastic activity of the sludge. Although in these tests the specific acetoclastic activity was significantly different (Figure 7.4*c*), the formaldehyde conversion rate was similar (Figure 7.4*d*), thus indicating that the concentration of bacteria capable of formaldehyde conversion very likely was comparable in both sludges. This suggests that formaldehyde conversion is non-specific, and hence depends on the concentration present of various trophic groups with the capacity to convert formaldehyde. Hence, the biomass diversity may play a key role in the outcome of toxicity tests, particularly in the case of reactive toxicants like formaldehyde. The diverse active bacterial compositions of various anaerobic sludges may contribute to the explanation of the controversies with respect to toxicity values reported in literature.

In view of the above statement, we suggest that metabolic characterization of the sludge should become an integral component of toxicity studies. In Chapter 6 we described a newly developed methodology that can be used for this purpose.

We described in chapter 5 that formaldehyde is converted into methanol and formate (and/or hydrogen). Like in the experiments described in chapter 5, here also, conspicuous methane production rate peaks due to methanogenesis from formate were observed just after the inhibition period. In addition, methane production rate peaks from methanol were observed in all tests (Figure 7.4*a* and Figure 7.4*c*) after 6 days of the experimental period. The exponential shape of the methane production rate peaks of methanogenesis from methanol suggest that the methanol-consuming bacteria were present at low numbers.

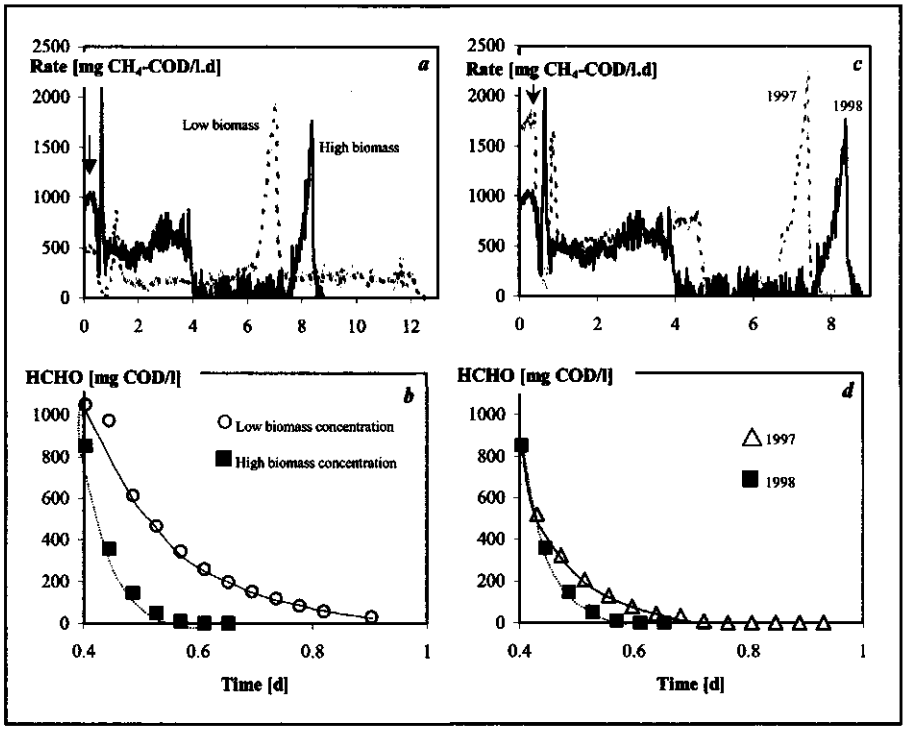

Figure 7.4 *Methane production rates showing the relationship between formaldehyde toxicity and the initial amount of active acetoclastic biomass. (**a**) The same type of sludge (i.e. sludge 1998) at initial sludge concentrations of 2 and 4 g VSS/l. (**c**) Different types of sludge, sampled in 1997 vs sampled in 1998 at initial sludge concentrations of 3 and 4 g VSS/l respectively. Panels (**b**) and (**d**) indicate the conversion of formaldehyde during the toxicity tests of the experiments shown in (**a**) and (**b**), respectively.*

Mathematical description of the anaerobic conversion and toxicity of formaldehyde by mixed methanogenic biomass

Based on the previous information, herewith a simple mathematical description of the conversion and toxicity of formaldehyde during methanogenesis from diverse methanotrophic substrates will be presented.

Substrate conversion and biomass growth

Acetate, formate and methanol are growth substrates for the biomass used, and their conversion can be described by Monod-type kinetics. Volumetric rates of substrate utilization (R_S), biomass growth (R_X), and product formation (R_P) in a batch system can be described by the following equations.

$$R_S = -\frac{\mu^{max} \cdot S}{K_S + S} \cdot \frac{X}{Y_{XS}} \tag{7.1}$$

$$R_X = -RS \cdot Y_{XS} \tag{7.2}$$

$$R_P = -(1 - Y_{XS}) \cdot R_S \tag{7.3}$$

where μ^{max} (d^{-1}) and K_S (mg COD/l) represent the maximum growth rate and the affinity constant, respectively. The COD-based yield, Y_{XS} (g X-COD/g S-COD), represents the biomass yield for growth of biomass X (mg X-COD/l) on substrate (S).

Formaldehyde conversion

Results of experiments with autoclaved and viable biomass indicated that formaldehyde transformation is a biological enzymatic conversion (Chapter 5). Based on measured CH_3OH/HCHO ratios of 0.7/2 to 0.9/2, we suggest that formaldehyde is converted to methanol and formate according to the following stoichiometry.

$$2HCHO + H_2O \rightarrow CH_3OH + HCOOH$$

Since it's conversion does not directly result in biomass growth, the rate of formaldehyde (R_{Fo}) conversion can be described by Michaelis-Menten kinetics. Our experiments furthermore showed that the formaldehyde conversion rate directly depends on the total concentration of active biomass present in the system (Figure 7.4*b*). Then, the following relation can describe the volumetric rate of formaldehyde conversion.

$$R_{Fo} = -\frac{V^{max} \cdot Fo}{K_m + Fo} \cdot X \tag{7.4}$$

where, Fo is the formaldehyde concentration (mg Fo-COD/l), V^{max} is the maximum specific formaldehyde conversion (mg Fo-COD/mg X-COD/d), and K_m is the Michaelis-Menten constant (mg Fo-COD/l).

Characteristics of formaldehyde toxicity

We observed that due to the presence of formaldehyde the anaerobic biomass becomes inhibited. This inhibition or toxicity is in part reversible since the methane production capacity is partially recovered after formaldehyde depletion. The reversible toxicity can be described by the following term, where Ki (mg Fo-COD/l) is the reversible inhibition constant.

$$\frac{Ki}{Ki + Fo}$$

We furthermore observed that formaldehyde toxicity is also in part irreversible since part of the methanogenic capacity of the biomass is lost after formaldehyde additions. Furthermore, our results show that (i) this irreversible toxicity depends on the amount or load of formaldehyde added (Figure 7.3), and that, (ii) this irreversible toxicity can likely be attributed to formaldehyde-enhanced decay. We approximate this enhanced decay by the following term, where k_D is the formaldehyde-related decay (l/mg Fo-COD.d) and X_T (mg X-COD/l) is the total biomass concentration.

$$k_D \cdot Fo \cdot X_T$$

Mathematical description

Based on the previous considerations, the next differential equations describing formaldehyde toxicity and conversion in a methanogenic batch system can be derived.

$$\frac{dS_{Ac}}{dt} = -\left[\frac{\mu_{Ac}^{max} \cdot S_{Ac}}{K_{S\,Ac} + S_{Ac}} \cdot \frac{X_{Ac}}{Y_{Ac}}\right] \cdot \left[\frac{Ki_{Ac}}{Ki_{Ac} + Fo}\right] \tag{7.5}$$

$$\frac{dS_{Moh}}{dt} = -\left[\frac{\mu_{Moh}^{max} \cdot S_{Moh}}{K_{S_{Moh}} + S_{Moh}} \cdot \frac{X_{Moh}}{Y_{Moh}}\right] \cdot \left[\frac{Ki_{Moh}}{Ki_{Moh} + Fo}\right] + 0.75 \cdot \frac{V^{max} \cdot Fo}{K_m + Fo} \cdot X_T \tag{7.6}$$

$$\frac{dS_{Fmate}}{dt} = -\left[\frac{\mu_{Fmate}^{max} \cdot S_{Fmate}}{K_{S_{Fmate}} + S_{Fmate}} \cdot \frac{X_{Fmate}}{Y_{Fmate}}\right] \cdot \left[\frac{Ki_{Fmate}}{Ki_{Fmate} + Fo}\right] + 0.25 \cdot \frac{V^{max} \cdot Fo}{K_m + Fo} \cdot X_T \tag{7.7}$$

$$\frac{dFo}{dt} = -\frac{V^{max} \cdot Fo}{K_m + Fo} \cdot X_T \tag{7.8}$$

$$\frac{dCH4}{dt} = (1 - Y_{Ac}) \cdot \frac{dS_{Ac}}{dt} + (1 - Y_{Moh}) \cdot \frac{dS_{Moh}}{dt} + (1 - Y_{Fmate}) \cdot \frac{dS_{Fmate}}{dt} \tag{7.9}$$

$$\frac{dX_{Ac}}{dt} = X_{Ac} \cdot \left[\frac{\mu_{Ac}^{max} \cdot S_{Ac}}{K_{S_{Ac}} + S_{Ac}} \cdot \left(\frac{Ki_{Ac}}{Ki_{Ac} + Fo}\right) - k_D \cdot Fo\right] \tag{7.10}$$

$$\frac{dX_{Moh}}{dt} = X_{Moh} \cdot \left[\frac{\mu_{Moh}^{max} \cdot S_{Moh}}{K_{S_{Moh}} + S_{Moh}} \cdot \left(\frac{Ki_{Moh}}{Ki_{Moh} + Fo}\right) - k_D \cdot Fo\right] \tag{7.11}$$

$$\frac{dX_{Fmate}}{dt} = X_{Fmate} \cdot \left[\frac{\mu_{Fmate}^{max} \cdot S_{Fmate}}{K_{S_{Fmate}} + S_{Fmate}} \cdot \left(\frac{Ki_{Fmate}}{Ki_{Fmate} + Fo} \right) - k_D \cdot Fo \right] \tag{7.12}$$

Model simulations

Simulations in order to describe several toxicity tests were conducted. In these toxicity tests dispersed granular sludge was used unless stated otherwise. This sludge originated from a full scale reactor from a brewery factory. The biomass capacity for acetate and formate conversion was relatively high, while its capacity to convert methanol was low. Acetate was added at the start of each toxicity experiment as main methanogenic substrate unless stated otherwise.

By measuring (i) the methane production rate on-line and (ii) the measurement of the liquid contents of formaldehyde, methanol, formate, and acetate, the experimental results could be simulated with the aid of the mathematical model. The parameter values used to run the mathematical model for the different simulations are shown in the appendix. Table 7.1 shows the estimated initial amount of active biomass for the different trophic groups involved.

Table 7.1 *Estimated biomass concentrations(mg X-COD/l) for the different simulations. The different methanotrophic substrates (S) and formaldehyde (Fo) are expressed in mg COD/l.*

	Different initial sludge concentration		Different initial formaldehyde addition		Formate as main substrate	Granular sludge with low formate activity
The simulation is shown in→	Figure 7.5		Figure 7.6		Figure 7.7	
S_{Ac}	2600	2400	3000	3000	0	3000
S_{Moh}	202.7	202.7	405	202.7	405	243
S_{Fmate}	0	0	0	0	3000	0
Fo	533.3	533.3	1066	533.3	1066	640
X_{Ac}	140	260	280	250	310	280
X_{Moh}	1.5 E-8	2.5 E-4	3 E-6	3 E-6	1.5 E-4	2 E-7
X_{Fmate}	110	200	290	240	135	15

The results of the simulation of tests with the same type of sludge and at different initial concentration of active biomass are shown in Figure 7.5. Formaldehyde was added at 533 mg COD/l (16.5 mM).

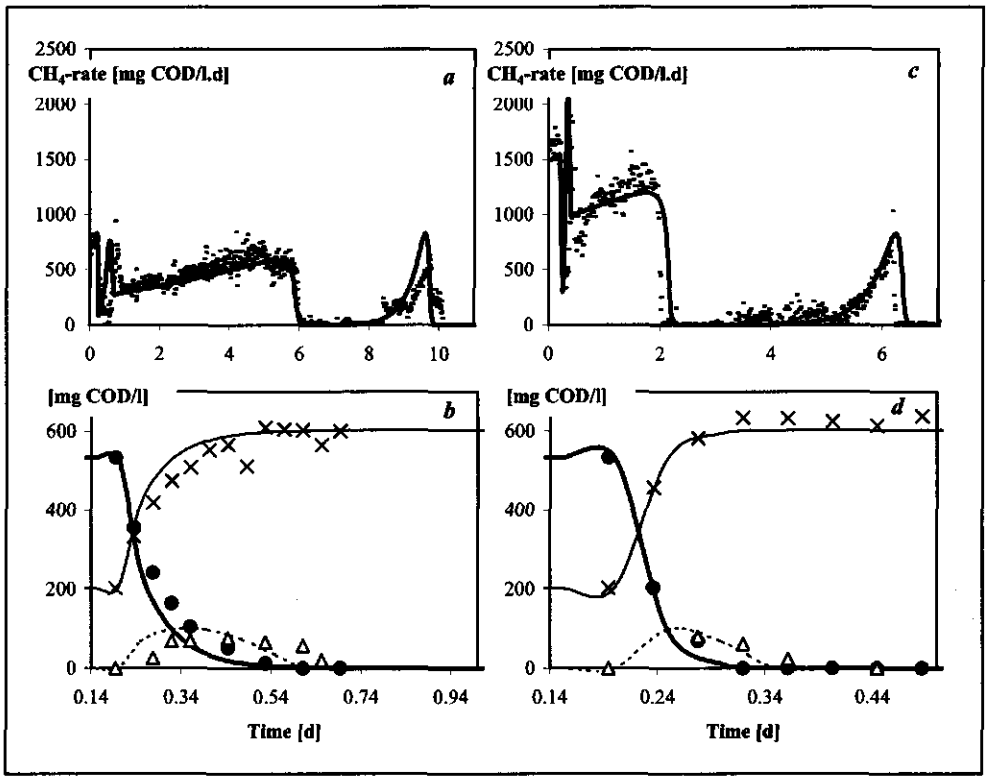

Figure 7.5 *Effect of slug addition of formaldehyde to the methane production rate at (a) low and (c) high active biomass concentration. (b) and (d) are respectively, for those tests, the liquid concentration of formaldehyde (●), methanol (×), and formate (Δ). In all cases markers correspond to measured data and continuous lines represent the output of the model. Formaldehyde in both tests was added at 533 mg COD/l (16.5 mM).*

The results of another set of toxicity experiments are shown in Figure 7.6. The initial amount of active biomass was similar, and formaldehyde was added at 1066 and 533 mg COD/l (33.3 and 16.5 mM, respectively).

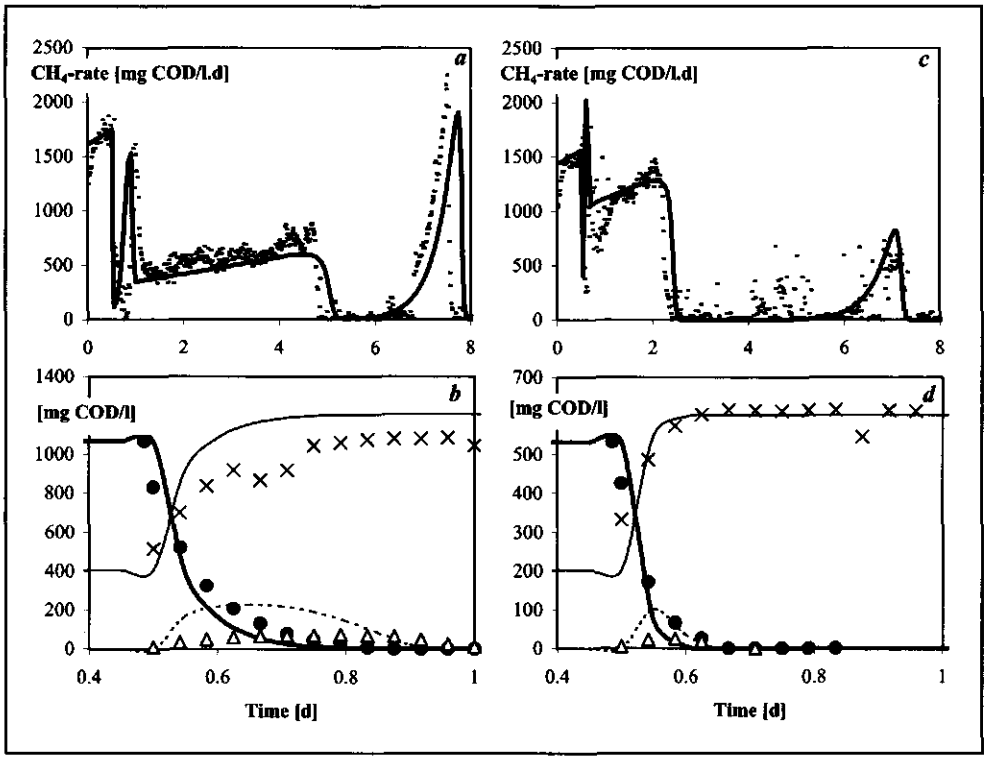

Figure 7.6 *Effect of slug addition of different formaldehyde concentrations to the methane production rate. Formaldehyde addition (**a**) at 1066 mg COD/l (33 mM), and (**c**) at 533 mg COD/l (16.5 mM). (**b**) and (**d**) are respectively for those tests, the liquid concentration of formaldehyde (●), methanol (×), and formate (Δ). In all cases markers correspond to measured data and continuous lines represent the output of the model.*

Interesting to note from Figures 7.6*b* and 7.6*d* is that the measured formate concentrations are lower than the expected based on the model calculations. However, the area of the calculated, and the measured methane production rate from the conversion of formate are similar. It is possible that the lower formate concentrations measured are the result of formate and H_2/CO_2

interconversion. Methanogens and some acetogens are capable of catalyzing this conversion [3]. Whether or not formate can be detected during formaldehyde toxicity test may depend on (i) the amount of formate and/or hydrogen consuming biomass and (ii) the rate of formate and H_2/CO_2 interconversion.

Furthermore, the methane production rate peak from formate consumption may not be observed if the formate consuming biomass present in the sludge is low. This is illustrated in Figure 7.7*a* in which granular sludge with low formate activity was used in a toxicity test on acetoclastic methanogenesis.

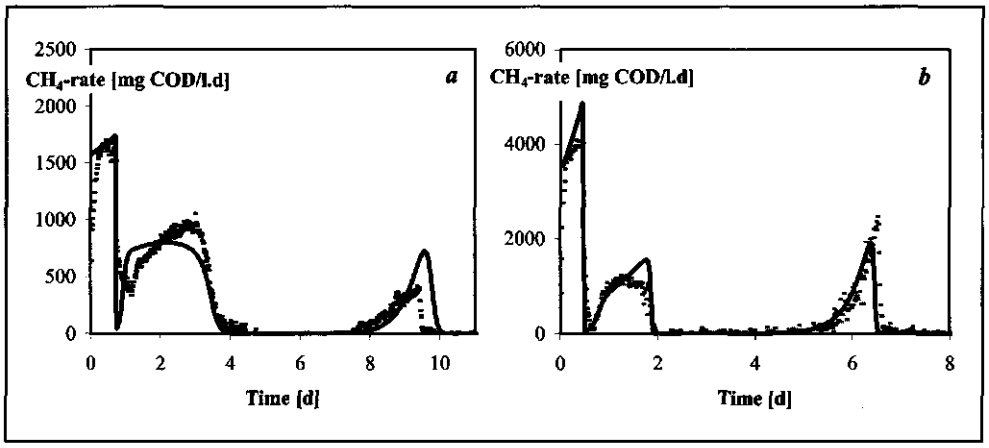

Figure 7.7 *Methane production rate during a toxicity test of (**a**) acetoclastic methanogenesis using granular sludge and (**b**) methanogenesis from formate using crushed sludge with high formate activity. In these tests, the liquid contents were not measured.*

In general we can conclude that the model gives a good description of the experimental results. However, we suggest that parameter values like Ki, should be estimated from experiments design specifically for that purpose and for different trophic groups.

We have stated that the ratio formaldehyde/non-toxic-substrate is of importance to avoid failure of the system (Chapter 4 and 5), hence it would be of interest to extrapolate the information obtained from the batch systems to a continuous system in order to enable a stable operation of such a system.

Appendix

Parameter		Value*	Units
μ_{Ac}^{max}		0.18	d^{-1}
μ_{Moh}^{max}	maximum growth rate	2.7	d^{-1}
μ_{Fmate}^{max}		0.8	d^{-1}
K_{SAc}		30 (100)	mg COD/l
K_{SMoh}	affinity constant for substrate	40 (130)	mg COD/l
K_{SFmate}		10 (100)	mg COD/l
Y_{Ac}		0.03	mg X-COD/mg COD
Y_{Moh}	yield coefficient	0.15	mg X-COD/mg COD
Y_{Fmate}		0.03	mg X-COD/mg COD
Ki_{Ac}		15	mg Fo-COD/l
Ki_{Moh}	inhibition constant	15	mg Fo-COD/l
Ki_{Fmate}		15	mg Fo-COD/l
K_D	formaldehyde- enhanced decay	0.03	l/mg Fo-COD.d
V^{max}	maximum formaldehyde specific conversion rate	62 (70)	mg Fo-COD/mg X-COD.d
K_m	Michaelis-Menten constant	400	mg Fo-COD/l
subscripts			
Ac	acetate		
Moh	methanol		
Fmate	formate		

* Parameter values used for the simulations. The values in parenthesis were used for the simulations of the experiments with granular sludge.

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8

Summary

Samenvatting

Resumen

Summary

The main objectives of the work described in this thesis were to assess the kinetic impact of (i) nickel and cobalt limitations on the methanogenic degradation of methanol, (ii) the relative importance of mass transport phenomena in methanogenic granular sludge, and (iii) to characterize the toxicity and biodegradation of formaldehyde in the anaerobic conversion of methanotrophic substrates.

Metal limitations

Trace metals like iron nickel and cobalt are main components of several enzymes involved in the metabolism of methanogenic archaea. Particularly in the case of anaerobic systems, the ubiquitous presence of sulfides resulting from sulfate reduction and organic matter mineralization will lead to strong metal precipitation as metal sulfides. These precipitated metals are not directly available for the biomass.

In **Chapter 2**, batch experiments are described that demonstrated that the bioavailable concentration of metals were limited during methanogenesis from methanol. If the metal uptake rate is higher than the rate of dissolution of metal sulfide precipitates, kinetic limitations for the biomass may prevail.

The results of batch experiments in which Ni and Co were added continuously showed that *nutrient limitations can be overcome if the essential metals are added continuously at a proper rate so that their availability in solution can fulfill the requirement for biomass activity and growth.* The metal dosing rates utilized range from 0.05 to 0.2 μmol/h corresponding to metal to methanol ratios of 0.1-0.4 (μmol/g methanol-COD) and these values agree well with the calculated metal requirements based on the biomass yield and Ni and Co content of methylotrophic methanogens (*Methanosarcina* sp.) grown on methanol. While in the experiments where metals were added in a slug mode, the methane production rate was limited by the dissolution rate kinetics of the concerning metal sulfide precipitates. This apparently is not the case in the experiments where the metals were supplied in continuous mode despite the fact that the total amount of metals added was significantly lower in that case.

In practice, in view of the evidence that the supply of metals enhances the treatment of several effluents, there exists a tendency to supply metals in excessive amounts. This could lead to inhibitory effects to the biomass and/or to metal accumulation in the sludge, and any how to a waste of chemicals and to higher costs of treatment. We have clearly shown in our experiments that *a much more rational supply of metals can be achieved, which in turn may*

also open the possibility to either enhance or diminish the production of biomass depending on the established treatment needs.

Mass transport

Kinetic properties of acetate degrading methanogenic granular sludge of different mean diameters were assessed at different upflow velocities (**Chapter 3**). Using this approach, the influence of internal and external mass transport could be assessed. It was found that *at liquid upflow velocities exceeding 1 m/h liquid-film (external) mass transfer limitations normally can be neglected for acetate degrading methanogenic granular sludge.* The reported reduction of the value of the apparent affinity constant (K_S) at upflow velocities exceeding 1 m/h in previous research, may be attributed to the reduction of preferential channeling of the wastewater and not to a direct effect on transport phenomena in anaerobic biofilms.

On the contrary, a clear increase in apparent K_S-value was found at increasing mean granule diameters. Herewith we have clearly shown that *anaerobic biofilms can be internally transport limited.* In addition we also demonstrated that substrate transport in the biofilm can be described by diffusion, and that *there was no evidence of convective flow due to biogas production in the anaerobic granules.*

Formaldehyde toxicity

Experimental results described in **Chapter 4** and **Chapter 5**, demonstrated that *methanogenesis from formaldehyde mainly occurred after intermediate formation of methanol and formate.* Since the formaldehyde conversion rate declined at increasing initial formaldehyde concentrations, we hypothesized that some enzymes and/or cofactors involved in formaldehyde conversion were denaturated at elevated formaldehyde concentrations.

Furthermore, the results described in these two chapters indicate that the characteristics of formaldehyde toxicity were independent of the methanotrophic substrate (methanol or acetate) used. *Formaldehyde toxicity was found to be in part reversible* because the methane production rate partially recovered after formaldehyde depletion from the system. The length of the time period for reversible inhibition of the methane production rate was dependent on the formaldehyde concentration imposed to the system. Since the recovery of the methane production rate after formaldehyde depletion was not complete, we conclude that *formaldehyde toxicity is also in part irreversible.* The degree of irreversible toxicity as indicated by the loss in the methane production rate was directly related to the amount of formaldehyde added.

Independent of the mode of dosing, either in continuous or in slug mode, the final decrease in the methane production rate was linearly related to the amount of formaldehyde dosed and

independent of the sludge concentration or the initial formaldehyde concentration utilized. Despite the low formaldehyde concentrations in the bulk in the experiments where formaldehyde was dosed continuously, apparently *a certain amount of formaldehyde added to the system irrevocably reduces the methane production rate to a certain extent.* This finding suggests that in order to avoid complete failure of the system, a balance between formaldehyde-related decay and biomass growth should be attained.

Results (**Chapter 7**) of toxicity experiments furthermore showed that *the degree or irreversible toxicity was proportionally related to the initial sludge concentration.*

The results of experiments conducted with different types of sludge showed that *the degree of irreversible toxicity was independent of the initial specific acetoclastic activity of the sludge.* Although in these tests the specific acetoclastic activity was significantly different, the formaldehyde conversion rate was similar, thus indicating that the concentration of bacteria capable of formaldehyde conversion very likely was comparable in both sludges. This suggests that formaldehyde conversion is non-specific, and hence depends on the concentration present of various trophic groups with the capacity to convert formaldehyde. Hence, *the biomass diversity may play a key role in the outcome of toxicity tests*, particularly in the case of reactive toxicants like formaldehyde. The diverse active bacterial compositions of various anaerobic sludges may contribute to the explanation of the controversies with respect to toxicity values reported in literature.

In view of the above we recommend that metabolic characterization of methanogenic sludge should be an integral component of toxicity studies conducted with mixed methanogenic populations. In **Chapter 6** we described a newly developed methodology that can be used for this purpose. The two main features of the method are that the methane production is measured on-line using a relatively cheap system, and that the methane production data can be plotted as rate *vs.* time curves. The case studies presented in this chapter show that very accurate kinetic data can be obtained. The method is specifically useful in experiments where strong changes in the methane production occur, and it is proposed as a powerful tool to study methanogenic systems. Furthermore, the method is simple and could be implemented by industry to be used as a routine analysis for the sludge.

Samenvatting

Het doel van het in dit proefschrift beschreven onderzoek was (i) het karakteriseren van de invloed van nikkel- en kobaltlimitaties op de methanogene afbraak van methanol, (ii) het kwantitatief karakteriseren van het relatieve belang van massatransport-limitaties in methanogeen korrelslib, en (iii) het karakteriseren van de toxiciteit en afbreekbaarheid van formaldehyde gedurende de anaërobe afbraak van methanotrofe substraten.

Metaal-limitaties

Sporenelementen als ijzer, nikkel en kobalt zijn belangrijke componenten van verschillende enzymen van methanogene archaea. De aanwezigheid van sulfides in methanogene systemen als gevolg van sulfaatreductie en de afbraak van organisch materiaal, leidt tot de vorming van zeer slecht oplosbare metaalsulfiden. De geprecipiteerde metalen zijn niet direct beschikbaar voor bacteriële opname.

De in **Hoofdstuk 2** gepresenteerde resultaten laten zien dat in batch-systemen de concentratie metalen limiterend was gedurende de methanogene afbraak van methanol. Indien de opnamesnelheid van metalen hoger is dan de snelheid waarmee metaalsulfide neerslagen oplossen, wordt de omzetting van substraat gelimiteerd door de beschikbare hoeveelheid opneembaar metaal. De resultaten van experimenten waarbij nikkel en kobalt continue aan een batch reactor werden gedoseerd toonden aan dat *limitaties in de biobeschikbare concentratie metalen kunnen worden voorkomen door continue dosering van deze metalen.* De toegepaste doseringssnelheden gedurende deze experimenten varieerden van 0.005 tot 0.2 μM/uur, hetgeen overeenkomt met metaal tot methanol verhoudingen tussen 0.1 en 0.4 (μmol/g methanol). Deze waarden komen overeen met de uit de biomassa opbrengst en de nikkel- en kobaltgehaltes van methylotrofe methanogenen (*Methanosarcina* sp.) berekende metaalbehoeften. Beperkte methaanproductiesnelheden werden waargenomen bij experimenten waarbij de metalen puntsgewijs werden gedoseerd, waarschijnlijk als gevolg van limitaties in de snelheid van oplossen van de metaalsulfide neergeslagen. Continue dosering van de metalen gaf geen aanleiding tot voornoemde kinetische limitaties, ondanks dat de bij continue dosering toegevoegde concentraties aanzienlijk lager waren dan bij batch-dosering.

In de praktijk van anaërobe afvalwaterzuivering worden sporenelementen veelal in overmaat gedoseerd, teneinde limitaties in bacteriële groei te voorkomen. In principe kan dit leiden tot inhibitie van de biomassa en/of tot accumulatie van metalen in het slib, en bovendien draagt het doseren van sporenelementen in belangrijke mate bij aan de kosten van de bedrijfsvoering van anaërobe reactoren. Ons onderzoek heeft aangetoond dat *een op rationele grondslagen*

gebaseerde dosering van metalen mogelijk is, hetgeen het mogelijk maakt om bacteriële groei te stimuleren dan wel te beperken, afhankelijk van de operationele behoefte.

Massatransport

De kinetische eigenschappen van acetaat afbrekend methanogeen korrelslib van verschillende diameters is onderzocht bij verschillende vloeistofopstroomsnelheden (**Hoofdstuk 3**). Op deze wijze konden de invloeden van zowel interne als externe massatransportlimitaties worden onderzocht. De resultaten hebben aangetoond dat *bij toepassing van vloeistofopstroomsnelheden hoger dan 1 m/h, externe massatransportlimitaties normaal gesproken kunnen worden verwaarloosd voor acetaat afbrekend methanogeen korrelslib.* De in de literatuur beschreven afname van de schijnbare affiniteitsconstante (K_S) bij opstroomsnelheden hoger dan 1 m/u is het gevolg van de afname van de vorming van voorkeursstromen in het slibbed, en niet van een direct effect op extern massatransport rond de slibkorrel.

Daarentegen werd een duidelijke toename in de schijnbare K_S-waarde waargenomen bij toenemende diameters van de slibkorrel. Hiermee hebben we aangetoond dat *interne diffusie-limitaties een belangrijke rol kunnen spelen bij methanogene biofilms.* Bovendien konden de experimentele resultaten worden beschreven met een op moleculaire diffusie gebaseerd model, en zodoende blijkt dat *de kwantitatieve invloed van convectie als gevolg van biogasvorming in de slibkorrel is te verwaarlozen.*

Formaldehyde-toxiciteit

In **Hoofdstuk 4** en **Hoofdstuk 5** beschreven experimentele resultaten hebben aangetoond dat de anaërobe afbraak van formaldehyde plaatsvindt via de intermediairen formiaat en methanol. Aangezien de snelheid van formaldehyde omzetting afneemt bij een toenemende dosering, stellen wij voor dat enzymen en/of co-factoren die betrokken zijn bij de omzetting van formaldehyde, worden gedenatureerd bij hoge formaldehyde concentraties.

Bovendien is gebleken dat de karakteristieken van formaldehyde-toxiciteit onafhankelijk waren van het gebruikte methanogene co-substraat (methanol of acetaat). Formaldehyde-toxiciteit is zowel reversibel als irreversibel. *Reversibele toxiciteit van formaldehyde blijkt uit een duidelijke toename in de methaanproductiesnelheid na omzetting van formaldehyde.* De lengte van de tijdsperiode van reversibele inhibitie van de methaanproductiesnelheid was afhankelijk van de gebruikte concentratie formaldehyde. Aangezien het herstel van de methaanproductiesnelheid na volledige omzetting van formaldehyde niet volledig was, *is formaldehyde-toxiciteit bovendien gedeeltelijk irreversibel.* De mate van irreversibele toxiciteit bleek direct gerelateerd aan de gedoseerde hoeveelheid formaldehyde.

De uiteindelijke afname in de methaanproductiesnelheid bleek onafhankelijk van de wijze waarop formaldehyde wordt gedoseerd (continue dosering of puntdosering). Ondanks de lage formaldehyde-concentraties bij continue dosering werd een vergelijkbare afname in de methaanproduktiesnelheid waargenomen als bij puntdosering. Dit suggereert dat *dosering van een bepaalde hoeveelheid formaldehyde in alle gevallen zal leiden tot een bepaalde afname in de methaanproductiesnelheid.* Hierop gebaseerd kan worden gesteld dat teneinde een stabiel systeem voor anaërobe behandeling van formaldehyde-houdend afvalwater te verkrijgen, een balans moet worden verkregen tussen formaldehyde gerelateerde afsterving en groei van biomassa.

Resultaten verkregen middels toxiciteitsexperimenten (**Hoofdstuk 7**) geven aan dat *de mate van irreversibele toxiciteit lineair is gerelateerd aan de toegepaste initiële slibconcentratie.* Bij toepassing van verschillende typen slib bleek dat *de mate van irreversibele toxiciteit onafhankelijk was van de initiële specifieke acetoclastische methanogene activiteit van het slib.* Ondanks dat gedurende deze experimenten de specifieke acetoclastische methanogene activiteit van beide slibsoorten sterk verschilde, werd formaldehyde met een vergelijkbare snelheid omgezet, hetgeen aangeeft dat de concentratie micro-organismen met de specifieke capaciteit om formaldehyde om te zetten eveneens vergelijkbaar was. Dit suggereert dat de omzetting van formaldehyde een niet-specifieke reactie is en dat daarmee de omzettingssnelheid van formaldehyde afhankelijk is van de concentratie van verschillende typen organismen in het slib. *De bacteriële diversiteit van het slib kan dus een belangrijke rol spelen bij toxiciteitsexperimenten,* met name in geval van reactieve toxische verbindingen zoals formaldehyde. Dit aspect kan mogelijk een bijdrage leveren aan de grote diversiteit in toxiciteitsdata beschreven in de literatuur.

Uitgaande van de hierboven genoemde argumenten stellen wij voor dat karakterisering van de metabole eigenschappen van methanogeen slib een integraal onderdeel zou moeten zijn van toxiciteitsexperimenten met methanogeen slib. In **Hoofdstuk 6** wordt een nieuwe methode beschreven die kan worden gebruikt voor dit doel. De twee belangrijkste eigenschappen van de beschreven methode zijn dat de methaanproductiesnelheid continue wordt geregistreerd en dat de methaanproductiesnelheid als functie van de tijd grafisch kan worden weergegeven. De in Hoofdstuk 6 gepresenteerde voorbeelden tonen aan dat met deze methode nauwkeurige kinetische data kunnen worden verkregen. De methode is met name nuttig voor experimenten waarbij sterke fluctuaties in de methaanproductiesnelheid optreden. De beschreven methode is bovendien simpel en kan ook in de praktijk worden toegepast voor slibkarakterisering.

Resumen

Los objetivos principales del trabajo descrito en esta tésis fue valorar el impacto en la cinética de (i) las limitaciones de níquel y cobalto durante la degradación metanogénica de metanol, (ii) la importancia relativa de los fenómeno del transporte de masa en lodo granular metanogénico, y (iii) de caracterizar la toxicidad y la biodegradación de formaldehido durante la conversión anaeróbica de substratos metanotróficos.

Limitación de Metales

Los metales traza como el hierro, níquel y cobalto son principales componentes de diversas enzimas involucradas en el metabolismo de microorganismos metanogénicos. Particularmente en el caso de sistemas anaeróbicos, la ubicua presencia de sulfuros que resultan del proceso de sulfato reducción, asi como de la mineralización de material orgánico, conduce a una alta precipitación de metales como sulfuros metálicos. Estos metales precipitados no estan directamente disponibles para ser utilizados por la biomasa.

En el **Capítulo 2** se describen experimentos en lote que demostraron que la concentracion de metales bio-disponibles fue limitante durante la metanogénesis a partir de metanol. Cuando la velocidad de consumo de metales es mayor que la velocidad de disolución de los precipitados de sulfuros metálicos, pueden prevalecer condiciones de cinética limitantes para la biomasa.

Los resultados de experimentos en lote en los que Ni y Co fueron añadidos contínuamente mostraron que *las limitaciones de nutrientes pueden ser paliadas si los metales esenciales son suministrados continuamente y a una tasa apropiada de forma que su disponibilidad en la solución satisfaga los requerimientos de la biomasa para su actividad y crecimiento.* Las tasas de dosificación de metales utilizadas fueron de 0.05 a 0.2 μmol/h, correspondiendo con relaciones de metal a metanol de 0.1 a 0.4 (μmol/g metanol-COD) y éstos valores están de acuerdo con los requerimientos de metal calculados a partir del rendimiento de la biomasa y del contenido de Ni y Co de microorganismos metanogénicos metilotróficos (*Methanosarcina* sp.) cultivados en metanol. En los experimentos en que los metales fueron añadidos en una sola dosis, la velocidad de producción de metano estuvo limitada por la cinética de disolución de los sulfuros metálicos precipitados. Esto aparentemente no fue el caso en los experimentos de dósis de metal continua pese al hecho de que, en este caso, la cantidad de metales dosificada fue significativamente menor.

En vista de la evidencia de que el suministro de metales mejora el tratamiento de muchos efluentes, hay en la práctica una tendencia a añadir metales en cantidades excesivas. Esto puede traer efectos inhibitorios a la biomasa y/o a una acumulación de metales en el lodo, a un malgasto de compuestos químicos y por tanto a mayores costos en el tratamiento. Hemos

mostrado con nuestra investigación que *un suministro mas racional de los metales puede ser calculado, el cual puede abrir la posibilidad ya sea de estimular o disminuir la producción de biomasa dependiendo de las necesidades durante el tratamiento.*

Transporte de masa

Se estimaron las propiedades cinéticas del lodo granular que degrada acetato variando, en diversas corridas, el diametro promedio del lodo granular y usando diferentes velocidades de flujo ascendente (**Capítulo 3**). Con este diseño experimental, se pudo estimar la influencia del transporte de masa externo e interno. Se encontró que *a velocidades de flujo ascendente mayores de 1 m/h las limitaciones en la transferencia de masa líquido-biopelícula (externa) normalmente pueden ser despreciadas, esto para el caso de lodo metanogénico granular durante la degradación de acetato.* La reducción en los valores de la constante de afinidad aparente (K_S), a velocidades de flujo ascendente mayores a 1 m/h reportados en investigaciones previas, podria ser atribuido a la disminución de canales preferenciales del agua residual y no a un efecto directo en los fenómenos de transporte en biopelículas anaeróbicas.

Por el contrario, se encontró un incremento claro en el valor de K_S aparente a medida que se incremento el diametro promedio del gránulo. Por consiguiente, hemos mostrado claramente que *las biopelículas anaeróbicas pueden estar limitadas en cuanto al transporte de masa interno.* Adicionalmente, también demostramos que el transporte del substrato en la biopelícula puede ser descrito por difusión y que *no hubo evidencia de flujo convectivo debido a la producción de biogas en los gránulos anaeróbicos.*

Toxicidad del formaldehido

Los resultados experimentales descritos en el **Capítulo 4** y **Capítulo 5**, demuestran que la metanogénesis a partir del formaldehido ocurre principalmente vía la formación intermedia de metanol y formato. Debido a que la velocidad de conversión del formaldehido disminuyó a medida que se incrementaron las concentraciones iniciales de formaldehido, se hipotetizó que algunas enzimas y/o cofactores enzimáticos podrían haberse desnaturalizado a concentraciones elevadas de formaldehido. Mas aún, los resultados presentados en estos dos capítulos indican que las características de la toxicidad del formaldehido fueron independientes del substrato metanotrófico utilizado (metanol o acetato). *La toxicidad del formaldhído es en parte reversible* porque la velocidad de producción de metano se recuperó parcialmente una vez que la concentración de formaldehido en el sistema fue baja o nula. La magnitud del periodo de la inhibición reversible fue dependiente de la concentración de formaldehido impuesta en el sistema. Debido a que la recuperación de la velocidad de producción de metano después del consumo de formaldehido no fue completa, concluímos

que *la toxicidad del formaldehido es también en parte irreversible.* La toxicidad irreversible indicada por la pérdida en la velocidad de producción de metano, esta directamente relacionada con la cantidad de formaldehido dosificado. Independientemente de la forma de dosificación (en continuo o en un pulso) la pérdida final en la velocidad de producción de metano fue la misma. Pese a las bajas concentraciones de formaldehido en el liquido del reactor, aparentemente, *una cierta cantidad de formaldehido dosificado al sistema irrevocablemente reduce la velocidad de la producción de metano en un cierto grado.* Este hallazgo sugiere que si se quiere evitar el fallo completo del sistema, es necesario obtener un balance entre el crecimiento de la biomasa y la mortandad causada por el formaldehido.

Resultados (**Capítulo 7**) de experimentos de toxicidad también mostraron que *el grado de toxicidad irreversible esta proporcionalmente relacionado a la concentración inicial de biomasa.* Los resultados de experimentos de toxicidad en los que se utilizaron diferentes tipos de lodo mostraron que *el grado de toxicidad irreversible fue independiente de la actividad especifica acetoclástica inicial del lodo.* Aun cuando en estas pruebas la actividad especifica acetoclástica fue significativamente diferente, la velocidad de conversion de formaldehido fue semejante, sugiriendo que, la concentracion de bacterias capaz de convertir el formaldehido estaban probablemente en cantidades comparables en ambos lodos. Esto sugiere que la conversión de formaldehido es no-específica y por tanto depende de la concentracion de biomasa de todos los grupos tróficos en el lodo. De aqui que, *la diversidad en la biomasa puede jugar un papel clave en los resultados de las pruebas de toxicidad,* particularmente en el caso de tóxicos muy reactivos como el formaldehido. Este aspecto puede contribuir a la explicación de las controversias que asoman cuando se comparan valores de toxicidad reportados en la literatura.

En vista de lo anteriormente expuesto, recomendamos que la caracterización metabólica del lodo debe convertirse en un componente importante en los estudios de toxicidad. En el **Capítulo 6** describimos una nueva metodología que puede ser usada para este propósito. Dos características importantes del método son que, la producción de metano es medida en linea empleando un sistema relativamente barato y que los datos capturados pueden ser graficados directamente como curvas de velocidad de producción de metano en función del tiempo. Los casos presentados en este capítulo muestran que con este sistema se pueden obtener datos cinéticos muy precisos. El método es específicamente útil en experimentos en los que ocurren cambios fuertes en la producción de metano y es propuesto como una poderosa herramienta para estudiar sistemas metanogénicos. Además es un método simple que podría ser implementado en la industria para ser usado de rutina como un método de caracterización de lodos.

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Finally, I hope that the readers find useful information in this thesis.

About the author

The author of this dissertation received a B.S. in biology from the Universidad Autónoma de México in 1990. She was afterwards involved in short research projects at the Engineering Institute of the same university, and at the Universidad Autónoma Metropolitana-Iztapalapa. In 1994 she received a M.S. degree from the Institute for Infrastructural Hydraulic and Environmental Engineering in Delft. The research of her M.S. was conducted at the University of Wageningen, and the subject was the effects of calcium precipitation on the biological activity and physical-chemical structure of anaerobic sludge. From 1996 to 1999 she worked as guest researcher in the Environmental Technology group of the Wageningen University. The results of her research are presented in this dissertation. The author will work at the Wageningen University as post-doc in a project dealing with the processes involved for the optimization of heavy metals for anaerobic treatment.