

# **Thermophilic Anaerobic Wastewater Treatment; Temperature Aspects and Process Stability**

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VERGELIJKEND ONDERZOEK  
VAN DE WERKING VAN  
MAGNETIT

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Temperature Aspects and Process Stability**

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## STELLINGEN:

- 1 Het herhaaldelijk genoemde euvel dat thermofiele hoogbelaste anaërobe zuiverings-systemen per definitie samengaan met een hoge concentratie aan vetzuren in het effluent, is te wijten aan onvoldoende inzicht ten aanzien van de toe te passen procestechnologie.  
Dit proefschrift
- 2 Niet de anaërobe omzetting van acetaat, maar de afbraak van propionaat is limiterend voor de maximaal toelaatbare temperatuur voor thermofiele anaërobe mineralisatie processen.  
Zinder SH (1990). FEMS Microbiol. Rev., 75: 125-138.  
Dit proefschrift
- 3 Het feit dat de acetoclastische methanogene populatie van bij extreem lage substraat concentraties gekweekt thermofiel korrelslib vrijwel uitsluitend bestaat uit *Methanosarcina spec.*, is voornamelijk toe te schrijven aan het gebruikte entmateriaal en de toegepaste lage vloeistof- en gasbelastingen. Het belang van dit type korrelslib voor reactoren op praktijkschaal is dubieus.  
Ahring *et al.* (1993). Appl. Environ. Microbiol., 59: 2538-2545.  
Schmidt *et al.* (1992). Appl. Environ. Microbiol., 58: 862-868.
- 4 Het gebruik van de term "adaptatie" voor een microbiologische respons op een grote verandering in milieucondities (bijvoorbeeld van mesofiel naar thermofiel) maskeert een ernstig gebrek aan microbiologische inzicht.
- 5 Indien "het gezin" wordt gezien als het belangrijkste zorgsysteem voor de opvoeding van kinderen, blijft het, ongeacht haar samenstelling, de hoeksteen van de samenleving.
- 6 Het niet voldoende uiting hebben kunnen geven aan de liefde voor een dierbaar persoon wordt het pijnlijks gevoeld indien de dood je van hem/haar scheidt.
- 7 Aangezien het in het leven niet eens zo zeer gaat om de levensduur maar om de kwaliteit van het bestaan, is de enorme druk uitgeoefend op "dertigers" om super te presteren binnen de werkkring, familieverband, alsmede op het sociale vlak, uiterst laakbaar.
- 8 Het najagen van economisch groei-herstel in de zeer welvarende geïndustrialiseerde landen (waaronder Nederland) en het streven naar de ontwikkeling van een duurzame samenleving zoals verwoord in het manifest van de VN wereld-milieu-conferentie in Rio de Janeiro (1992), vormt een onoplosbare paradox binnen de geldende economische principes van die landen.
- 9 Het bevorderen van het aanbrengen van een wijdvertakt rioolstelsel tezamen met decentrale conventionele aërobe zuiveringsinstallaties in een armoedig gebied zoals de Fayoum, Egypte, staat lijnrecht op de intenties welke de minister van Ontwikkelingssamenwerking, Pronk, uitspreekt in zijn beleidsnota's.

- 10 De kosten van onderzoek (analyses, apparatuur, salaris, enz.) zouden aanzienlijk omlaag kunnen indien onderzoekers zich beter zouden realiseren dat "Meten = Weten" alleen geldt indien je weet wat je meet.
- 11 Het boven de rivieren niet (kunnen) erkennen van Carnaval als een volwaardig cultureel volksvermaak is karakteristiek voor een bekrompen calvinistische zienswijze. Door deze tekortkoming blijft dit landsdeel verstoken van "nonsense-plezier" waarvoor daar op soms krampachtige wijze compensatie wordt gezocht.
- 12 Het getuigt van commercieel opportunisme van de LUW dat men levensgrote "bill boards" in openbare LUW gebouwen wel toe laat, maar reglementair verbiedt dat gelijksoortige advertenties in proefschriften worden afgedrukt.
- 13 Om in een land als Nederland op grond van het argument "de wereld is te vol" af te zien van het krijgen van kinderen getuigt van weinig realiteitszin.
- 14 Een effectieve integratie van migranten-minderheden is alleen te realiseren indien de migranten de Nederlandse taal in voldoende mate leren beheersen, hetgeen in belangrijke mate tot hun eigen verantwoordelijkheid behoort.
- 15 Het contact tussen de sexen zal pas veel meer worden dan snuffelen en confronteren, wanneer men kenmerken van de andere sexe in zichzelf ontdekt en gebruikt.
- 16 Snuffelen en confronteren is best lekker.

Stellingen behorende bij het proefschrift "Thermophilic anaerobic wastewater treatment; temperature aspects and process stability".

Jules B. van Lier  
Wageningen, 13 september 1995

*Aan mijn ouders*

*Voor Ivonne, Luc, Lieveke en Janine*

## VOORWOORD

Aan de vooravond van een 1.5 jarig 'vooronderzoek' en een overladen onderzoekprogramma is de belangrijkste vraag die door je hoofd speelt: "hoe is hier een verlenging uit te slepen". Gelukkig stond ik niet alleen om wat licht te scheppen in het nog relatief onontgonnen gebied van de thermofiele anaërobe afvalwaterzuivering. De eerste inspiratie kwam van Wim Wiegant die wellicht gezien mag worden als de nestor van de thermofiele anaërobe vergisting op de vakgroep. Ik kreeg meer dan voldoende steun van Katja Grolle die naast 'stront roeren' succesvolle experimenten uitvoerde met gedefinieerde mengcultures bij de vakgroep Microbiologie. Voorts wil ik de vele studenten en stagiaires bedanken die in min of meerdere mate hebben bijgedragen tot de totstandkoming van dit proefschrift: Arno Kleine Schaars, Carla Frijters, Feico Boersma, Frans Visser, Jacqueline Resink, Janneke Koerts, Jeroen Hulsbeek, Marc Debets, Nico Groeneveld, Peter Wielaard en Rene Dijkstra.

Behalve met de Nederlandse studenten heb ik zeer prettige samen gewerkt met José Luis Sanz Martin, Jukka Rintala, Jon Iza, Xu Yangsheng en Alla Nozhevnikova die voor langere en kortere periodes bij de vakgroep te gast waren. Een goede samenwerking op 'afstand' werd verkregen met Alberto Macario en Everly Conway de Macario te Albany, New York. Hun bijdrage tot het immunologische werk hebben zeer interessante resultaten opgeleverd.

Met betrekking tot de analytische ondersteuning ben ik Johannes van der Laan, Martin de Wit en Ilse Bennehey dankbaar. Verder zijn de mensen van de centrale dienst en de fotolocatie onmisbaar voor de uitvoer van de experimenten en de uiteindelijke presentatie van de behaalde resultaten. Iedereen bedankt.

Praktische informatie op ook andere gebieden dan anaërobe zuivering kwam naar boven bij de 'jonge vaders club', met André Visser en Arne Alphenaar als belangrijke pijlers; later versterkt door Renze van Houten en Sjon Kortekaas. Periodiek verraadde rood doorlopen ogen de nachtelijke escapades van één onzer spruiten, hetgeen tot een broodnodige relativering van het onderzoek leidde.

Rest nog de begeleiding:

Gatez, werk moet wel je hobby zijn en aan hobby's raak je verslaafd. Jouw niet voor te stellen hoeveelheid energie werpt in steeds grotere mate zijn vruchten af. Je had heus niet naar Friesland hoeven te vluchten. Binnen het onderzoek heb je mij zeer veel vrijheid gegeven waarvoor ik je zeer erkentelijk ben. Dankzij het vakkundig omzetten van een onvoorziene omstandigheid in een 'geschenk van Onze Lieve Heer' zijn zeer veel resultaten benut. Bedankt voor de samenwerking.

Fons Stams wil ik bedanken voor een Macro samenwerking op Micro niveau. Je weet altijd tijd te vinden voor discussies en correcties op artikelen. Mede dankzij jou heb ik publikaties leren schrijven en het zwembadfeest zal mij nog lang heugen...

Naast de wetenschappelijke discussies en experimenteel werk is een goede werksfeer onontbeerlijk voor een succesvolle afronding van een promotie-onderzoek. Hiervoor wil ik alle mensen van de vakgroep en een ieder die ik vergeten ben van harte bedanken.

Last but not least wil ik Ivonne bedanken die mij ieder keer weer wist duidelijk te maken dat de wereld niet ophoudt bij het wel of niet slagen van een thermofiel anaëroob experiment.

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## ABSTRACT

**Van Lier, J.B. (1995).** Thermophilic Anaerobic Wastewater Treatment; Temperature Aspects and Process Stability. Ph.D. thesis, Wageningen Agricultural University, Wageningen, The Netherlands.

The main objective of this thesis was to assess the thermostability of thermophilic anaerobic wastewater treatment processes and the possibility to optimize the performance of thermophilic high-rate systems.

Experiments were conducted to study the suitability of two types of seed material to start a thermophilic anaerobic process. Both mesophilic granular sludge and digested organic fraction of municipal solid waste were used as inoculum. The fate of mesophilic granular sludge under thermophilic conditions was studied in detail. Due to the temperature increase the mesophilic methanogens are replaced by thermophiles. In fact, the mesophilic granules appeared to serve mainly as carrier material for the thermophilic bacteria during the start-up. Since the thermophilic organisms attach quite well, the thermophilic specific methanogenic activity increased very rapidly in this period. Treatment of completely acidified wastewater leads to a deterioration of the 'mesophilic-thermophilic' granules. It therefore appeared extremely difficult to develop thermophilic granular sludge on this type of wastewater for both types of inocula. However, the thermophilic granulation process proceeded easily when sucrose was added to the influent.

The temperature sensitivity of the various types of thermophilic anaerobic sludge depends strongly on the process conditions applied, such as temperature and reactor type. Thermophilic sludge cultivated in high-rate reactors with high solids retention shows a high thermostability. Therefore, thermophilic anaerobic treatment in high-rate reactors can be applied in a wide temperature range, even under mesophilic conditions. In contrast, sludge cultivated in batch reactors is very sensitive to relatively small temperature variations. Regarding the thermostability of the process, application of high-rate reactors is preferred over batch reactors or completely mixed reactors. The presence of granular sludge enhances the stability towards temperature fluctuations quite substantially. The maximum specific activity of the cultivated granules appeared to be limited by the mass transfer rate. Consequently, a 'biomass buffer' is created which can be drawn on if the specific activity drops as a result of a temperature decrease.

A high process stability and high removal efficiencies were obtained in upflow staged sludge bed (USSB) reactors under extreme loading conditions. This USSB reactor consisted of 5 compartments along the reactor height. From each separate compartment of this reactor the produced biogas is withdrawn. The major effect of staging the thermophilic process is a very low concentration of intermediate products, such as hydrogen and acetate, in the last compartments of the system. A low concentration of these products enhances the anaerobic thermophilic degradation of all fatty acids. The properties of the sludge grown in the various compartments of the staged reactor depend on the environmental conditions prevailing in each compartment. Therefore, withdrawal of the produced excess sludge should be performed from each compartment or from the first compartment when an upflow reactor is used. Otherwise, a stable operation on the long term cannot be guaranteed because the voluminous acidifying sludge will eventually force out the extremely active acetogenic and methanogenic consortia in the subsequent compartments.

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# Chapter 1

## Introduction

## 1.1 Thermophilic Anaerobic Wastewater Treatment, General Introduction

Anaerobic treatment of waste and wastewater is presently accepted as a proven technology (Nyns, 1994; Wheatley, 1990). The applicability of the anaerobic treatment technology is growing each year. Several kinds of wastewaters which were believed to be unsuitable for anaerobic treatment are now treated with advanced reactor systems (Frankin *et al.*, 1992; Frankin *et al.*, 1994a, 1994b; Tseng and Yang, 1994; Nagano *et al.*, 1992; Narayanan *et al.*, 1993a, 1993b; Vellinga *et al.*, 1986). In addition, recent research shows that various recalcitrant compounds, like chlorinated aliphates, chlorinated aromates, nitroaromates and other xenobiotics, can be degraded under either anaerobic conditions or in aerobic-anaerobic sequences (Pavlostathis, 1994; Field *et al.*, 1994). These recent developments show that the full potentials of anaerobic digestion are still underestimated.

One of the major contributors to the success of anaerobic wastewater treatment is the introduction of high-rate reactors in which biomass retention and liquid retention are uncoupled, such as the upflow anaerobic sludge bed (UASB) reactor (Lettinga *et al.*, 1980) the (upflow) anaerobic filter ([UJAF) (Young and McCarty, 1969; Young and Yang, 1989), the downflow stationary fixed film reactor (DSFF) (Duff and Kennedy, 1982), the fluidized bed (FB) reactor (Jeris, 1983), the anaerobic baffled reactor (ABR) (Bachmann *et al.*, 1985), the anaerobic attached film expanded bed (AAFEB) reactor (Switzenbaum and Jewell, 1980), and the anaerobic gas lift reactor (AGLR) (Beefink and Staugaard, 1986). High-rate reactors can accommodate very high organic loading rates as a result of the high concentration of bacterial mass and the sufficient sludge-water contact. The biomass is generally present as biofilms and/or granular aggregates (Hulshoff Pol, 1989; Jeris, 1983; MacCleod *et al.*, 1990; Wu *et al.*, 1993; Young and McCarty, 1969). Among the above reactor systems, the UASB reactor is undoubtedly the most successful, and the total number of UASB reactors in use is increasing every year (De Zeeuw, 1987; Lin and Yang, 1991; Nyns, 1994). In most cases, the anaerobic reactor is only a pre-treatment unit of a complete wastewater treatment system. Competition between aerobic, anaerobic and other treatment technologies should, therefore, be replaced by taking stock of the advantages of each of the systems for the different types of waste(water) (Field *et al.*, 1994; Jianrong *et al.*, 1994; Kortekaas *et al.*, 1994).

The possibilities of the anaerobic treatment technology could be increased if the process could also be applied at high temperatures. Thermophilic treatment could be an attractive alternative (§ 1.3), particularly when the wastewater is discharged at high temperatures. Furthermore, in the thermophilic range ( $> 45^{\circ}\text{C}$ ) reaction rates proceed much faster than under mesophilic conditions ( $25\text{--}40^{\circ}\text{C}$ ) so that the loading potentials of anaerobic bioreactors can be significantly higher (Buhr and Andrews, 1977; Zinder, 1986). This may lead to considerably shorter retention times and/or smaller reactor units (e.g. Cecchi *et al.*, 1991; Lo *et al.*, 1985; Harris and Dague, 1993). The feasibility of anaerobic thermophilic treatment of waste and wastewater has been researched in many laboratories over the past 100 years.

The interested reader is referred to the literature reviews of e.g. Buhr and Andrews (1977), Cooney and Wise (1975), Wiegant (1986), Zinder (1986), and Ahring (1994). Over the last decade(s) much research has been done on thermophilic high-rate reactors with solids retention. A concise literature review is given in Table 1.5. The results obtained with the various kinds of wastewaters are very promising. However, due to the different types of wastewaters treated and the different kinds of systems used, it is impossible to judge whether the mesophilic or the thermophilic temperature range is more appropriate for the various cases. More defined laboratory-scale experiments showed that higher conversion rates were achieved under thermophilic conditions than under mesophilic conditions (Borja *et al.*, 1995; Rintala and Lepistö, 1992; Romero *et al.*, 1993). On the other hand, comparative studies on mesophilic and thermophilic wastewater treatment processes demonstrated that thermophilic processes were less stable (Disley *et al.*, 1992; Fernandez and Forster, 1993; Seif *et al.*, 1992; Soto *et al.*, 1992). While some investigations revealed the higher susceptibility of thermophilic systems to temperature fluctuations (Zinder *et al.*, 1984b; Zinder, 1986), other studies revealed that thermophilic high-rate processes were characterized by high concentrations of volatile fatty acids (VFA) in effluents, particularly under high loading conditions (Rudd *et al.*, 1985; Wiegant and Lettinga, 1985; Wiegant *et al.*, 1985). For instance, Wiegant *et al.* (1985) found a clear correlation between the strength of the wastewater from an alcohol distillery process and the VFA concentration in the effluent of a thermophilic UASB reactor. Organic loading rates (OLR) of 25-40 kg chemical oxygen demand (COD).m<sup>-3</sup> reactor.day<sup>-1</sup> resulted in total effluent VFA concentrations of 3,000-8,000 mg COD.l<sup>-1</sup> (Wiegant *et al.*, 1985). On the other hand, moderately low loaded thermophilic high-rate reactors perform much better in removing residual VFA (Ahring *et al.*, 1993; Schraa and Jewell, 1984). It was also found that immobilization of anaerobic biomass is more difficult to achieve under thermophilic conditions than under mesophilic conditions (see § 1.5.3). Nevertheless, immobilization of anaerobic biomass by formation of granular sludge is a common phenomenon in thermophilic methanogenic upflow reactors (Ohtsuki *et al.*, 1992, 1994; Schmidt and Ahring, 1993; Souza *et al.*, 1992; Uemura and Harada, 1993, 1995; Wiegant and Lettinga, 1985; Wiegant and De Man, 1986). Due to the conflicting and sometimes disappointing results found by the various researchers (Disley *et al.*, 1992; Soto *et al.*, 1992), thermophilic anaerobic wastewater treatment has, so far, hardly been applied.

In this thesis, results are described of experiments which were conducted on the stability of thermophilic wastewater treatment in high-rate reactors under defined conditions. With respect to the temperature sensitivity, several aspects were studied which may influence the thermostability of the sludge: i) the height of the process temperature, ii) the mode of reactor operation, and iii) the presence of immobilized biomass. Also, the response of the bacterial population to an upward temperature shift from mesophilic to thermophilic conditions was investigated using immunological methods. In the second part of the thesis, experiments are presented which deal with the frequently cited phenomenon of high concentrations of volatile fatty acids in effluents of thermophilic reactors. A high VFA level indicates a high degree

of substrate-, product-, or non-competitive inhibition in the thermophilic sludge bed. In order to minimize possible inhibition effects, experiments were conducted using plug-flow reactors. In contrast to (partly) mixed sludge bed reactors, like the UASB reactor, high VFA concentrations are only experienced in the first and separated part of the sludge bed.

## 1.2 Effects of temperature on biotechnological processes

Temperature has a considerable effect on the intracellular and extracellular environment of bacteria. Temperature acts as an accelerator of conversion processes, and it also determines whether or not a reaction can be performed by specific bacteria. The intercellular environment requires several adaptations to resist the high temperatures. This is discussed in more detail in § 1.2.1. The effects of temperature on the physical-chemical properties of the solution are discussed in § 1.2.2.

### 1.2.1 Factors determining the optimum growth temperature

Denaturation of enzymes and nucleic acids (DNA, RNA) are examples of possible limitations of bacteria at elevated temperatures (Brock, 1986). Generally, the nucleic acids of thermophilic organisms are much more stable and contain relatively more Guanosine (G) - Cytidine (C) bonds with 3 hydrogen bridges instead of Adenosine (A) and Thymidine (T) bonds, with only 2 hydrogen bridges. However, a clear correlation between the optimum growth temperature and the G-C content was never found (Winter and Zellner, 1990). The nucleic acids can also be stabilized by DNA-binding proteins, which are more frequently observed in thermophiles than in mesophiles (Brock, 1986). Enzymes and other proteins of thermophiles generally appear to be more stable because of a more stable tertiary structure due to the presence of more S-containing amino acids like cysteine, leading to S-S bond interactions.

In addition to the above adaptations on the molecular level, various cell organelles and/or cell structures of thermophilic bacteria also need extra provisions to increase the thermostability of these vital components. For example, the membranes of thermophilic bacteria have to be much stronger because of the increased fluidity and the possible loss of selective permeability at high temperatures. In general, the glycolipid content of the bacterial membranes increases with the increasing growth temperature from psychrophiles to thermophiles (Russell and Fukunaga, 1990). It was hypothesized that a higher degree of sugar-containing lipids increases the hydrogen-bonding capacity of the lipid bilayer surface, stabilizing the membrane at high temperatures, perhaps through additional interactions with cell wall components. Also, membranes of thermophilic bacteria generally have a higher content of saturated fatty acids. Thermal adaptations were also found for stabilizing the structure of ribosomes (Brock, 1986).

The degree of thermostability of the various cell components determines the temperature span of a specific bacterium. In general, a temperature span of 20-40°C is common for (micro)organisms. However, some bacteria have a much wider span, like the thermophilic hydrogen-consuming methanogen *Methanobacterium thermoautotrophicum* ΔH which is able to grow between 22 and 78°C (Wiegel, 1990). It is obvious that such a bacterium needs various adaptations to stabilize proteins, nucleic acids, membrane lipids, etc. The number of high temperature requirements needed indicates that an adaptation of common mesophilic bacteria to the thermophilic temperature range is impossible. However, various authors claim a 'conversion' or adaptation of mesophiles into thermophiles or extreme thermophiles, as mentioned in the review of Wiegel (1990). These so-called cryptic thermophilic organisms possess: i) thermostable enzymes, lipids, membrane components, and the means to stabilize their DNA at high temperatures; or ii) a facultative system for synthesizing thermostable isoenzymes and/or the capability to alter their lipids into the thermostable form but apparently are not using it (Wiegel, 1990, Tsien *et al.*, 1980). Another mechanism of thermo-adaptation was proposed by Hensel and König (1988) who observed an upward shift of the optimum growth temperature of methanogenic bacteria. This phenomenon was attributed to a high intracellular ion concentration, and it was concluded that potassium 2,3-diphosphoglycerate enhanced the thermostability of thermolabile enzymes. Similarly, Zellner and Kneifel (1993) found an increase in the proportion of long-chain polyamines with increasing growth temperature (50-85°C) in cells of *Thermotoga* species, a thermophilic anaerobic eubacterium. According to Woese (1987) the extreme thermophilic *Thermotoga maritima* is one of the most primeval strains of anaerobic bacteria found on earth today, suggesting that all original eubacteria were thermophiles (Achenbach-Richter *et al.*, 1987). An upward shift of the optimum growth temperature was also described by Pledger *et al.* (1994), who could successfully elevate the optimum temperature of some specific hyperthermophiles by increasing the hydrostatic pressure on the culture.

(Micro)organisms are classified into 'temperature classes' on the basis of the optimum temperature and the temperature span of the species (Table 1.1). Although it is very convenient to classify organisms into thermal groups, the overlapping growth temperature ranges in Table 1.1 indicate that there are no real boundaries between these groups. Generally, 43-45°C is considered to be the upper temperature limit for mesophilic processes and the lower limit for thermophilic processes. Most studies on the effect of temperature on anaerobic digestion show a sharp transition in the digestion process beyond 45°C (Chapter 3; Feilden, 1981; Henze and Harremoës, 1983; Rintala and Lettinga, 1992; Speece and Kem, 1970; Van Lier *et al.*, 1990). However, in some cases, a gradual increase in the methanogenic activity was observed with a temperature increase from the mesophilic to the thermophilic range (Chen, 1983). Nevertheless, a rough classification in mesophiles and thermophiles, with 45°C as the border temperature, suffices for reactor design purposes.

**Table 1.1** Definition of organisms according to their cardinal growth temperatures (in °C), after Wiegel (1990)

	$T_{\min}$	$T_{\text{opt}}$	$T_{\max}$
Psychrophiles (Cryophiles)	< 0	< 15	< 20
Temperature tolerant mesophiles (psychotrophs)	< 5	> 15	> 20
Mesophiles	> 5	< 45	< 50
Thermotolerants (thermoduric)	-	< 45	> 50
Temperature tolerant thermophiles	< 25	> 45	> 50
Thermophiles	> 25	> 45	> 50
Temperature tolerant extreme thermophiles	< 45	> 65	> 70
Extreme thermophiles	> 45	> 65	> 70
Barothermotolerants (hyperthermophilic)	?	< 100	> 100
Barothermophiles (hyperthermophilic)	?	> 100	> 100

### 1.2.2 Influence of temperature on the physical-chemical aspects of anaerobic conversion processes

#### Thermodynamics

At high temperatures, chemical and biological reaction rates proceed much faster than at low temperatures. Biological reactions, however, are dependent on the possible growth temperature of the organisms performing the reaction (§ 1.2.1). Within the temperature span of the organisms the thermodynamics of the conversion reaction  $aA + bB \leftrightarrow cC + dD$  are accelerated by increasing temperatures. Free energies of formation as well as  $\Delta G^\circ$  will change (equations 1.1 and 1.2).

$$\frac{\Delta G_2}{T_2} - \frac{\Delta G_1}{T_1} = -\Delta H \left( \frac{T_2 - T_1}{T_1 \cdot T_2} \right) \quad (1.1)$$

$$\Delta G = \Delta G^\circ + RT \cdot \ln \left( \frac{A^a \cdot B^b}{C^c \cdot D^d} \right) \quad (1.2)$$

where  $\Delta G$  = the Gibbs free-energy change ( $\text{kJ} \cdot \text{mole}^{-1}$ ),  $T$  = temperature (K),  $\Delta H$  = change in enthalpy ( $\text{kJ} \cdot \text{mole}^{-1}$ ),  $R$  = gas constant ( $8.31 \cdot 10^{-3} \text{ kJ} \cdot \text{mole}^{-1} \cdot \text{K}^{-1}$ ),  $A$  and  $B$  = reactants,  $C$  and  $D$  = products and  $a, b, c$  and  $d$  = moles of reactants and products per reaction. The subscripts 1 and 2 refer to two different temperatures.

Most reactions in the biodegradation of organic matter require less energy to proceed at high

temperatures (Table 1.2), which results in a faster digestion. Reactions dependent on the  $H_2$  partial pressure, such as the oxidation of propionate and butyrate, are possible at higher  $H_2$  concentrations in the biogas (Zinder, 1990).

**Table 1.2** Methanogenic and acetogenic reactions involved in the anaerobic conversion of organic matter and the Gibbs free-energy changes<sup>a</sup>. Table includes some possible acidification reactions with sucrose as model compound.

Reaction	$\Delta G^{\circ}$ , 25°C (kJ.mole <sup>-1</sup> )	$\Delta G^{\circ}$ , 55°C (kJ.mole <sup>-1</sup> )
$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O$	-135.6	-122.5
$4 \text{ Formate}^- + H_2O + H^+ \rightarrow CH_4 + 3HCO_3^-$	-130.4	-118.9
$\text{Acetate}^- + H_2O \rightarrow HCO_3^- + CH_4$	-31.0	-34.7
$\text{Acetate}^- + 4H_2O \rightarrow 2HCO_3^- + H^+ + 4H_2$	+104.2	+89.8
$\text{Acetate}^- + 2HCO_3^- \rightarrow 4 \text{ Formate}^- + H^+$	+99.1	+86.1
$\text{Propionate}^- + 3H_2O \rightarrow \text{Acetate}^- + HCO_3^- + H^+ + 3H_2$	+76.1	+62.3
$\text{Propionate}^- + 2HCO_3^- \rightarrow \text{Acetate}^- + 3 \text{ Formate}^- + H^+$	+72.2	+59.7
$\text{Butyrate}^- + 2H_2O \rightarrow 2 \text{ Acetate}^- + H^+ + 2H_2$	+48.1	+37.9
$\text{Butyrate}^- + 2HCO_3^- \rightarrow 2 \text{ Acetate}^- + 2 \text{ Formate}^- + H^+$	+45.5	+36.1
$\text{Sucrose} + 9H_2O \rightarrow 4HCO_3^- + 4 \text{ Acetate}^- + 8H^+ + 8H_2$	-457.5	-511.8
$\text{Sucrose} + 3H_2O \rightarrow 2HCO_3^- + 2 \text{ Acetate}^- + 2 \text{ Propionate}^- + 6H^+ + 2H_2$	-610.5	-641.2
$\text{Sucrose} + 5H_2O \rightarrow 4HCO_3^- + 2 \text{ Butyrate}^- + 6H^+ + 4H_2$	-554.1	-590.7

<sup>a</sup> Energy changes were calculated by using the van 't Hoff equation, standard enthalpy values of compounds (Chang, 1977), and Gibbs free-energy changes at 25°C (Thauer *et al.*, 1977).

### Solubility of gases and (in)organic compounds

The solubility of gaseous compounds decreases with increasing temperature (Fig.1.1). This lower solubility implicates that gases like  $NH_3$ ,  $H_2S$  and  $H_2$ , which have a negative (or even toxic) effect on the digestion process, are easily stripped from the solution. Consequently, the concentration of these gases, as well as that of methane, is lower in the effluent of thermophilic reactors than in mesophilic digesters. The decreased solubility of  $CO_2$  indicates a higher reactor pH under thermophilic conditions.

The solubility of most salts increases with increasing temperature, while the solubility constants of precipitates like  $CaCO_3$  decreases (Sillen and Martell, 1964). If organic salts are more soluble at high temperatures (e.g. neutralized long-chain fatty acids), the organic matter is more accessible to the microorganisms, which might improve the overall conversion efficiency of an anaerobic treatment process. On the other hand, changes in chemical equilibria sometimes result in a higher fraction of undissociated compounds like  $NH_3$  (Weast,

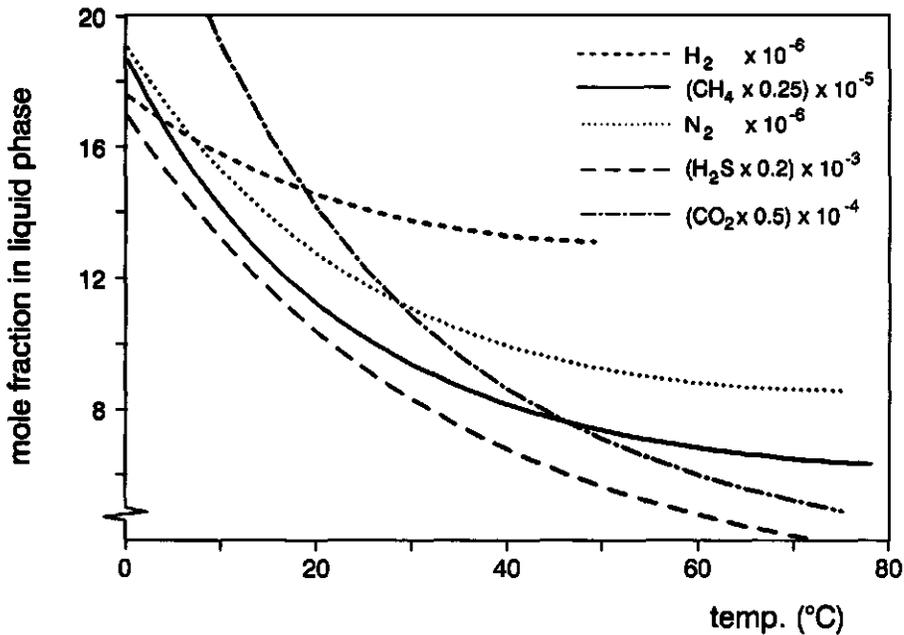
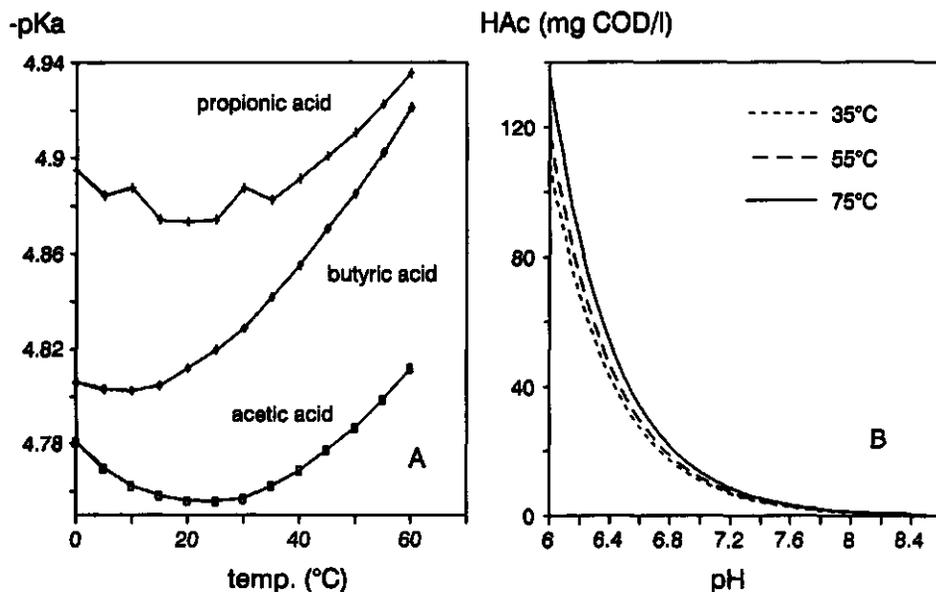


Fig. 1.1 Gas solubility in pure water at various temperatures, after Lide (1992).

1976), which limits the biodegradability of specific wastes under thermophilic conditions (Angelidaki and Ahring, 1994; Wiegant, 1986). For most 'small' molecules, like fatty acids, H<sub>2</sub>S, NH<sub>3</sub>, etc., it is believed that the toxic effect is caused by the undissociated form which diffuses more easily across the bacterial membrane (see also § 5.4). The effects of temperature on the chemical equilibria and its implications for anaerobic treatment are most pronounced for those compounds with a dissociation constant of approximately 10<sup>-8</sup> - 10<sup>-7</sup>, i.e. at neutral pH. Consequently, with respect to VFA, this effect is of much less importance since the digester pH is always far above the pK<sub>a</sub> values of these acids (Fig. 1.2). Only in acid reactors, e.g. pre-acidification under low pH conditions, is a substantially higher fraction of undissociated VFA present.

### Liquid viscosity

A physical impact of high temperatures is the lower viscosity of liquid and semi-solid slurries. This implies that less energy is required for mixing and that sludge bed reactors are more easily mixed at relatively low gas productions. In such reactors, particles will settle faster because of an improved liquid-solids separation under high temperature conditions. On the other hand, the same particles will be lifted from the sludge bed more easily by the evolving gas bubbles and/or sudden biogas eruptions.



**Fig. 1.2** A) pKa of acetic acid, propionic acid and butyric acid at various temperatures, after Sillen and Martell (1964). B) Undissociated acetic acid concentration at 36, 55 and 75°C. Values were calculated using the extrapolated data of Sillen and Martell (1964), assuming an acetate concentration of 2000 mg COD.l<sup>-1</sup> in pure water.

Related to liquid viscosity is the diffusivity of soluble compounds which increases with increasing temperature (Perry and Green, 1984):

$$D_2 = D_1 \cdot \left( \frac{\eta_1}{T_1} \right) \cdot \left( \frac{T_2}{\eta_2} \right) \quad (1.3)$$

where  $D$  = diffusion coefficients of a specific compound ( $\text{m}^2 \cdot \text{s}^{-1}$ ),  $T$  = temperature (K), and  $\eta$  = the liquid viscosity of the solution ( $\text{N} \cdot \text{s} \cdot \text{m}^{-2}$ ). The subscripts 1 and 2 refer to two different temperatures. The diffusivity of soluble compounds at various temperatures relative to the diffusivity at 30°C is given in Table 1.3.

**Table 1.3** The diffusivity of soluble compounds at various temperatures relative to the diffusivity at 30°C<sup>a</sup>

temp. (°C)	10	20	30	40	50	60	70	80	90
$D_{\text{temp.}}/D_{30}$ (-)	0.57	0.77	1.00	1.26	1.55	1.88	2.24	2.62	3.04

<sup>a</sup> Values were calculated using equation 1.3 and the viscosity of pure water at the various temperatures (Lide, 1992).

From Table 1.3 follows that diffusion constants of soluble compounds are about 50% higher under thermophilic conditions (50-60°C) than under mesophilic conditions (30-40°C). The fact that bacterial growth rates are generally a factor 2-3 higher (§ 1.3.1) implies that thermophilic biofilm processes are easily limited by diffusion limitation of the substrate (see also Chapter 4.2). It is clear that with gaseous compounds the effect of temperature on the diffusivity in liquids and biofilms is even much greater. The occurrence of mass transfer limitation reduces the conversion capacity of thermophilic sludge but enhances the stability of the overall process.

### 1.3 Advantages and disadvantages of thermophilic treatment

#### 1.3.1 Advantages

##### Higher metabolic rates

The bacterial growth rates of thermophiles are generally higher than the growth rates of their mesophilic homologues. Table 1.4 lists the most important methanogenic and acetogenic bacteria which are involved in the anaerobic conversion of organic matter at both temperature ranges. Optimization of anaerobic digestion by applying the process at high temperatures is used in its optimal sense in completely mixed systems where the overall conversion rate is determined by the maximum specific growth rate. Within the temperature range of one species the growth rate increases exponentially with temperature (Brock, 1986; Heitzer *et al.*, 1991). However, the rate of increase is considerably lower if the growth rates of different species are compared at their optimal growth temperature (Brock, 1986). Apparently, the growth efficiency of thermophiles is much lower than that of mesophiles, which may be attributed to the higher maintenance energy demands with increasing temperatures. Nonetheless, an increase in growth rate by a factor of 2 to 3 (Fig. 1.3, Table 1.4) is generally found between thermophiles and their mesophilic homologues in the anaerobic digestion process. For hydrogenotrophic methanogens a factor of 10 is almost reached. In principle, therefore, in applying the process at high temperatures the digestion time can be substantially reduced, and a more complete degradation can be achieved. This is confirmed by the results of many small- and large-scale digesters as reviewed by various authors (Cooney and Wise, 1975; Buhr and Andrews, 1977; Varel, 1983; Wiegant, 1986; Zinder, 1986). On the other hand, in the digestion of sewage sludge, some reports have been published in which thermophilic treatment did not show any advantage over mesophilic digestion (Zeeman and Van Veen, 1990). Thermophilic digestion seems to be less effective particularly when the  $\text{NH}_4^+$ -N concentration is higher than  $1.1 \text{ g.l}^{-1}$ . This is probably due to the toxic effect of free  $\text{NH}_3$  (Wiegant, 1986; Angelidaki and Ahring, 1994; Zeeman and Van Veen, 1990). Differences in experimental set-up, accuracy and interpretation of the

Table 1.4 Comparison of the maximum growth rate of some mesophiles and their thermophilic homologues

Substrate	Genus	Mesophiles		Thermophiles	
		$\mu_{\max}$ (day <sup>-1</sup> )	ref.	$\mu_{\max}$ (day <sup>-1</sup> )	ref.
H <sub>2</sub> /CO <sub>2</sub>	<i>Methanobacterium</i>	0.26	1	4.80-16.6	2,20-23
	<i>Methanococcus</i>	2.16-5.52	2	18.2-51.1	24-27
	<i>Methanosarcina</i>	0.48-1.44	3,4		
	<i>Methanobrevibacter</i>	1.44-4.08	1,5,6		
	Enrichment			7.92 <sup>b</sup>	28
Formate	<i>Methanobacterium</i>	1.44-1.92	7	13.4	29
Acetate	<i>Methanotherix</i>	0.10-0.22 <sup>a</sup>	8-10	0.48-0.72	30
		0.60-0.72	11,12		
	<i>Methanosarcina</i>	0.24-0.67	13	1.27-2.04	30
	Enrichment	0.10-0.34	1	0.96 <sup>b</sup>	28
Propionate	<i>Syntrophobacter</i>	0.10-0.19	14		
	Enrichment	0.10-0.14	6,15,16	0.14-0.31	31,32
		0.31 <sup>c</sup>	17	0.72 <sup>b</sup>	28
Butyrate	<i>Syntrophomonas</i>	0.19-0.31	18,19		
	Enrichment	0.36 <sup>c</sup>	17	0.48-0.77	33
				2.62 <sup>b</sup>	28

<sup>a</sup> *M. soehngeni*, predominant bacterium in mesophilic granular sludge (ref. 8)

<sup>b</sup> Thermophilic methanogenic consortia (see also ref. 28)

<sup>c</sup> Mesophilic mixed cultures (see also ref. 17)

Ref: 1 Pavlostahis and Giraldo Gomez (1991); 2, Balch *et al.* (1979); 3, Weimer and Zeikus (1978); 4, Smith and Mah (1978); 5, Zehnder and Wuhrmann (1977); 6, Gujer and Zehnder (1983); 7, Schauer *et al.* (1980); 8, Huser *et al.* (1982); 9, Van den Berg *et al.* (1977); 10, Zehnder *et al.* (1980); 11, Fathepure (1983); 12, Patel (1984); 13, Mah *et al.* (1978); 14, Boone and Bryant (1980); 15, Koch *et al.* (1983); 16, Wu *et al.* (1992); 17, Lawrence and McCarty (1969); 18, McInerney *et al.* (1981); 19, Zhao *et al.* (1993); 20, Brandis *et al.* (1981); 21, Gerhard *et al.* (1993); 22, Kitaura *et al.* (1992); 23, Winter *et al.* (1984); 24, Huber *et al.* (1982); 25, Peillex *et al.* (1989); 26, Jones *et al.* (1983); 27, Zhao *et al.* (1988); 28, Wiegant *et al.* (1986); 29, König and Stetter (1987); 30, See Table 4.2; 31, Stams *et al.* (1992); 32, Zinder *et al.* (1984a); 33, Ahring and Westermann (1987).

results of the various experiments could influence the conclusion of whether or not thermophilic digestion is superior.

With the exception of the start-up period, the maximum specific growth rate of the bacteria is obviously of minor importance when thermophilic treatment is applied in high-rate systems with high solids retention times. The principal advantage of the higher temperature in such reactors is the higher maintenance energy demand of the thermophilic biomass, as explained previously by Wiegant (1986):

Growth Rate Methanogens (%)

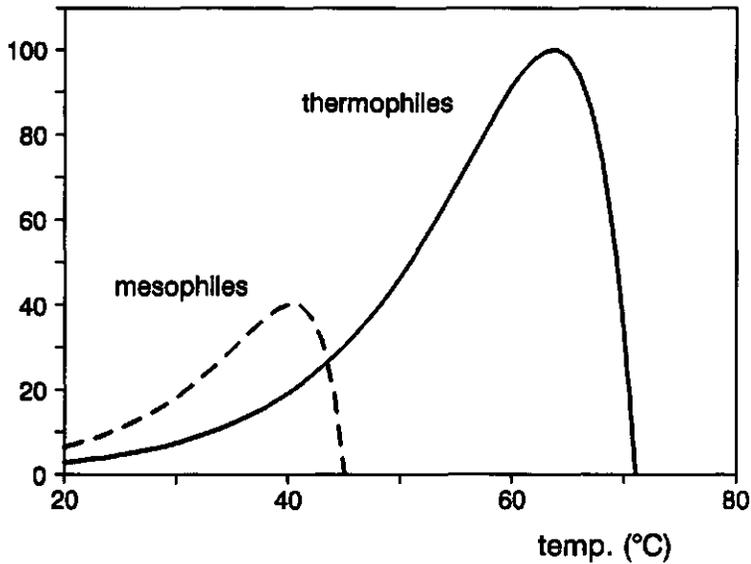


Fig. 1.3 Relative growth rate of mesophilic and thermophilic methanogens.

$$\mu - b = \theta_c^{-1} \tag{1.5}$$

$$\mu = A \cdot Y \tag{1.6}$$

where  $\mu$  = specific growth rate ( $\text{day}^{-1}$ ),  $b$  = maintenance and decay rate ( $\text{day}^{-1}$ ),  $\theta_c$  = cell residence time (days),  $A$  = specific sludge activity ( $\text{day}^{-1}$ ), and  $Y$  = bacterial growth yield ( $\text{g.mole}^{-1}$ ). Combining equations 1.5 and 1.6 we obtain:

$$A = \frac{\theta_c^{-1} + b}{Y} \tag{1.7}$$

From equation 1.7 it is clear that both the maintenance energy demand and the cell residence time (solids retention time) affect the specific activity of the biomass.

Due to the high metabolic activities, the net biomass yield per mole of substrate is less, resulting in a very low production of excess sludge (Zinder, 1986). Moreover, in sludge digestion, the residue seems to be better stabilized and the dewatering capacity improved (Torpey *et al.*, 1984; Garber, 1977, 1982; Garber *et al.*, 1975; Rimkus, 1982).

### Pathogen removal

The decimation of pathogenic bacteria is described as a first-order process and is related to the die-off rate of an indicator bacterium, mostly *E.coli* (e.g. Catunda *et al.*, 1994):

$$\frac{dN}{dt} = -K_b \cdot N \quad (1.8)$$

The die-off constant,  $K_b$ , increases with increasing temperature. Under thermophilic conditions the death rate of pathogenic organisms is extremely high and as a result the required retention times can be reduced substantially. For most pathogenic bacteria, a contact period of several weeks is required at ambient temperatures (20°C), which can be reduced to several days at 35°C. However, at 53-55°C, contact times of less than 1 hour are sufficient to meet the prevailing sanitation standards in e.g. Denmark (Bendixen, 1994). Comparative studies with specific pathogenic bacteria as indicator showed the superiority of thermophilic treatment over mesophilic digestion (Olsen *et al.*, 1985; Olsen and Larsen, 1987; Bendixen, 1994; Lund *et al.*, 1995). Thermophilic treatment makes a separate hygienization step superfluous particularly in the cases of manure or sewage sludge digestion. In fact, pathogen removal is the driving force behind the Danish Biogas Programme to implement centralized thermophilic treatment for manure digestion, including industrial and household waste (e.g. Bendixen, 1994; Mathrani *et al.*, 1994; Tafdrup, 1994). After the high temperature treatment, the residue can be safely used as soil conditioner. The latter is also of interest in the USA where new legislation regarding disposal of biosolids will restrict land use based on pathogen destruction criteria, favouring thermophilic treatment (Aitken and Mullennix, 1992). A high degree of destruction during thermophilic (55°C) treatment was also found for plant pathogens and weed seeds (Engeli *et al.*, 1992).

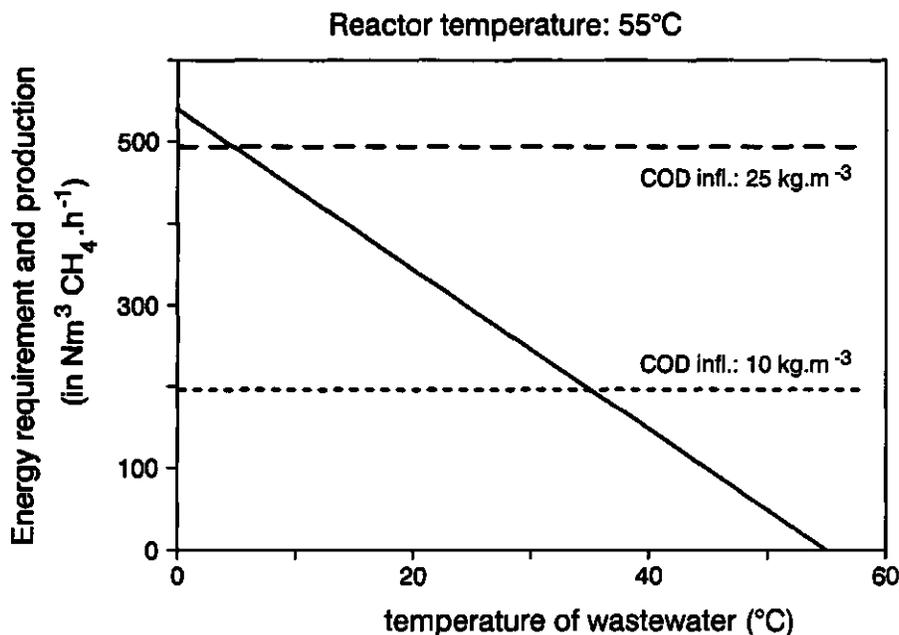
### Improved physical-chemical properties

The effects of temperature on the various physical-chemical parameters are already discussed in § 1.2.2. In most cases, the effects of temperature are advantageous to the anaerobic digestion process.

### 1.3.2 Disadvantages

#### Energy requirements

The energy requirements of thermophilic systems are obviously much higher than those of mesophilic reactors. However, the actual energy consumption for heating depends on the temperature of the incoming wastewater (Fig. 1.4), type of insulation and the retention time. An economic evaluation should be made as to whether or not it is beneficial to heat the wastewater using the produced biogas as fuel. Considering the fact that under thermophilic conditions the applicable loading rate will be higher (§ 1.3.1), heating of the wastewater may be a feasible alternative (Fig. 1.4).



**Fig. 1.4** Theoretical energy requirement for heating (—) of a thermophilic (55°C) continuous flow reactor versus wastewater temperature. The horizontal bars represent the methane production in case of an influent concentration of (—) 25 g COD.l<sup>-1</sup> (OLR: 60 kg.m<sup>-3</sup>.day<sup>-1</sup>) and (- - -) 10 g COD.l<sup>-1</sup> (OLR: 24 kg.m<sup>-3</sup>.day<sup>-1</sup>). For the theoretical calculations the following assumptions were made: Degree of bioconversion (methane recovery): 75%; Bacterial yield: 5%; Reactor volume (cylinder, r = 5m, h = 10m): 785 m<sup>3</sup>; Influent flow: 79 m<sup>3</sup>.hr<sup>-1</sup>; Hydraulic retention time: 10 hr; Reactor temperature: 55°C; Ambient temperature: 15°C; Reactor side and insulation: 1.5 cm steel and 10 cm glass wool with heat conductivity coefficients of 39 and 0.035 kcal.m<sup>-1</sup>.h<sup>-1</sup>.°C<sup>-1</sup>, respectively (Forsythe, 1954).

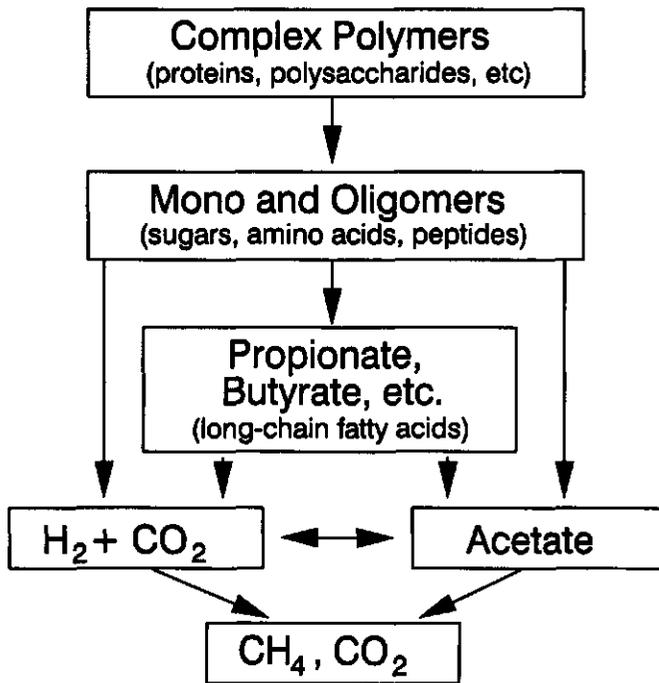
The example in Fig. 1.4 illustrates that thermophilic treatment is more advantageous if the wastewater is discharged at high temperatures. The loss of heat during thermophilic treatment is much less during solid waste digestion. For full-scale thermophilic co-digestion of manure and industrial waste, the extra energy for heating is less than 5% of the produced biogas (Ahling, pers. comm.). The large-scale reactors are operated at 55°C and at retention times of approximately 15 days.

### Process stability

Thermophilic anaerobic treatment of waste and wastewater is often regarded as less stable than mesophilic treatment (Buhr and Andrews, 1977; Rudd *et al.*, 1985), restraining most industries and constructors from implementing this new technology. Literature reports mention several drawbacks of thermophilic digesters, such as high susceptibility to: i) temperature increases (Varel *et al.*, 1977; Schraa, 1983; Zinder *et al.*, 1984b), ii) feed interruptions (Wiegant, 1986), and iii) shock loadings (Duff and Kennedy, 1982; Seif *et al.*, 1992; Soto *et al.*, 1992; see also § 1.1). In comparison to mesophilic sludge digesters, thermophilic digesters are generally characterized by relatively high effluent VFA concentrations indicating a lower degree of process stability, as reviewed by Wiegant (1986). In contrast, in other studies, very low effluent VFA concentrations were found in thermophilic sludge digesters (Aoki and Kawase, 1991; Ghosh *et al.*, 1980), as well as in thermophilic wastewater treatment plants (Chapters 3 and 6; Schraa and Jewell, 1984; Cail and Barford, 1985; Wiegant and De Man, 1986). In addition, Ahring (1994) recently made a comparison between mesophilic and thermophilic full-scale digesters operating under more or less similar conditions and concluded that the VFA concentrations were very similar. Yet, it remains unclear as to what extent the frequently cited process instabilities are intrinsic disadvantages of thermophilic digestion, and what measures must be taken to adapt the current technology in order to most optimally apply thermophilic treatment. For these reasons, process stability is investigated in detail in the present research. Our results show that intermediate compounds may limit high-rate conversion of biomass (Chapters 5 and 6). However, if a more appropriate process technology is chosen the conversion rate may be considerably enhanced (Chapter 6) with a temperature susceptibility similar to that of mesophilic reactors (Chapter 4).

## 1.4 Microbiology of thermophilic anaerobic bioconversion of organic matter

The anaerobic decomposition of organic matter is characterized by a sequence of reactions which are performed by different physiological types of anaerobic bacteria (Bryant, 1979; McInerney and Bryant, 1981; Mah, 1982). In the first step, complex organic compounds are hydrolysed to monomers like sugars, long-chain fatty acids and amino acids. Next, sugars and amino acids are fermented to volatile fatty acids, alcohols, lactate, carbon dioxide and hydrogen. In the third step, the produced intermediates and long-chain fatty acids are further degraded to acetate, carbon dioxide and hydrogen, followed by a subsequent conversion to methane. Obviously, a complete anaerobic conversion of organic matter requires a complex community of different anaerobic bacteria. The sequence of general reactions occurs both under mesophilic and thermophilic conditions and is schematically shown in Fig. 1.5.



**Fig. 1.5** Flow of carbon in the anaerobic degradation of organic matter, after Gujer and Zehnder (1983).

The main difference between mesophilic and thermophilic bioconversion is the reaction rate, which is much higher at high temperatures (§ 1.3.1). For many mesophilic bacteria involved in anaerobic digestion a thermophilic homologue can be found. The number of new thermophilic isolates is still increasing. Both the microbiology and technology of thermophilic digestion has been reviewed by various authors over the last decades, e.g. Cooney and Wise (1975), Buhr and Andrews (1977), Sonnleitner (1983), Varel (1983), Wiegant (1986), Zinder (1986, 1990), Winter and Zellner (1990), Rintala (1992), Lowe *et al.* (1993) and Ahring (1994). In this section, some of the most important microbiological characteristics of thermophilic digestion are discussed.

#### Non-aceticlastic methanogenesis

Following the sequence of anaerobic biotransformation (Fig. 1.5), complex organic material is converted into more simple compounds with similar COD equivalents. Actual COD removal from waste and wastewater is achieved by the formation of reduced gaseous compounds like  $\text{CH}_4$ ,  $\text{H}_2$ ,  $\text{H}_2\text{S}$ , etc., among which  $\text{CH}_4$  is the most important. Acetate is the major direct precursor for methanogenesis in both mesophilic and thermophilic waste treatment systems, accounting for approximately 70-80% of the total  $\text{CH}_4$  produced (Mackie

and Bryant, 1981; Gujer and Zehnder, 1983; Zinder *et al.*, 1984a; Jeris and McCarty, 1965; Smith and Mah, 1966; Mountfort and Asher, 1978). Based on tracer experiments, it was concluded that acetate is split in a so-called aceticlastic reaction in which the methyl-group of the acetate molecule is reduced to CH<sub>4</sub>, while the carboxyl-group is oxidized to HCO<sub>3</sub><sup>-</sup> (e.g. Zehnder *et al.*, 1980; Zehnder *et al.*, 1982):



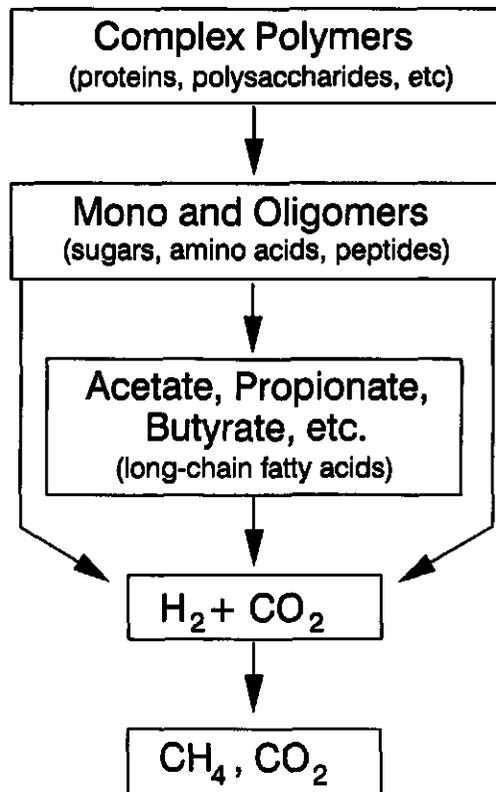
So far, only two genera of methanogenic bacteria, i.e. *Methanosarcina* and *Methanotherix* ("*Methanosaeta*"; Patel and Sprott, 1990), are capable of catabolizing acetate to CH<sub>4</sub>, both under mesophilic and thermophilic conditions (Zinder 1990). The role of the various species of aceticlastic methanogens in thermophilic waste(water) treatment processes is further discussed in Chapters 3 and 4.1. Thermophilic aceticlastic methanogens and some of their kinetic properties are listed in Table 4.2.

In addition to the above reaction, Zinder and Koch (1984) described the occurrence of a two-step reaction in which acetate is first oxidized to H<sub>2</sub>/CO<sub>2</sub>, followed by a subsequent conversion to CH<sub>4</sub>. The reaction is performed by a homo-acetogenic bacterium in co-culture with a methanogen:



The co-culture grew optimally at 60°C and was further characterized by Lee and Zinder (1988a, 1988b). Independently, Weber *et al.* (1984) found that in a mesophilic methanogenic culture, syntrophic acetate conversion can take place in an order similar to the aceticlastic reaction. The culture was enriched from various anaerobic environments including sewage treatment plants. The acetate oxidation reaction, being the first step of the sequence, is thermodynamically difficult ( $\Delta G^{\circ\prime} = +104 \text{ kJ/mol}$ ). For this reason, Wiegant (1986) considered a possible contribution of syntrophic acetate conversion to the overall digestion process to not be very important. However, recent findings reveal that the two-step reaction might become important under specific 'stress' conditions like high ammonium concentrations (Blomgren *et al.*, 1990) and, interestingly, at high temperatures. Petersen and Ahring (1991) demonstrated that syntrophic acetate oxidation might contribute to up to 14% of total acetotrophic methanogenesis in a thermophilic (60°C) digester. Moreover, results of Ahring (1995) show that when the acetate concentration drops to below the threshold level for the dominating aceticlastic methanogen in a 55°C digester, the two step reaction becomes the predominant mechanisms for acetate conversion. Uemura and Harada (1993) and Ahring *et al.* (1993) recently reported that a relatively large fraction of granular sludge grown in UASB reactors at 55°C, also consisted of such syntrophic consortia. An even more dominant role

of non-aceticlastic acetate conversion is expected at temperatures higher than 65°C (Ahring et al. 1995; Uemura & Harada 1993 1995), which is beyond the temperature range of the aceticlastic methanogens known thus far (Table 4.2; Zinder, 1990). The addition of acetate to methanogenic sludge cultivated at 70-75°C in UASB reactors resulted in a rapid build-up of H<sub>2</sub> in the head-space of closed serum vials (Rintala et al., 1993; Van Lier et al., 1993). Moreover, recent investigations with the 75°C-sludge using <sup>13</sup>C-labelled acetate as the substrate showed that approximately 95% of the total acetate conversion was performed by the syntrophic acetate oxidation reaction (Van Lier et al., unpublished results). The predominance of the latter reaction in extreme thermophilic (70°C) UASB reactors was recently confirmed by Ahring et al. (1995). Furthermore, syntrophic oxidation of acetate was shown to be the principal pathway to acetate conversion in Islandic hot springs of 70°C (Ahring, 1992). These results indicate that under extreme thermophilic conditions, hydrogen-consuming methanogens are mainly responsible for the final COD removal from waste and wastewater (Fig. 1.6).



**Fig. 1.6** Hypothetical carbon flow in the anaerobic degradation of organic matter under extreme thermophilic conditions, assuming that aceticlastic methanogenesis does not contribute to the ultimate COD removal.

The kinetics and biochemical characteristics of various thermophilic hydrogenotrophic methanogens are discussed in recent review papers of e.g. Vogels *et al.* (1988), Winter and Zellner (1990) and Lowe *et al.* (1993). The number of new isolates of these types of methanogens is still increasing (Zhao *et al.*, 1988; Nishimura *et al.*, 1991; Lowe *et al.*, 1993) and a maximum growth temperature of up to 110°C is reported (Mukund *et al.*, 1992; Huber *et al.*, 1989). In general, thermophilic hydrogenotrophic methanogens possess very high maximum specific growth rates with doubling times of 1-4 hours, while the apparent substrate half-saturation constants ( $K_s$ ) are higher than those of comparative mesophilic organisms as reviewed by Wiegant (1986). When  $\text{SO}_4^-$  is the final electron acceptor, the produced  $\text{H}_2$  can alternatively be used by sulphate reducing bacteria (SRB). The latter organisms generally have a much higher affinity for  $\text{H}_2$  than the methanogens (Oude Elferink *et al.*, 1994; Rinzema and Lettinga, 1988). Under sulphate reducing conditions, methanogens and sulphate reducers compete for the same substrate (Visser, 1995). While acetate is merely converted by the acetoclastic methanogens, the hydrogen is used by the SRB (Isa *et al.*, 1986; Yoda *et al.*, 1987; Rinzema, 1988; Rinzema and Lettinga, 1988; Visser, 1995). However, if syntrophic acetate conversion is becoming more important, methanogenesis is more easily suppressed, which will result in the predominance of SRB. A very rapid recovery of sulphate reduction after a shift from mesophilic to thermophilic conditions was observed by Rintala and Lettinga (1991). The same researchers found that sulphate reduction contributed to more than 50% of the total COD removal during thermophilic (55°C) treatment of sulphate-rich TMP whitewater (Rintala *et al.*, 1991). Also, acetate oxidation to  $\text{H}_2/\text{CO}_2$  seemed to play a larger role in thermophilic sewage sludge digesters which were adapted to relatively high sulphate concentrations (Petersen and Ahring, 1992). The above results indicate that, due to syntrophic acetate oxidation, complete COD conversion under thermophilic conditions can be acquired with  $\text{SO}_4^-$  as the final electron acceptor.

#### **Role of $\text{H}_2$ as intermediate electron carrier**

Hydrogen is an important intermediate in anaerobic conversion processes and is mainly produced during the acidification of complex organic matter (e.g. carbohydrates, proteins, lipids, etc.).  $\text{H}_2$  is also generated during the subsequent conversion of the produced fermentation products to acetate and  $\text{CO}_2$ . Particularly the latter acetogenic reactions, which are performed by obligate proton-reducing bacteria, depend on the  $\text{H}_2$  partial pressure (Table 1.1). For thermodynamic reasons a further oxidation of the produced intermediates to acetate and  $\text{CO}_2$  can only occur at extremely low  $\text{H}_2$  concentrations. Therefore, a complete mineralization of carbon compounds is only possible if  $\text{H}_2$  is effectively removed by e.g. the methanogenic bacteria (Boone and Bryant, 1980; Bryant, 1979; McInerney *et al.*, 1981; Wolin, 1982). This phenomenon of interspecies transfer of reducing equivalents was recently reviewed by Schink (1992) and Stams (1994). While acetogenic reactions are inhibited by high concentrations of  $\text{H}_2$  (Ahring and Westermann, 1988; Stams *et al.*, 1992), the  $\text{H}_2$  partial pressure also determines the types of fermentation products of the fermentative bacteria

(Zinder, 1986; Winter and Zellner, 1990). At high  $H_2$  partial pressures further reduced compounds are formed, such as ethanol, lactate, and the VFAs propionate and butyrate. On the other hand, if the  $H_2$  concentration is low, carbohydrates are merely converted into acetate,  $CO_2$  and  $H_2$ , which especially applies under thermophilic conditions (Zinder, 1986b; Winter and Zellner, 1990). In addition, the spectrum of fermentation products of thermophilic acidifying bacteria is generally more restricted than of mesophilic acidifiers, as reviewed by Winter and Zellner (1990).

Obviously, the effectiveness of interspecies hydrogen transfer plays a crucial role in the product distribution pattern of the fermentative phase, i.e. it determines the stability of the overall digestion process. As an alternative to hydrogen, various researchers have postulated that formate may be a more suitable electron carrier due to its high solubility (Thiele and Zeikus, 1988a, b; Ozturk *et al.*, 1988; Boone *et al.*, 1989). Recently, Dong (1994) demonstrated that interspecies formate transfer was the predominant mechanism during propionate and butyrate oxidation in dispersed defined mesophilic cultures. The occurrence of interspecies formate transfer is also supported by the results of Grobicky and Stucky (1991) who reported that overloading of a mesophilic anaerobic baffled reactor resulted in an accumulation of formate, while the  $H_2$  concentration remained constant. On the other hand, in various cases, syntrophic associations were studied in which the methanogen was not able to use formate as a substrate, excluding the involvement of interspecies formate transfer (Grotenhuis, 1992; Stams, 1994). Furthermore, in balanced microbial consortia like granular anaerobic sludge, the distances between the various bacteria are small enough to explain the high biodegradation rates through interspecies hydrogen transfer (Grotenhuis *et al.*, 1990; Stams, 1994). Finally, according to recent results of Bleicher and Winter (1994) formate transfer is only of importance if hydrogen consumption is actively suppressed or whenever perturbation requires an extra electron sink. The role of formate under thermophilic conditions is even less clear. Up to now, formate was not observed to be an important intermediate in thermophilic digesters. In one of our own investigations, a sudden increase in the organic loading rate of a thermophilic reactor led to an immediate build-up of  $H_2$  while the formate concentration remained unchanged (Chapter 6.1). Therefore, whether  $H_2$  or formate is the predominant electron carrier between acetogens and methanogens may also depend on the process temperature. Interestingly, *Methanobacterium thermoautotrophicum* is one of the predominant hydrogenotrophic methanogens in many thermophilic methanogenic consortia. This bacterium is unable to use formate as a substrate (Chapter 3.2; König and Stetter, 1989; Zeikus and Wolfe, 1972; Schönheit *et al.*, 1980; Zehnder *et al.*, 1982). Recently, we described the enrichment of a thermophilic propionate oxidizer, with *M. thermoautotrophicum*  $\Delta H$  as the only syntrophic partner, demonstrating that the electrons could be channelled exclusively by hydrogen (Stams *et al.*, 1992). The hypothesis that reducing equivalents are channelled through  $H_2$  as an intermediate rather than through formate, particularly under thermophilic conditions, is also supported by the recent results of Schmidt and Ahring (1993). The reason for this prominent contribution of  $H_2$  to

thermophilic anaerobic conversion processes is not yet clear. However, interspecies  $H_2$  transfer may be favoured at high temperatures because the effect of temperature on the diffusivity of gaseous compounds is higher than on soluble compounds such as formate (§ 1.2.2).

The involvement of hydrogen-consuming methanogens in thermophilic systems would be even considerably larger if non-aceticlastic acetate conversion were an important route in the mineralization process, with hydrogen as the predominant electron carrier. Interestingly, a reversed observation was recently made by Westermann (1994) under psychrophilic conditions. A decreased contribution of hydrogenotrophic methanogens in swamp slurries with decreasing temperatures from the mesophilic ( $37^\circ C$ ) to the psychrophilic ( $2^\circ C$ ) range was observed. Combining these results with the observations made under thermophilic conditions, one can speculate on a biological rule that the importance of  $H_2$  in methanogenic ecosystems increases with increasing temperatures from below  $0^\circ C$  to higher than  $100^\circ C$ .

## 1.5 Thermophilic anaerobic wastewater treatment

Anaerobic treatment at high temperatures has been investigated for more than a century, particularly for the treatment of slurries and solid wastes. The first full-scale applications consisted of thermophilic sewage sludge digesters. The interested reader is referred to the excellent review papers of Cooney and Wise (1975), Buhr and Andrews (1977), Varel (1983), Wiegant (1986) and Zinder (1986). More recently, research has also been performed on the feasibility of thermophilic treatment of municipal solid waste (Deboosere *et al.*, 1986; Cecchi *et al.*, 1991, 1992; Pavan *et al.*, 1994; Pera *et al.*, 1992; Rintala and Ahring, 1994; Vallini *et al.*, 1993), and thermophilic digestion of manure with or without the addition of industrial waste (Ahring, 1994; Angelidaki and Ahring, 1994; Lo *et al.*, 1985; Zeeman *et al.*, 1985). Results from the experimental studies and large-scale applications demonstrate that the thermophilic treatment of waste is promising and that it is becoming an accepted technology.

In this section attention will be given to the state-of-the-art and perspectives of thermophilic wastewater treatment systems. Research on thermophilic treatment of wastewaters is mainly performed with model compounds, such as VFAs and carbohydrates, and with industrial wastewaters which are discharged at high temperatures, e.g. effluents from pulp and paper industries and wastewaters from food processing industries such as alcohol distilleries, palm oil production plants, and canneries. Research on thermophilic anaerobic wastewater treatment started with completely mixed systems (§ 1.5.1) while in the last decades there has been an increasing interest in applying high-rate reactors for this purpose (§ 1.5.2).

### 1.5.1 Completely mixed systems

In completely mixed thermophilic systems the high growth rates of thermophilic bacteria generally result in shorter retention times and/or higher conversion rates compared to mesophilic systems (Borja *et al.*, 1995; Ono, 1965; Romero *et al.*, 1990, 1993). Applying a thermophilic contact process, i.e. a CSTR-type digester with solids recycle, a reduction in the retention time from 14 to 3 days was found, with similar COD removal rates (Brune *et al.*, 1982). Other research showed no benefits of thermophilic treatment over conventional mesophilic digestion (Sen and Bhaskaran, 1962; Basu and Leclerc, 1975). Experiences with thermophilic lab-, pilot-, and full-scale CSTR-type reactors are reviewed by Rintala (1992), Wiegant (1986) and Zinder (1986). Among the various types of wastewaters investigated, effluents from distilleries received the most attention (Basu and Leclerc, 1975; Ono, 1965; Romero *et al.*, 1990, 1993; Sen and Bhaskaran, 1962; Vlissidis and Zouboulis, 1993), followed by palm-oil production wastewaters (Cail and Barford, 1985; Chin and Wong, 1983; Yeoh, 1986). In general, the obtained results are very promising, but the overall performance of thermophilic CSTR-type reactors is worse than the well developed high-rate reactors under mesophilic conditions.

### 1.5.2 Thermophilic anaerobic high-rate reactors

Since the seventies there has been an increasing interest in researching the potentials of thermophilic high-rate reactors with immobilized biomass. Thermophilic methanogenic sludge cultivated in such reactors exerts a very high specific activity. The high activity of immobilized sludge is attributed to the high energy consumption for the maintenance of thermophiles (see also § 1.3.1). A summary of the research on thermophilic high-rate reactors is presented in Table 1.5. Part of these data are derived from literature reviews of Wiegant (1986), Zinder (1986) and Rintala (1992). The different substrates listed were tested under various loading conditions, however, Table 1.5 shows only the most optimal values. Going through the literature results, it looks as though extreme loading rates are generally accompanied by decreased removal efficiencies, particularly in the studies performed with industrial (non-synthetic) wastewaters (e.g. Rintala and Lepistö, 1992; Wiegant *et al.*, 1985). However, due to the variety of wastewaters studied and the different processes applied it is very difficult to make a conclusion from the obtained results. Roughly, the results reveal that a high degree of VFA removal can only be achieved in moderately low-loaded thermophilic high-rate reactors (e.g. Ahring *et al.*, 1993; Schraa and Jewell, 1984; Chapter 3). The occurrence of (substrate) toxicity and/or other rate-limiting steps under thermophilic conditions is still poorly understood. In addition to high effluent VFA concentrations, perturbed reactors were in some cases also characterized by excessive wash-out of active thermophilic biomass (§ 1.5.3). Problems with high effluent VFA concentrations and a high

biomass wash-out could be solved by applying a staged plug-flow reactor as suggested in Chapter 6.

### Seed sludge

So far, suitable thermophilic sludge for seeding high-rate reactors is hardly available. However, because of the natural distribution of thermophilic methanogens, any inoculum source with a reasonable mesophilic methanogenic activity can be used as seed sludge. Once the temperature is increased beyond the mesophilic range ( $\approx 45^\circ\text{C}$ ) a rapid die-off of mesophiles occurs followed by growth of thermophilic organisms present in the seed sludge. The higher the newly installed process temperature, the faster the deterioration of the mesophilic inoculum (Van Lier *et al.*, 1990). On the other hand, various authors showed that anaerobic digestion of the organic fraction of municipal solid waste (OFMSW) could be shifted from the mesophilic to the thermophilic temperature range in a relatively short period of time (Deboosere *et al.*, 1986; Cecchi *et al.*, 1992). Interestingly, we found that OFMSW, digested under mesophilic conditions, exhibited a more or less similar activity under mesophilic ( $37^\circ\text{C}$ ) as well as under thermophilic ( $55^\circ\text{C}$ ) conditions (Van Lier *et al.*, 1993). Apparently, with OFMSW as seed sludge, a rapid transition from mesophilic to thermophilic conditions can be expected. The suitability of fresh and digested cow manure for the thermophilic start-up was studied extensively by Wiegant (1986). Cow manure was used successfully in the early studies of Varel *et al.* (1977). In this thesis the use of mesophilic granular sludge for the start-up of thermophilic high-rate reactors is discussed in more detail (Chapter 3).

The application of thermophilic methanogenic seed sludge for starting high-rate thermophilic wastewater treatment reactors was recently studied by Ahring (1991). The inoculum was derived from a CSTR-type large-scale biogas plant treating manure and industrial waste at  $53\text{--}55^\circ\text{C}$  (Ahring, 1991; Ahring *et al.*, 1995). The researchers indeed found very short periods for reactor start-up. Most interestingly was the observation that *Methanosarcina* species were the only acetate utilizing methanogens in the cultivated granular sludge (Ahring, 1991; Ahring *et al.*, 1995). On the contrary, *Methanotherix* species are generally observed as the predominant acetate-utilizing methanogens in thermophilic granular sludge grown in UASB reactors which are seeded with a mesophilic inoculum (Chapters 3.1 and 4.1). Wiegant and De Man (1986) deliberately cultivated '*Methanosarcina* granules' at high effluent acetate concentrations. They observed that UASB reactors with this type of granular sludge performs distinctly worse than UASB reactors with '*Methanotherix* granules'. So far, the '*Methanosarcina* granules' cultivated by Ahring (1991) have not been tested under high loading conditions. The predominance of *Methanosarcina* species in the latter sludge granules can be easily explained by the fact that the seed material from the large-scale thermophilic digesters is predominated by *Methanosarcina* species as well (Ahring, 1991), see also § 4.1.4.

**Table 1.5** Thermophilic anaerobic wastewater treatment in high-rate reactors. Reactor volumes refer to the active working volume.

Substrate	Temp. °C	Process	Volume <i>l</i>	Influent g COD. <i>l</i> <sup>-1</sup>	Loading rate kg COD.m <sup>-3</sup> .day <sup>-1</sup>	HRT h	COD reduc %	Ref.
sucrose	55	AAFEB	2.0 <sup>c</sup>	2-16	30-40	0.5-5	70	1
sucrose-VFA	55	UASB	5.75	8	35	5.7	84.5	2
VFA	55	UASB	5.75	14.7	104	3.2	78	2
Ac/ Ac-Bu	55	UASB	5.75	3-4.5	120-150	0.6	84-93	3
sucrose-Ac	55	UASB	5.5	3	87	0.8	96	4
sucrose-Ac	65	UASB	5.5	3	41	1.8	77	4
coffee waste	55	UASB	10 <sup>d</sup>	4-8	6-10	32	70	5
coffee waste	53	CSTR-FB <sup>b</sup>	5+0.45	23	7	78	20	6
coffee waste	55	UAF	1.14	4	4	24	60	7
corn steep	53	AFR	1.1	5.5	11	12	90	8
liquor/glucose								
beer brewing process	55	UASB (two stage)	1400	20	30-40 <sup>e</sup>	< 24	80-90	9
fish and sea food	55	UAF	0.92	10-26	12	53	74	10
non-fat dry milk	56	UAF	16.8	25	50	12	67-78	11
wood hydrolysate	55	UFFR	0.45 <sup>c</sup>	22.5	10	54	84.4	12
bean blanching	55	DSFF	1.25 <sup>c</sup>	10	6.6-17.4	13-31	84-91	13
meat waste	57-60	FB	4	2.5	4.6	13	49-59	14
boiled soya bean	54	UFF/AF	lab	32-66	47	20	90	15
boiled soya bean	54	UFF/AF	pilot	32-66	40	n.r.	90	15
ice cream	55	UAF	1.1	10	36	6.6	85	16
distillery	42	UAF	500	45-50	38 <sup>f</sup>	29	40-50	17
vinasse <sup>a</sup>	55	UASB	5.75	diluted	100	2.5	60	18
vinasse <sup>a</sup>	55	UASB	75000	31.5	25-30	11	72	19
TMP-white water	55	UASB	0.25	2-3.5	14-22	6	65-75	20
				3	max.80	1	60	
TMP white water	70	UASB	0.25	3	13	7	60	20
pharmaceutical- glucose	55	UFF	35 <sup>c</sup>	2.5-7	0.5-1.5	113	51-58	21

a effluents of alcohol distillery

<sup>b</sup> batch digestion of coffee waste, flow is kept by recirculation, COD reduction based on CH<sub>4</sub> yield

<sup>c</sup> total volume

<sup>d</sup> results from 6 m<sup>3</sup> UASB pilot study were slightly worse

<sup>e</sup> a load of 50 kg COD.m<sup>-3</sup>.day<sup>-1</sup> was reached after replacement of 30% of the COD load with sugar

<sup>f</sup> loading rate in kg VS.m<sup>-3</sup>.day<sup>-1</sup>

Ref: 1, Schraa and Jewell (1984); 2, Wiegant and Lettinga (1985); 3, Wiegant and De Man (1986); 4, Uemura and Harada (1993); 5, Lanting *et al.* (1989); 6, Kida *et al.* (1992); 7, Fernandez and Forster (1993); 8, Yang *et al.* (1992); 9, Ohtsuki *et al.* (1994); 10, Lema *et al.* (1988); 11, Harris and Dague (1993); 12, Good *et al.* (1982); 13, Kennedy and Van de Berg (1982); 14, Rudd *et al.* (1985); 15, Kawase *et al.* (1989); 16, Ugurlu and Forster (1992); 17, Braun and Huss (1982); 18, Wiegant *et al.* (1985); 19, Souza *et al.* (1992); 20, Rintala and Lepistö (1992); 21, Seif *et al.* (1992).

### 1.5.3 Biomass retention

Effective retention of biomass is a prerequisite for a successful application of anaerobic high-rate reactors. In general, two approaches are followed to achieve a high biomass hold-up:

- i) auto-immobilization of anaerobic bacteria which leads to the formation of granular sludge. The process of granulation is characteristic for upflow (hybrid) sludge bed reactors which are operated in the mesophilic or thermophilic temperature range;
- ii) adherence of the microorganisms to inert support media. This mechanism is essential in fixed film reactors such as anaerobic filters and fluidized bed reactors.

From Table 1.5 it can be seen that various types of high-rate reactors were studied under thermophilic conditions, among which the (U)AF and UASB received the most interest. In several studies it was found that immobilization of thermophilic bacteria is more difficult to achieve under thermophilic than under mesophilic conditions. Duff and Kennedy (1982) studied the performance of mesophilic and thermophilic downflow stationary fixed film reactors under conditions of hydraulic and organic overloads. The low stability of the thermophilic reactor to the shock loadings was attributed to the large fraction of loosely attached biomass. Contrary to their results, Ugurlu and Forster (1992) observed a relatively high stability of thermophilic upflow filters which were subjected to organic and hydraulic shocks. The filters were most affected by increased COD concentrations. Problems with biomass retention were also observed by Soto *et al.* (1992) who investigated the feasibility of mesophilic and thermophilic anaerobic filters for the treatment of saline wastewaters from mussel cooking factories. While the mesophilic filter could be operated satisfactory at an OLR of up to  $25 \text{ kg} \cdot \text{m}^{-3} \cdot \text{day}^{-1}$ , the thermophilic reactor was very unstable at loading rates beyond  $12 \text{ kg} \cdot \text{m}^{-3} \cdot \text{day}^{-1}$ . They found that significantly less biomass could be retained by the thermophilic filter compared to the mesophilic reactor, where the largest fraction of the biomass was present as non-attached flocs. Instead of flocs or aggregates, dispersed sludge with a poor settling ability was formed in the thermophilic filter (Soto *et al.*, 1992). Growth of a similar type of sludge was also observed during the start-up of thermophilic UASB reactors, fed with VFA-mixtures as the substrate, see also Chapter 3.1 (Uemura and Harada, 1993; Wiegant and Lettinga, 1985). The formation of dispersed sludge might partly be attributed to the higher degree of sludge mineralization under thermophilic conditions (Soto *et al.*, 1992), which, consequently, results in a lower amount of extracellular polymers. Schmidt and Ahring (1994) recently indeed found that the content of extracellular polysaccharides and protein in thermophilic granular sludge was much lower than in mesophilic granules. A low content of these polymers may hinder the formation of dense and firm granules. While dispersed biomass was formed using VFA mixtures as the sole substrate, addition of sucrose or glucose to the influent resulted in the formation of thermophilic granular sludge with a high specific activity and a good settling ability (Chapters 3.1 and 6; Wiegant and Lettinga, 1985; Uemura and Harada, 1993). Vanderhaegen *et al.*

(1992) previously discussed the important role of acidifiers and/or their excretion products, such as extracellular polysaccharides, for the cultivation of sludge granules in the mesophilic temperature range. Due to the higher mineralization rate, the requirement for extracellular polymers may even be higher under thermophilic conditions. Another reason explaining dispersed growth, might be the physiological appearance of the predominant methane bacteria at high temperatures. In contrast to mesophilic conditions, long filamentous bacteria are less frequently observed, while often short rod-shaped or even coccoid methane bacteria dominate, see also Chapter 3 (Uemura and Harada 1993; Schmidt and Ahring 1993). A low surface to volume ratio of thermophilic methanogens may negatively influence the adherence capacity of the cells, assuming that the cell-hydrophobicity also plays an important role in the initial attachment under thermophilic conditions (Grotenhuis et al. 1992). Finally, due to the low liquid viscosity under thermophilic conditions (§ 1.2.2), the cells which are only weakly bound, are more easily rinsed from the system. Nonetheless, as previously mentioned, formation of granular sludge with a high activity and a good settling ability, generally occurs in thermophilic methanogenic upflow reactors (Chapter 6, Ohtsuki *et al.*, 1992, 1994; Schmidt and Ahring, 1993; Souza *et al.*, 1992; Uemura and Harada, 1993, 1995; Wiegant and Lettinga, 1985). Interestingly, growth of thermophilic granular sludge was also observed when acetate and acetate/butyrate mixtures were used as the sole substrate (Ahring, 1991; Wiegant and De Man, 1986). In the latter investigations other types of inocula were used for seeding the UASB reactors, such as digested cow manure and digested residue of large-scale thermophilic biogas plants. Likely, in addition to the substrate composition, the source of the inoculum may also play a role in the granulation process. Factors affecting granulation of thermophilic methanogenic sludge are discussed further in Chapters 3 and 6.

In case (auto)immobilization of thermophilic biomass is difficult to achieve, the use of reactors with inert support material (fixed film reactors) should be reconsidered. Moreover, also the use of single stage sludge bed reactors is questionable, because high loading conditions may result in a high wash-out of the dispersed sludge. In such cases, the use of compartmentalized reactor systems is apparently more appropriate (Chapter 6). Bachman *et al.* (1985) introduced the anaerobic baffled reactor and claimed a good retention of non-granular biomass under relatively high loading conditions. So far, the ABR has not been tested under high temperatures. However, omission of the gas-solid separators, as proposed in the ABR design, may become the bottleneck under the extreme loading conditions at high temperatures.

## 1.6 Outline of the thesis

**Chapter 3** describes the effect of a temperature increase from mesophilic to thermophilic conditions on the performance of UASB reactors inoculated with mesophilic granular sludge. As a result of the temperature increase from 38°C to the range between 45-65°C, mesophilic bacteria are replaced by thermophiles. A more gradual shift can be expected at temperatures just above the mesophilic range, i.e. 45°C. Application of mesophilic granular sludge for starting a thermophilic process is beneficial if the granular structure is conserved while the bacterial population is replaced.

The temperature susceptibility of thermophilic sludge is investigated in **Chapter 4**. The optimum temperature for substrate conversion of sludge cultivated in experiments in Chapters 3 and 6 is assessed. Selection criteria other than process temperature may determine the temperature sensitivity of the cultivated thermophilic biomass. The temperature susceptibility is relatively low, particularly for high-rate reactors which are characterized by immobilized biomass. In such systems a mild temperature dependence is attributed to substrate diffusion limitation in the biofilm.

Thermophilic reactor systems are often characterized by relatively high concentrations of propionate in the effluent. **Chapter 5** presents results on the effects of volatile fatty acids on the thermophilic conversion of propionate. The effects are studied in UASB reactors as well as in defined cultures of a highly enriched propionate oxidizer in syntrophy with a hydrogen-consuming methanogen.

Thermophilic UASB reactors are not very stable during long-term operation under high loading conditions. Application of plug-flow conditions enhances the stability of the process considerably. In **Chapter 6** a staged upflow process is investigated for the thermophilic treatment of mixtures of volatile fatty acids with and without the addition of a large fraction of sucrose to the influent. Wash-out of viable biomass is minimized by applying various gas-solid separators along the height of the reactor. For long-term operation a 'steady-state' in the segregated sludge development must be pursued. Otherwise, voluminous acidifying sludge will eventually force out the more active methanogenic granules.

The thesis is concluded by a general **Summary and Discussion** of the obtained results.

## Chapter 2

### Materials & Methods

In this chapter the general materials and methods are listed which are used throughout the research. Specific methods for particular experiments are discussed in each chapter.

## 2.1 Continuous Flow Experiments

Continuous flow experiments were performed by using Upflow Anaerobic Sludge Bed (UASB) reactors and modified UASB reactors, equipped with various gas-solid separators along the height of the reactor. Latter reactors were denominated as the Upflow Staged Sludge Bed (USSB) reactor.

### UASB reactors

The total volume of the poly-acrylate UASB reactors was 7.1 litre (settler included), the working volume was 5.75 litre, and the inner diameter of the reactor 0.09 m (Fig. 2.1). Temperature was controlled by a thermostat-bath-circulator (Haake D1-L, FRG) connected to the double wall of the reactor. Temperature in the sludge bed was checked with a temperature control unit (West mini, Brighton, UK) connected to a thermocouple. Methane production was measured by a wet-test gas meter (Meterfabriek Dordrecht, The Netherlands) at 20°C after the biogas had been led through a NaOH solution (1 M) and a column of soda lime pellets with indicator (Merck Art. no. 6839, Darmstadt, FRG).

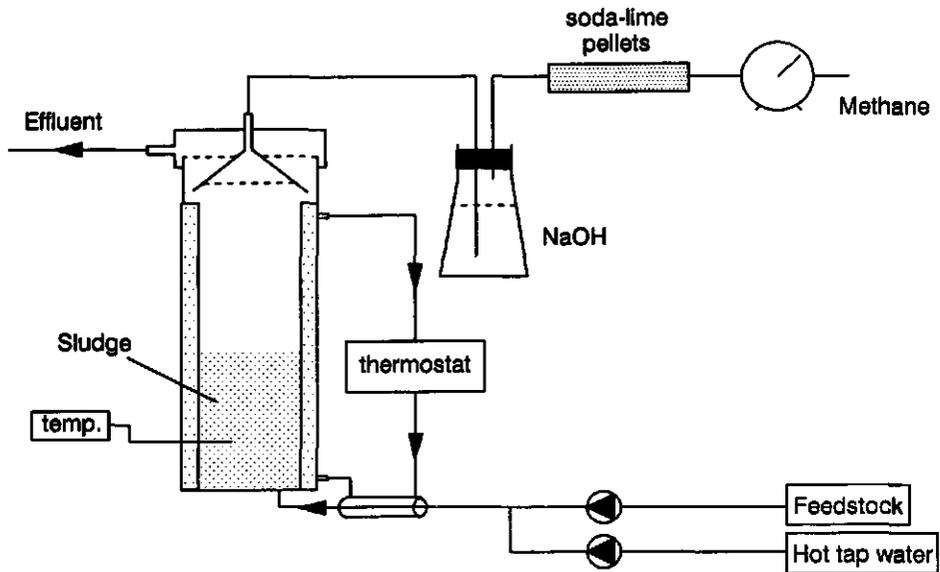
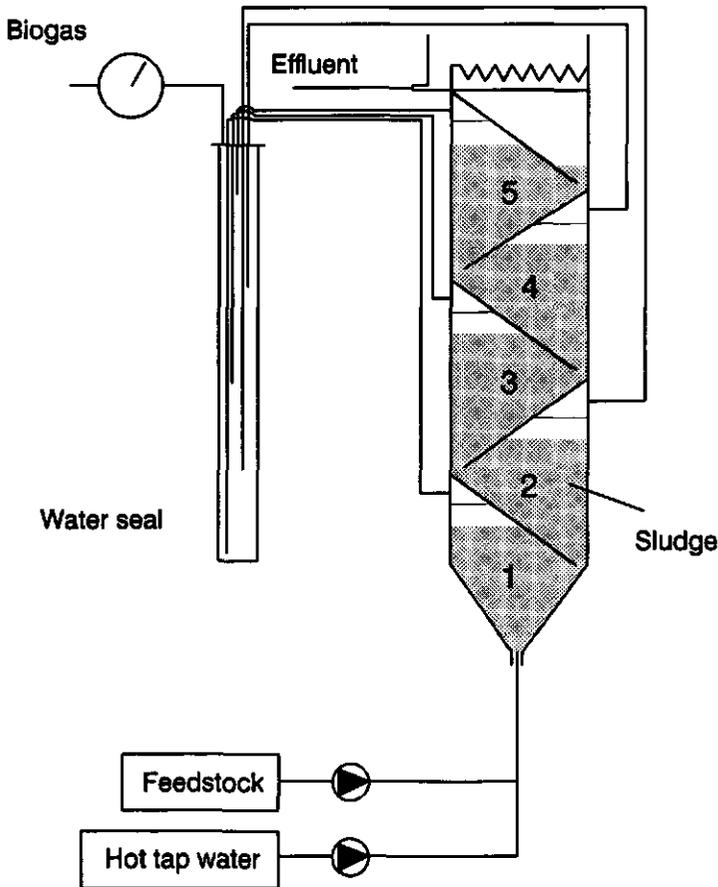


Fig. 2.1 Scheme of the 7.1 litre Upflow Anaerobic Sludge Bed (UASB) reactor.

All computations with respect to process performance were calculated based on the working volume of 5.75 litre. The influent flow was composed of a dilution flow consisting of tap water and a substrate flow consisting of a concentrated feed stock. Before the influent was fed to the reactor, both flows were combined and brought to reactor temperature. Peristaltic pumps (Watson Marlow 202 and 302, Falmouth, Cornwall, UK) were used.



**Fig. 2.2** Experimental set-up of the 5.1 litre Upflow Staged Sludge Bed (USSB) reactor.

### USSB reactors

A schematic representation of the USSB reactor and the experimental set-up is presented in Fig. 2.2. The dimensions of the poly-acrylate reactors are 0.1 x 0.1 x 0.54 m with a pyramid-shape at the bottom. The total volume was 5.1 litre and the liquid volume was approximately 4.2 litre. The volumes of the compartments 1, 2 to 5, and the settler were

approximately 0.7, 0.9, and 0.8 l, respectively. The opening between each gas-solid separator and reactor wall is 0.012 m, which results in a maximum superficial liquid velocity in this opening of about 2 m/h when a HRT of 2 h is applied. The liquid-gas interface of each compartment is controlled by a central water seal. Biogas production is measured by wet-test gas meters (Meterfabriek Dordrecht, The Netherlands) at 20°C. All computations with respect to process performance are calculated based on the total volume. The reactors were fed with either a VFA mixture or with a sucrose-VFA mixture (Chapter 6). Concentrated stock solutions were diluted with hot tap water (55°C) and then led into the reactor. In case reactors were fed with a sucrose-VFA mixture the feed stock solutions were stored at 4°C to prevent acidification of the substrate. Peristaltic pumps (Watson Marlow 202 and 503, Falmouth, Cornwall, UK) were used.

## 2.2 Mesophilic Seed Material

Two types of mesophilic seed material were used to inoculate the thermophilic digesters:

- I Mesophilic granular sludge (MGS). The mesophilic methanogenic granules originated from a 1700-m<sup>3</sup> UASB reactor (36°C) of the Aviko potato-processing factory at Steenderen, The Netherlands. The sludge is further referred to as Aviko-MGS.
- II Digested organic fraction of municipal solid waste (OFMSW). Digested OFMSW was derived from a 450 m<sup>3</sup> dry anaerobic batch digestion pilot plant, the so called BIOCEL reactor, which was operated at 25-30°C, at 't Zand, The Netherlands (Ten Brummeler *et al.* 1991, 1992). The fresh OFMSW was separated at the source and was composed of vegetable, fruit and yard waste. Digested OFMSW was manually sieved prior to use in two steps (pore size 5 mm and 2 mm) to remove the large particles.

## 2.3 Medium Composition

### Continuous flow experiments

The influent flow of all reactors was composed of a dilution flow consisting of tap water with approximately 30 mg Ca<sup>++</sup>.l<sup>-1</sup> and a substrate flow consisting of a concentrated feed stock. The reactors were fed with either a VFA mixture (pH 6.5) or with a sucrose-VFA mixture. The latter feed stock solution was stored at 4°C which prevented acidification. The exact composition of the basal nutrients and carbon source is indicated in each chapter. The composition of the trace element solution used in each study was (mg.l<sup>-1</sup>): FeCl<sub>2</sub>.4H<sub>2</sub>O (2000), H<sub>3</sub>BO<sub>3</sub> (50), ZnCl<sub>2</sub> (50), CuCl<sub>2</sub>.2H<sub>2</sub>O (30), MnCl<sub>2</sub>.4H<sub>2</sub>O (500), (NH<sub>4</sub>)<sub>2</sub>Mo<sub>7</sub>O<sub>24</sub>.4H<sub>2</sub>O (50), AlCl<sub>3</sub>.6H<sub>2</sub>O (90), CoCl<sub>2</sub>.6H<sub>2</sub>O (2000), NiCl<sub>2</sub>.6H<sub>2</sub>O (92), Na<sub>2</sub>SeO<sub>3</sub>.5H<sub>2</sub>O (164), EDTA (1000), resazurin (200), 36% HCl (1 ml.l<sup>-1</sup>). All chemicals were of analytical grade and were purchased from Merck (Darmstadt, FRG) except yeast

extract which was purchased from Gibco BRL (Paisly, UK) and resazurin which was purchased from Fluka Chemie (Buchs, Switzerland).

### Batch experiments

The mineral medium of the batch experiments consisted of (g.l<sup>-1</sup>): NH<sub>4</sub>Cl, 0.28; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.11; K<sub>2</sub>HPO<sub>4</sub> 2.0; NaH<sub>2</sub>PO<sub>4</sub>·2H<sub>2</sub>O, 3.33; yeast extract, 0.10 and; 1 ml.l<sup>-1</sup> trace element solution. The carbon source is indicated in each chapter.

## 2.4 Activity Tests

### Substrate-depletion activity test

The substrate-depletion activity tests were performed in 120 ml serum bottles which were filled with 100 ml medium (§ 2.3) and brought to appropriate temperatures. Sludge samples from the thermophilic reactors were used directly. The sludge was introduced into the bottles beneath the liquid surface by means of 5 ml automatic pipet (Gilson, Villiers, France) with a plastic tip of which the narrow opening was cut off. After closing the bottles and changing the gas phase composition to N<sub>2</sub>-CO<sub>2</sub> (70:30; vol/vol), 1 ml Na<sub>2</sub>S from a 1 M stock solution was added per 1 litre medium, to obtain completely anaerobic conditions. After various periods, samples were taken and analyzed for acetate. After each experiment the exact amount of VSS per bottle was measured.

### Specific methanogenic activity test: the "headspace method"

The specific methanogenic activity using the "headspace method" was assessed by following the increase in CH<sub>4</sub> concentration in the headspace of 320 ml serum bottles according to the tests described by Rinzema *et al.* (1988). In this way, the methanogenic activity can be assessed in a short time period (1-3 h) with only a little decrease in substrate concentration and without any nominal pressure build up in the headspace of the bottle. Serum bottles were filled with 100 ml of the mineral medium (§ 2.3) at pH 6.8 and brought to the appropriate temperature in a GFL-1083 waterbath-shaking incubator (Burgwedel, FRG). The procedure for adding the methanogenic sludge was similar as in the substrate-depletion activity tests. After addition of sodium acetate the bottles were incubated under anaerobic conditions for an overnight acclimatization period. Acetate concentrations were measured after 16 hours of incubation and the acetate consumed was replenished. Thereafter, the bottles were incubated at 55°C for 2 hours, followed by flushing the headspace of each bottle with N<sub>2</sub>-CO<sub>2</sub> (80:20; vol/vol) before starting the actual activity measurements. During the tests, bottles were continuously shaken in an incubator at 50 rpm. The GC was calibrated with a 55°C gas mixture consisting of 3% CH<sub>4</sub> and 97% N<sub>2</sub> at a temperature similar to the incubation temperature in order to avoid unexpected errors (Kim and Daniels, 1991). Within a period of 1-3 hours, samples of 100 µl were taken periodically from the headspace and analyzed for

CH<sub>4</sub>. Sampling was stopped after a CH<sub>4</sub> concentration of 3% in the headspace was achieved. At 55°C, this concentration is equivalent to a maximum acetate conversion of approximately 150 mg COD.l<sup>-1</sup>. The acetate concentration, the pH, as well as the exact amount of VSS were measured after the test was completed. The final pH was in the range 6.8-7.3, depending on the converted amount of acetate. The specific methanogenic activity was calculated using the slope of the linear increase of the CH<sub>4</sub> concentration, divided by the exact amount of VSS, which was in the range 1.5-2 g.l<sup>-1</sup>.

## 2.5 Analyses

The *immunological assays* (described in Chapter 3) were performed in the laboratory of Dr. A. Macario and Dr. E. Conway de Macario, Wadsworth Center for Laboratories and Research, New York State Department of Health, and School of Public Health, State University of New York, Albany, New York, 12201 - 0509, USA.

Samples for GC and HPLC analyses were centrifuged for 5 min. at 10,000 rpm in a Biofuge A (Heraeus Sepatech, Osterode, FRG).

*COD* was analyzed using the micromethod as described by Jirka and Carter (1975). The sample was oxidized with dichromat in H<sub>2</sub>SO<sub>4</sub> (18M) under pressure at 160°C in closed 20 ml glass vessels during 2 h. The sample was analyzed colorimetrically. From the effluent, both total COD as well as soluble COD were analyzed. Samples for soluble COD were obtained after centrifugation for 5 min. at 10,000 rpm in the Biofuge A.

*VFA* was determined by gas chromatography. The chromatograph (HP 5890A, Palo Alto, USA) was equipped with a 2 m x 4 mm glass column, packed with Supelcoport (100-120 mesh) coated with 10% Fluorad FC 431. Operating conditions are: column, 130°C, injection port, 200°C, flame ionization detector, 280°C. N<sub>2</sub> saturated with formic acid at 20°C is used as carrier gas (30 ml.min<sup>-1</sup>).

*Ethanol, Methanol* were analyzed in the same gas chromatograph which was used for VFA determination. Operational conditions were the same except for the oven temperature which was 70°C.

*Lactate, Formate* were measured by HPLC with a 300 x 6.5 mm organic acids column (Interaction, Mountainview, Calif. USA). The eluent was 0.01 N H<sub>2</sub>SO<sub>4</sub> with a flow rate of 0.8 ml.min<sup>-1</sup>. The column temperature is 35°C. 10 µl samples were analyzed with a 737 Kratos UV detector (Ramsey, N.J. USA) at 210 nm.

**Biogas composition** ( $\text{CH}_4$ ,  $\text{CO}_2$ ,  $\text{N}_2$ ) was determined with a Packard Becker gas chromatograph model 433 (Delft, The Netherlands) equipped with two columns connected in parallel (split 1:1); column 1: 1.5 m x 1/8" packed with chromosorb 108, 60-80 mesh (Johns Manville, USA) and column 2: 1.2 m x 1/8" stainless steel, packed with molecular sieve 5A (60-80 mesh) (Chrompack, Bergen op Zoom, The Netherlands). Helium was used as a carrier gas ( $45 \text{ ml}\cdot\text{min}^{-1}$ ). Temperatures were: column,  $40^\circ\text{C}$ , injection port,  $100^\circ\text{C}$ , and hot wire detector,  $100^\circ\text{C}$ . Injection volume was  $100 \mu\text{l}$ .

**Hydrogen** was determined by gas chromatography with a Hewlett Packard 5890 gas chromatograph equipped with a thermal conductivity detector and molecular sieve 25H (60-80 mesh). Column size is 1.5 m x 6.4 mm. Argon was used as carrier gas at a flow rate of  $25 \text{ ml}\cdot\text{min}^{-1}$ . Temperatures are: column,  $40^\circ\text{C}$ , injection port,  $110^\circ\text{C}$ , and detector,  $125^\circ\text{C}$ . Injection volume was 1 ml.

**Methane** in low concentrations (up to 3%) was determined in a Packard-Becker 438/S gas chromatograph (Delft, The Netherlands). Injection volume was  $100 \mu\text{l}$ . A 2 m x 2 mm stainless steel column was used packed with Poropak Q (80-100 mesh). The temperatures of the column, injection port and flame ionization detector were 60, 200 and  $220^\circ\text{C}$ , respectively.  $\text{N}_2$  was used as carrier gas ( $20 \text{ ml}\cdot\text{min}^{-1}$ ).

**$\text{Li}^+$**  was determined by flame atomic absorbance/emission spectrometry (AA/AES), Varian model SpectrAA 300 (Springvale Australia). A mixture of air/acetylene (2:1) was used as burning gas for the flame. Wavelength for emission was 670.8 nm. A burner of 10 cm was used and the slide width was 0.1 nm. The samples were diluted using a Gilson automatic diluter (Model 401, Villiers, France).

**VSS/TSS** were analyzed according the Dutch Standard Methods (NEN 32355.3). The total solids were measured after drying at  $105^\circ\text{C}$  (over night), the ash content after 3.5 h at  $550^\circ\text{C}$ . The VSS content was calculated as the difference between total solids and ash.

## Chapter 3

### Start-up of thermophilic UASB reactors with mesophilic granular sludge

Technological and microbiological aspects of a temperature shift from mesophilic to thermophilic conditions in UASB reactors. Reactors were fed with a mixture of volatile fatty acids and seeded with mesophilic granular sludge.

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*Published as:*

- (3.1) Van Lier, J.B., K.C.F. Grolle, A.J.M. Stams, E. Conway de Macario and G. Lettinga (1992). Start-up of a thermophilic Upflow Anaerobic Sludge Bed (UASB) reactor with mesophilic granular sludge. *Appl. Microbiol. and Biotechnol.*, 37: 130-135.
- (3.2) Visser, F.A., J.B. van Lier, A.J.L. Macario and E. Conway de Macario (1991). Diversity and population dynamics of methanogenic bacteria in a granular consortium. *Appl. Environ. Microbiol.*, 57: 1728-1734 (only partly used).  
Macario, A.J.L., F.A. Visser, J.B. van Lier and E. Conway de Macario (1991). Topography of methanogenic subpopulations in a microbial consortium adapting to thermophilic conditions. *J. Gen. Microbiol.*, 137: 2179-2189 (only partly used).

## 3.1 Increase of the UASB Process Temperature from 38°C to 46°C, 55°C, and 64°C.

### Abstract

Fast start-up of thermophilic UASB reactors was achieved at process temperatures of 46, 55 and 64°C, using mesophilic granular sludge as inoculum and fatty acid mixtures as feed. The start-up was brought about by increasing the temperature of mesophilic UASB reactors in a single step, which initially led to a sharp drop in the methane production rate. Thereafter, stable thermophilic methanogenesis was achieved within a period of 1 or 2 weeks depending on the temperature of operation. Mesophilic granules functioned initially as effective carrier material for thermophilic organisms. However, long-term operation led to disintegration of the granules, resulting in wash-out of thermophilic biomass. All the sludges examined were dominated by *Methanotherix*-like rods. These could be distinguished by antigenic fingerprinting into two subpopulations, one predominant at 36°C, and the other predominant at 46°C and above. The temperature optima for acetate-degrading methanogenic activity of the sludges cultivated at 46, 55 and 64°C, were similar, but differed significantly from the temperature optimum of the mesophilic inoculum.

### 3.1.1 Introduction

Many industries discharge wastewater at high temperatures, e.g. the food and pulp and paper industries. However, so far, practically all existing full-scale UASB reactors are operated at moderate temperatures (20-40°C). Therefore, anaerobic treatment of hot wastewaters is only possible after active or passive cooling, with the risk that a failure in the cooling system can lead to an irreversible deterioration of the digestion process (Speece and Kem, 1970, Van Lier *et al.*, 1990). Inactivation due to temperature increase has been reported for full-scale installations (Lescure *et al.*, 1988). In the past 10 years thermophilic treatment of waste and wastewaters has been studied in several laboratories (see § 1.1, § 1.5). More recently, Souza *et al.* (1992) presented their very promising results of a 75 m<sup>3</sup> pilot UASB reactor for the thermophilic treatment of distillery effluents. Nonetheless, full-scale thermophilic reactors are hardly applied and, so far, no reports mention the operation of high-rate systems for thermophilic wastewater treatment.

Nowadays the start-up period of a mesophilic UASB reactor can be reduced very substantially by using granular sludge as seed material. The present study was carried out to investigate the start-up of thermophilic bioreactors by increasing the process temperature of UASB reactors containing mesophilic granular sludge. Because the decay rate of mesophilic

bacteria in the thermophilic range (50-70°C) is extremely high (Van Lier *et al.*, 1990), the change in temperature will lead to a considerable bacterial die-off, which will possibly result in replacement of the mesophilic population by thermophilic organisms. The mesophilic granule may serve as a kind of carrier material in which or on which thermophilic bacteria can grow, maintaining the granular shape.

The assumed benefit of using mesophilic granular sludge for cultivating thermophilic granules obviously becomes bigger when more of the original mesophilic organisms can adapt to thermophilic conditions. However, in view of the big differences between thermophiles and mesophiles with respect to function, structure and composition (§ 1.2) adaptation is hardly to be expected. A kinetic study of the population shifts inside mesophilic granules when the process temperature was increased from 38°C to 55°C is described in Chapter 3.2. In this Chapter (3.1) results are presented of the operation of UASB reactors, started up at 38°C and shifted in a single step to 46, 55 and 64°C.

### 3.1.2 Materials and Methods

#### Experimental conditions

Experiments were performed using 5.75 l poly-acrylate UASB reactors (§ 2.1). During the whole study the hydraulic retention time was kept constant at about 8 h by means of a peristaltic pump (Watson Marlow 302, Falmouth, Cornwall, UK), pumping hot tap-water at a constant rate. Higher loading rates were imposed on the system by increasing the flow rate of a separate peristaltic pump (Watson Marlow 202) for the concentrated feedstock solution. Before the influent was fed to the reactor, both flows were combined and brought to reactor temperature.

#### Biomass

The reactors were inoculated with Aviko-MGS (§ 2.2) with a total amount of about 75 g volatile suspended solids (VSS) per reactor.

#### Medium

The reactors were fed with a diluted concentrated stock solution of 135 g COD.l<sup>-1</sup> at the appropriate organic loading rates. The substrate consisted of a partly neutralized (pH 6.5) VFA-mixture: acetate, propionate, butyrate = 3:1:1, based on COD. The concentrations of basal nutrients in the concentrated stock solution were (g.l<sup>-1</sup>): NH<sub>4</sub>Cl, 7.5; MgSO<sub>4</sub>.7H<sub>2</sub>O, 3.0; NaH<sub>2</sub>PO<sub>4</sub>.2H<sub>2</sub>O, 27.6; K<sub>2</sub>HPO<sub>4</sub>, 21.2, CaCl<sub>2</sub>.2H<sub>2</sub>O, 0.3; yeast extract, 0.5. To each litre of stock solution 26.7 ml of a trace element solution was added (§ 2.3). The dilution factor of the concentrated feed stock solution was dependent on the applied organic loading rate and ranged from 20 to 90.

### Start-up

Feeding of the reactors was started directly after inoculation with the mesophilic granular sludge. After 13, 28 and 62 days the temperature of reactors R2, R4 and R6 was shifted from 38°C to 64, 55 and 46°C, respectively. This moment was defined as zero time for the thermophilic start-up experiments. The organic loading rates of the reactors were decreased at time zero to prevent overloading. Thereafter increments were imposed only when the acetate level dropped below 200 mg COD. $l^{-1}$ . A similar start-up approach was followed by Wiegant and de Man (1986).

### Activity tests

The temperature dependence of the acetate-degrading activity of the cultivated sludges and the seed material was determined in duplicate using substrate-depletion activity tests in serum bottles (§ 2.4). Bottles (120 ml) were filled with 100 ml medium and brought to the desired temperature. The basal medium (§ 2.3) was supplemented with 2.56 g NaAc. $l^{-1}$ . The Aviko seed sludge which had been stored at 4°C, was first activated for some days at 36°C with acetate as feed (two feeds of 3 g acetate-COD. $l^{-1}$ ) before measuring the activity. Sludge samples from the thermophilic reactors were used directly.

### Identification of methanogens

The methanogens in the sludge samples were analyzed for antigenic relatedness (positive, negative or partially positive) with reference methanogens using immunological methods which are described elsewhere (Macario and Conway de Macario, 1985; Macario *et al.*, 1991; Visser *et al.*, 1991). Morphotype controls of the identified methanogens were performed as described previously (Visser *et al.*, 1991). A comprehensive panel of calibrated antibody probes for reference methanogens (Balch *et al.*, 1979; König and Stetter, 1989; Macario and Conway de Macario, 1983) was used for quantification of the methanogens in the sludge samples. The antibody probes were derived from antisera against the reference methanogens and were used for antigenic fingerprinting. The reference methanogens are listed below in the order prescribed by the antigenic fingerprinting method (Macario and Conway de Macario, 1983):

- 1, *Methanobrevibacter smithii* PS; 2, *Methanobacterium formicicum* MF; 3, *Methanosarcina barkeri* MS; 4, *Methanobacterium bryantii* MoH; 5, *M. bryantii* MoHG; 6, *M. barkeri* R1M3; 7, *Methanospirillum hungatei* (*Methanospirillum hungatii*) JF1; 8, *Methanobrevibacter ruminantium* M1; 9, *Methanobrevibacter arboriphilus* (*Methanobrevibacter arboriphilicus*) DH1; 10, *M. smithii* ALI; 11, *Methanobacterium thermoautotrophicum* GC1; 12, *M. thermoautotrophicum* ΔH; 13, *Methanococcus vanielii* SB; 14, *Methanococcus voltae* PS; 15, *Methanogenium marisnigri* JR1m; 16, *M. barkeri* 227; 17, *Methanogenium cariaci* JR1; 18, *Methanosarcina mazei* S6; 19, *M. barkeri* W; 20, *Methanosarcina thermophila* TM1; 21, *M. arboriphilus* AZ; 22, *M. arboriphilus* DC; 23, *Methanomicrobium mobile* BP; 24, *Methanothermus fervidus* V24S; 25, *Methanolobus*

*tindari* Tindari; 26, *Methanococcus maripaludis* JJ; 27, *Methanosphaera stadtmanae* MCB3; 28, *Methanoplanus limicola* M3; 29, *Methanococcus thermolithotrophicus* SN1; 30, *Methanotherix soehngenii* Opfikon; 31, *Methanotherix* sp. strain CALS-1; 32, *Methanococcoides methylutens* TMA-10; and 33, *Methanocorpusulum parvum* XII.

Granules of the Aviko-MGS as well as sludge cultivated at 64, 55 and 46°C for 98, 56 and 42 days, respectively, were prepared for the immunoassays. From reactor R4 (55°C) also the granules that were cultivated for a period of 119 days were analyzed. Periodically all sludge samples were examined with a phase contrast microscope (Olympus BHT, Tokyo, Japan).

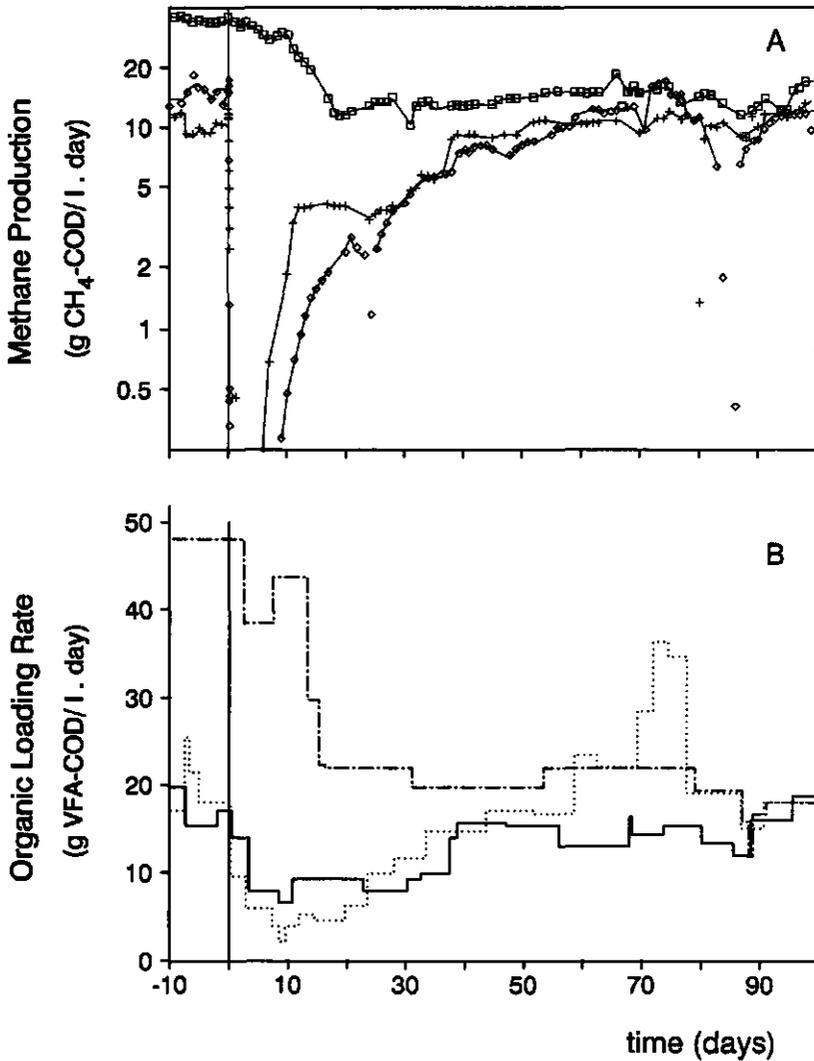
### 3.1.3 Results

#### Increase of process temperature to 46, 55 and 64°C

The performance data of reactors R2, R4 and R6, in which the temperature was shifted in a single step from 38°C to 64, 55 and 46°C respectively, are shown in the Figs. 3.1a,b and 3.2a-c. The sudden increase to temperatures above 45°C, led to an immediate more-or-less exponential decrease of the methane production rate in all reactors (Fig. 3.1a). From this decline apparent decay rates of the mesophilic methanogens of 0.004, 0.22 and 4.72 h<sup>-1</sup> at 46, 55 and 64°C, respectively, were estimated. These values correspond well with decay rates of mesophilic methanogens found during short-term temperature shocks in a mesophilic sludge bed (Van Lier *et al.*, 1990).

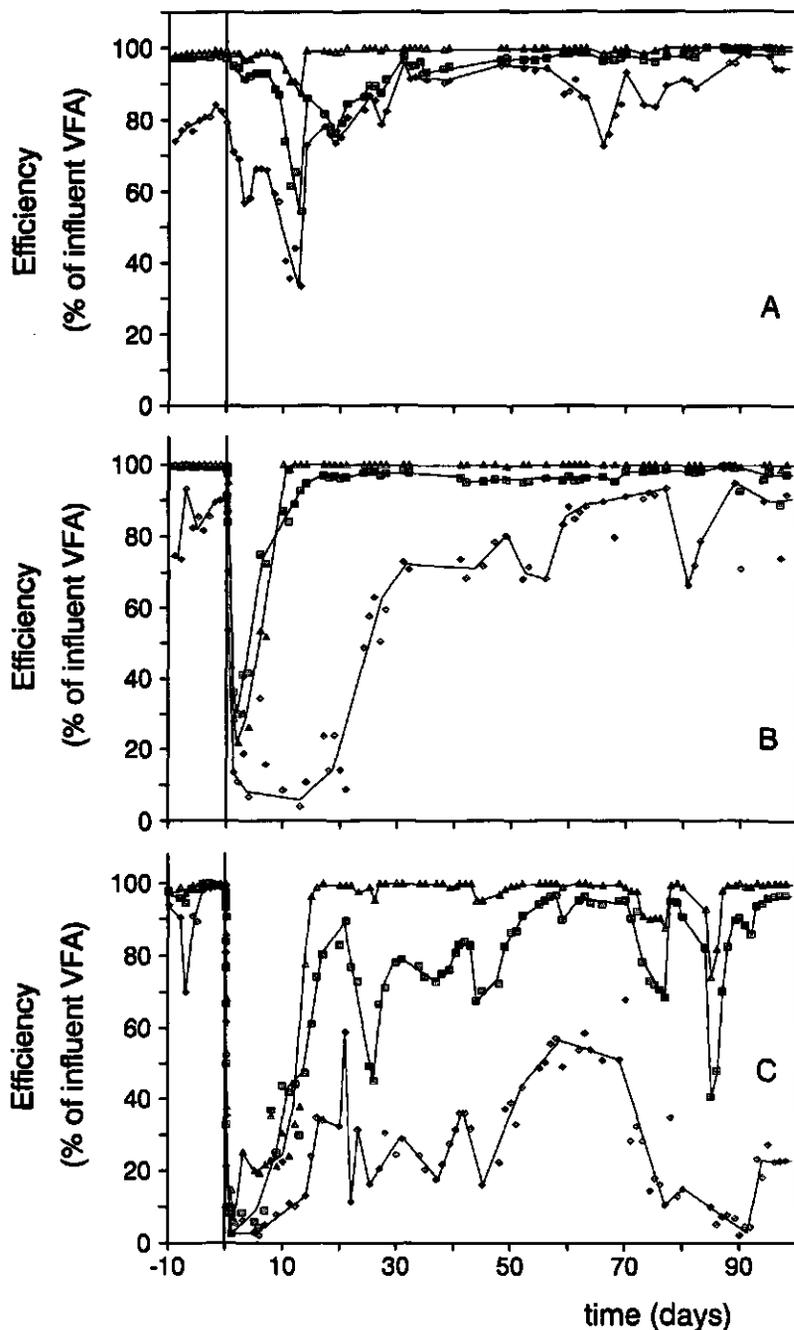
Roughly exponential increases in methane production rates occurred after 5 days (55°C) and 10 days (64°C) following the temperature increase. From the curves apparent growth rates of 0.02 h<sup>-1</sup> at 55°C and 0.01 h<sup>-1</sup> at 64°C were calculated. It was not possible to determine a growth rate at 46°C, owing to the slow decay of the mesophilic methanogens. The increase in the methane production rate after the temperature was increased proceeded very rapidly after the initial drop. After 2 and 4 weeks of operation almost 5 g CH<sub>4</sub>-COD.l<sup>-1</sup> reactor per day ( $\approx 2$  l CH<sub>4</sub>.l<sup>-1</sup> reactor.day<sup>-1</sup>) was produced in R4 (55°C) and R2 (64°C), respectively. In R6 (46°C), the methane production rate never dropped below 10 g CH<sub>4</sub>-COD.l<sup>-1</sup> reactor per day ( $\approx 4$  l CH<sub>4</sub>.l<sup>-1</sup> reactor.day<sup>-1</sup>).

The courses of the removal efficiencies for acetate, propionate and butyrate at 46, 55 and 64°C are represented in Fig. 3.2a-c. After the shift of the process temperature to the (sub)thermophilic range at t=0, similar recovery sequences occurred in all reactors. First, butyrate degradation recovered most rapidly at all temperatures. Next, acetate degradation recovered and almost complete acetate removal could be achieved, except at 64°C in reactor R2. For the calculation of the acetate removal efficiency, the stoichiometric amount of acetate produced during the oxidation of propionate and butyrate was taken into account. Propionate degradation dropped most rapidly of all the VFA after raising the temperature.



**Fig. 3.1** A) Course of the methane production rate in grams CH<sub>4</sub>-chemical oxygen demand (COD).l<sup>-1</sup> reactor per day, in reactor R6 (□, 46°C), R4 (+, 55°C) and R2 (◇, 64°C). Note: log-scale for methane formation.

B) Changes in organic loading rates of reactors R6 (---, 46°C), R4 (—, 55°C) and R2 (·····, 64°C), before and after the temperature increase. Organic loading rates were increased only if the acetate and propionate concentration was below 0.2 g COD.l<sup>-1</sup>. This was not possible in reactor R2 in which the propionate concentration was never below 0.4 g COD.l<sup>-1</sup>. In every reactor, the concentration of butyrate was < 10 mg COD.l<sup>-1</sup>. At t=0 the process temperature of the mesophilic reactors was set to 46, 55 or 64°C.



**Fig. 3.2** Removal efficiency of acetate ( $\square$ ), propionate ( $\diamond$ ) and butyrate ( $\Delta$ ) in reactor A) R6, B) R4, and C) R2. Efficiency is expressed as the percentage of the influent VFAs. At  $t=0$  the process temperature of the mesophilic reactors R6, R4 and R2 was set to 46, 55 and 64°C, respectively.

Its recovery proceeded slowly and apparently depended on the new process temperature. At 46°C propionate degradation recovered relatively quickly, but at 64°C recovery proceeded slowly and incompletely. A high propionate removal rate could only be obtained after the system was operated at a fixed loading rate over relatively long periods of time. This obviously sometimes led to stagnation of the increase in the methane production rate during the start-up period, e.g. in reactor R4 (55°C) at day 10 (Fig. 3.1a). After 1 month stable propionate degradation was achieved at loading rates of 15-20 g VFA-COD.l<sup>-1</sup> reactor.day<sup>-1</sup> except for reactor R2 operated at 64°C. The increase of the organic loading rate by 50-100% led to severe deterioration in the digestion process at 64°C (Fig. 3.2c, day 73). As a result propionate degradation dropped to almost zero.

### Effect of temperature on granule structure

During the first months of operation the granules in all cases maintained their original shape and apparently served as effective biological carriers for thermophilic organisms. During this period the start-up of each of the reactors was very promising with respect to the increase in the methane production rate. However, after 2-3 months of operation, the granules disintegrated in all reactors. The 'mesophilic-thermophilic' granules became spongy and finally fell apart. Only very small aggregates with a high ash content (about 70%) were retained in the reactors, but active dispersed sludge washed-out from the reactors, resulting in a decrease in the total sludge bed volume. Therefore, it was not possible to increase the organic loading rate any further without overloading the system. Apparently either the sludge retention capacity of the UASB reactors used was inadequate, or the settleability of the active sludge aggregates became too poor.

**Table 3.1** Acetate-degrading activity at various temperatures of Aviko-MGS and thermophilic sludge cultivated at 46, 55 and 64°C.

temp. (°C)	Aviko-MGS	thermophilic sludge cultivated at:		
		46°C	55°C	64°C
36	0.63 ± 0.01 <sup>a</sup>	0.40 ± 0.01	0.37 ± 0.02	0.11 ± 0.02
46	0.14 ± 0.02	1.09 ± 0.06	0.66 ± 0.04	0.25 ± 0.00
55	- 0.03 <sup>b</sup> ± 0.01	0.90 ± 0.05	0.91 ± 0.07	0.52 ± 0.06
64	- 0.05 ± 0.01	1.65 ± 0.25	1.96 ± 0.22	0.77 ± 0.16

<sup>a</sup> acetate-degrading activity in g acetate-COD.g<sup>-1</sup> VSS.day<sup>-1</sup> ± standard deviation

<sup>b</sup> negative values due to increase of acetate concentrations during the test

### Temperature characteristics of the cultivated sludge

After 4 months of operation the maximum acetate-degrading activity of each of the sludges, as well as that of the Aviko inoculum, was determined at 36, 46, 55 and 64°C. Although the sludges were grown at different temperatures (46, 55 and 64°C), only small differences in temperature optima of the acetate-degrading activity were found (Table 3.1). The mesophilic inoculum did not show net acetate conversion above 46°C. The other sludge types showed optimal acetate degradation at 64°C, irrespective of the temperature of cultivation. The sludge cultivated at 64°C showed a linear increase in acetate-degrading activity up to 64°C, and the activity was lower at all temperatures than the activity found with the other two thermophilic sludges. The temperature characteristics of the cultivated sludges are discussed in more detail in Chapter 4.1.

### Identification of methanogens

Microscopical examination demonstrated a dominance of *Methanotrix*-like bacteria in all sludge samples. In the sludges grown at high temperatures (46-64°C) these bacteria were of variable length, but the long filaments, usually observed in mesophilic methanogenic sludge from UASB reactors, were less abundant. The predominant methanogenic subpopulation in the mesophilic inoculum was morphologically very similar and strongly antigenically related to *M. soehngenii* strain Opfikon (further referred to as MTSO-1) (Table 3.2). This subpopulation was also present in the sludge cultivated at 46°C and, to a considerable lesser extent, in the sludge cultivated at 64°C. In all (sub)thermophilic sludge samples the predominant *Methanotrix*-like methanogen that could be detected immunologically was only weakly antigenically related to *M. soehngenii* strain Opfikon (further referred to as MTSO-2). Its highest concentration was detected in the sludge grown at 46°C.

The appearance of some methanogenic subpopulations was dependent on the cultivation temperature, as indicated by the data in Table 3.2. Some methanogens (e.g., *M. cariaci* JR1, ref.nr. 17) were only present in the mesophilic inoculum whereas others (such as *M. thermoautotrophicum* ΔH (ref.nr. 12) and, *M. arboriphilus* AZ (ref.nr. 21) grew only in the thermophilic temperature range. The highest numbers of the methanogens related to *M. thermoautotrophicum* ΔH and *M. arboriphilus* AZ were found in the sludges cultivated at 55°C or higher. Other subpopulations preferred the lower part of the thermophilic range. For example, the methanogen strongly related to *M. thermophila* TM1 (ref.nr. 20), the optimal growth temperature of which is 50°C (Zinder *et al.*, 1985), was more abundant at 46 and 55°C than at 64°C.

**Table 3.2** Concentration of methanogenic subpopulations that showed temperature dependence and that were measured immunologically in the sludge samples.

Methanogenic subpopulations antigenically related to:	Sludge sample				
	Aviko 36°C	R6 46°C (42 days) <sup>a</sup>	R4 55°C (56 days)	R4 55°C (119 days)	R2 64°C (98 days)
<i>Methanosarcina thermophila</i> TM1	0.30 ± 0.05 <sup>b</sup>	0.83 ± 0.04	5.20 ± 0.70	1.70 ± 0.04	0.18 ± 0.06
<i>Methanotherix soehngeni</i> Opfikon	280.3 ± 15.5	233.7 ± 18.0	- <sup>c</sup>	-	2.80 ± 0.09
<i>Methanotherix soehngeni</i> Opfikon <sup>d</sup>	68.8 ± 7.4	682.7 ± 11.0	99.0 ± 17.2	93.2 ± 15.3	118.8 ± 2.4
<i>Methanogenium cariaci</i> JRI	0.58 ± 0.10	-	-	-	-
<i>Methanobacterium thermoautotrophicum</i> ΔH	-	-	42.3 ± 2.8	236.8 ± 19.0	199.9 ± 4.7
<i>Methanobrevibacter arboriphilus</i> AZ	0.60 ± 0.09	1.90 ± 0.11	5.50 ± 0.32	13.2 ± 0.5	69.4 ± 5.9

<sup>a</sup> days of cultivation in the UASB reactor at the indicated temperature

<sup>b</sup> cells (or packets in the case of methanogens related to *Methanosarcina* sp.)·gram<sup>-1</sup> dry weight x 10<sup>7</sup> ± standard deviation.

<sup>c</sup> below the cut off level (see Visser et al, 1991).

<sup>d</sup> only weakly related to the reference organism

### 3.1.4 Discussion

A relatively fast thermophilic start-up of UASB reactors is possible over a temperature range of 46-64°C, by using mesophilic granular sludge as inoculum. The start-up period is much shorter than previously found in experiments conducted in our laboratory. In the latter experiments fresh cow manure and digested sewage sludge were used as inoculum (Wiegant and de Man, 1986). In the present study the rate of digestion initially deteriorated when the process temperature was raised above 45°C. However, stable thermophilic methanogenesis could be achieved within a period of 1 or 2 weeks depending on the temperature of operation. At 64°C, the process remained significantly less stable than at lower temperatures, particularly with respect to propionate oxidation. A temperature around 45°C is generally considered to be the worst process temperature for anaerobic digestion: too high for mesophilic processes (Henze and Harremoës, 1983), and probably too low for efficient thermophilic treatment. However, our results clearly show the quickest 'start-up' at 46°C without serious deterioration of the digestion process. Probably, this satisfactory start-up behaviour can be attributed to a more gradual shift of the mesophilic methanogenic population to a (sub)thermophilic population. A sudden increase in temperature to 55 or 64°C leads to a drastic population shift in the methanogenic consortium which is discussed in Chapter 3.2 for reactor R4 in more detail.

In our studies the temperature effect on the acetate-degrading methanogens is considered to be most important, because 88% of total methanogenesis was from acetate. Thermophilic acetate-utilizing methanogens have different temperature optima (Zinder, 1990). However, our results indicate a similar composition of the methanogenic sludge cultivated for 2-4 months at either 46, 55 and/or 64°C (Table 3.1, 3.2). In addition, the temperature dependence of acetate conversion of sludge samples taken after 6 months of cultivation was very similar (Chapter 4.1), irrespective of the cultivation temperature. This implies that process temperature is only of minor importance for selection of the thermophilic methanogenic bacteria in a UASB system. In treatment systems with a high sludge retention, i.e. high immobilization capacity, the composition of the methanogenic consortium probably also depends on other selection criteria, such as: i) the type of substrate (Grotenhuis *et al.*, 1991); ii) growth kinetic characteristics (Gujer and Zehnder, 1983); iii) syntrophic interactions (Stams *et al.*, 1990); and, iv) attachment properties (Wiegant, 1987). This hypothesis is supported by the fact that population shifts were also found during long-term operation of sludge bed systems at constant temperatures (Hulshoff Pol, 1989; Zinder, 1990). The authors attributed the population shifts to better kinetic properties and differences in adherence of the different methanogens.

Granular anaerobic sludge cultivated in mesophilic UASB reactors with low effluent concentrations is generally dominated by *Methanothrix spec.* (de Zeeuw, 1984; Dubourguier *et al.*, 1988; Grotenhuis *et al.*, 1991). Species from the genera *Methanothrix* grow in

filamentous rods and exert a high adherence capacity (Grotenhuis *et al.*, 1992). In thermophilic granules (55°C) filamentous *Methanothrix*-like bacteria also were observed (Uemura and Harada, 1993; Wiegant and de Man, 1986). Thermophilic bacteria of the genus *Methanothrix* are able to grow in a temperature range of 46-64°C, and exhibit a relatively high growth rate at very low acetate concentrations (Zinder *et al.*, 1987; Nozhevnikova and Yagodina, 1983; Zinder, 1990). The same accounts for the Thermophilic Acetate-utilizing Methanogen (TAM) organism isolated by Ahring and Westermann (1984), but the adherence capacity of this organism is probably significantly lower owing to its short rod-shaped configuration.

Until now no *Methanothrix*-like methanogens are known in the literature with an optimal growth temperature within the subthermophilic range (45-50°C). This indicates that after a considerable adaptation period a sludge bed may be cultivated with more or less the same properties, irrespective of differences in process temperature, i.e. 46, 55, and/or 64°C. Microscopic examinations showed that *Methanothrix*-like rods were predominant in all sludges. These rods could be identified immunologically, although some of them may have escaped identification if they did not react with the used probes. Those rods that did react belonged to two subpopulations: MTSO-1 and MTSO-2. The latter was in the minority in the Aviko seed sludge grown at 36°C, but this picture was reversed at all the other temperatures tested (Table 3.2). While MTSO-1 was scarce at 55 and 64°C, MTSO-2 was more abundant above 36°C. The highest concentration of MTSO-2 was detected in the sludge grown at 46°C. This might explain why the methane production rate in reactor R6 (46°C) was the highest of all thermophilic reactors during the test period (Fig. 3.1a). Even though the maximum acetate-degrading methanogenic activity at 46°C was lower compared to the activity at 55°C and particularly 64°C (Table 3.1). The total sludge volume in all reactors was more or less the same.

The thermophilic processes in the present study were limited by significant wash-out of active biomass. This probably was due to disintegration of the 'mesophilic-thermophilic' granules, which were characterized by a spongy structure (Chapter 3.2). In the present experiments as well as in a study previously performed in our laboratory (Wiegant and Lettinga, 1985) we were not able to cultivate thermophilic granules using mesophilic granular sludge as inoculum and VFA mixtures as substrate. Why no granulation occurred is not yet understood. However, both Wiegant and Lettinga (1985) as well as Hulshoff Pol (1989) have shown that the granulation process is enhanced considerably if partially acidified or non-acidified feed is used. On the other hand, thermophilic granulation on acetate alone, and on an acetate-butyrate mixture was found, when fresh cow manure was used as inoculum (Wiegant and de Man, 1986). In these sludges long filamentous *Methanothrix*-like bacteria were dominant. Instead of long filamentous organisms, rod-shaped bacteria dominated in the aggregates obtained in this study, as evidenced by microscopical examinations and immunological assays. Therefore, in addition to the feedstock composition the type of

inoculum might also have an effect on the granulation process.

**Acknowledgements**

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## 3.2 Population Dynamics of Methanogenic Bacteria in Mesophilic Granular Sludge After Shifting the Temperature to Thermophilic Conditions

### Abstract

Anaerobic sludge granules from UASB reactors were used as an experimental model microbial consortium to study the dynamics and distribution of methanogens. Immunologic methods revealed a considerable diversity of methanogens, greater in mesophilic granules than in the period four months after a temperature shift from 38 to 55°C. During this period, the size of the methanogenic subpopulations changed, with distinctive profiles after the initial reduction caused by the shift. Methanogens antigenically related to *Methanobrevibacter smithii* PS and ALI, *Methanobacterium hungatei* JF1 and *Methanosarcina thermophila* TM1 increased rapidly, reached a short plateau and then fell to lower concentrations that persisted for the duration of the experiment. A methanogen related to *Methanogenium cariaci* JR1 followed a similar profile at the beginning, but it soon diminished to below detection levels. *Methanothrix* rods weakly related to the strain Opfikon increased rapidly, reaching a high-level, long-lasting plateau. Two methanogens related to *Methanobrevibacter arboriphilus* AZ and *Methanobacterium thermoautotrophicum* ΔH emerged from very low levels before the temperature shift and multiplied to attain their highest numbers four months after the shift. Histo- and immunohistochemistry revealed thick layers and globular clusters or lawns of variable density distinctive of the methanogens related to *M. thermoautotrophicum* ΔH, *M. thermophila* TM1, and *M. arboriphilus* AZ and *M. soehngenii* Opfikon, respectively, in thin sections of granules grown at 55°C for four months. Mesophilic granules showed a different pattern of methanogenic subpopulations.

### 3.2.1 Introduction

Microbial consortia, which may appear as films or granules, play a key role in the bioconversion of wastes (Bochem *et al.*, 1982; Chartrain and Zeikus, 1986; Hulshoff Pol, 1989). Their cellular and molecular composition, mechanism of formation and functions are still not completely understood.

Granular consortia in anaerobic methanogenic ecosystems involve different groups of microbes among which methanogenic bacteria are predominant (Chartrain and Zeikus, 1986; Dolfing, 1987; Grotenhuis, 1992; Novaes, 1986; Zellner and Winter, 1987). Until a few years ago, the composition of the methanogenic contingent in the granules and its response to external changes were poorly understood. There were practically no methods available for

direct elucidation of the native methanogenic flora. Culture isolation procedures may select only some species, thus influencing the interpretation of which methanogens are actually present. Antibody probes and immunotechnology were developed to fill this methodologic void (Bryniok and Trösch, 1989; Kemp *et al.*, 1988; Macario and Conway de Macario, 1983; Prensier *et al.*, 1988; Robinson and Erdos, 1985). Various microbial communities were analyzed to elucidate the methanogens present, using the newly developed immunological means along with classic microbiological, biochemical and other auxiliary procedures (Macario and Conway de Macario, 1985). Among the ecosystems examined were anaerobic bioreactors of various types operated in different laboratories, which demonstrated a considerable diversity of methanogens (Kobayashi *et al.*, 1988; Macario and Conway de Macario, 1988). It was hypothesized that the microbial composition of a granular consortium is dependent on the total sum of selective forces, such as temperature or operating conditions, acting on the microbes of the original inoculum over a period of time (Macario *et al.*, 1989).

In this chapter we discuss the occurrence of specific methanogenic subpopulations and their quantitative variations in the granules of reactor R4 (see Chapter 3.1) after a shift from mesophilic (38°C) to thermophilic (55°C) conditions. The application of mesophilic granular sludge may be beneficial for the enhancement of a thermophilic start-up, either through the adaptive behaviour of the bacteria themselves or by the entrapment of the newly formed thermophilic organisms in or on the granule as a whole. The latter is particularly of interest if the mesophilic bacteria which do not survive the temperature shock are gradually replaced by the thermophiles, while the granular shape of the sludge is maintained after the temperature increase. This could lead to a fast thermophilic start-up, even at low hydraulic retention times. On the other hand, the high amount of dead mesophilic biomass might alter a thermophilic granulation process owing to structural limitations for microbial growth. The role of the mesophilic granules for the entrapment of the thermophiles is discussed.

### **3.2.2 Materials and Methods**

#### **Identification, quantification and localization of methanogens**

Immunological and immunohistochemical techniques were used to elucidate the effect of a temperature increase on the methanogens inside the granules and on the architectural plan of the granule as a whole. This work was mainly focused on the granules in reactor R4, in which the process temperature was increased to 55°C (Chapter 3.1). Sludge samples were taken at different time intervals following the start-up. Ten days before the temperature was increased, sample -10 was collected. Subsequent samples were collected 7, 21, 56, 76 and 119 days after increasing the temperature and the inoculum was also sampled. A dual approach was followed for a specific characterization of the methanogenic consortium:

- a) Identification and counting of methanogens in cell suspensions which were obtained from the disrupted granules. This method is necessary to identify and quantify the different

methanogenic species occurring in the granules. Because the sludge samples of reactor R4 were harvested at different time intervals, we were able to closely study the population dynamics of the methanogens in the granules.

- b) Mapping of methanogenic subpopulation, determined by the first method in cross sections of the whole granule. This method was applied to elucidate the textural changes inside the granules and to study the spatial arrangement of the different colonies. The latter method gives a better understanding of the architectural plan of the granule and the effect of temperature increase on the granular structure.

Preparation of the samples for immunologic testing was done according to techniques described elsewhere (Macario and Conway de Macario, 1985; Visser *et al.*, 1991). Methods for histochemistry and immunohistochemistry are described in detail by Macario *et al.* (1991). The panel of calibrated antibody probes is given in § 3.1.2. The results reported, with respect to the immunohistochemical work, were obtained using indirect immunofluorescence with probe numbers 12, 19, 20, and 30 (see reference list § 3.1.2). The other methanogens were absent or were present in only very low numbers and, therefore, probably not of crucial importance in the granule development at the high temperatures tested.

All sludge samples were periodically examined with a phase contrast microscope (Olympus BHT, Tokyo, Japan).

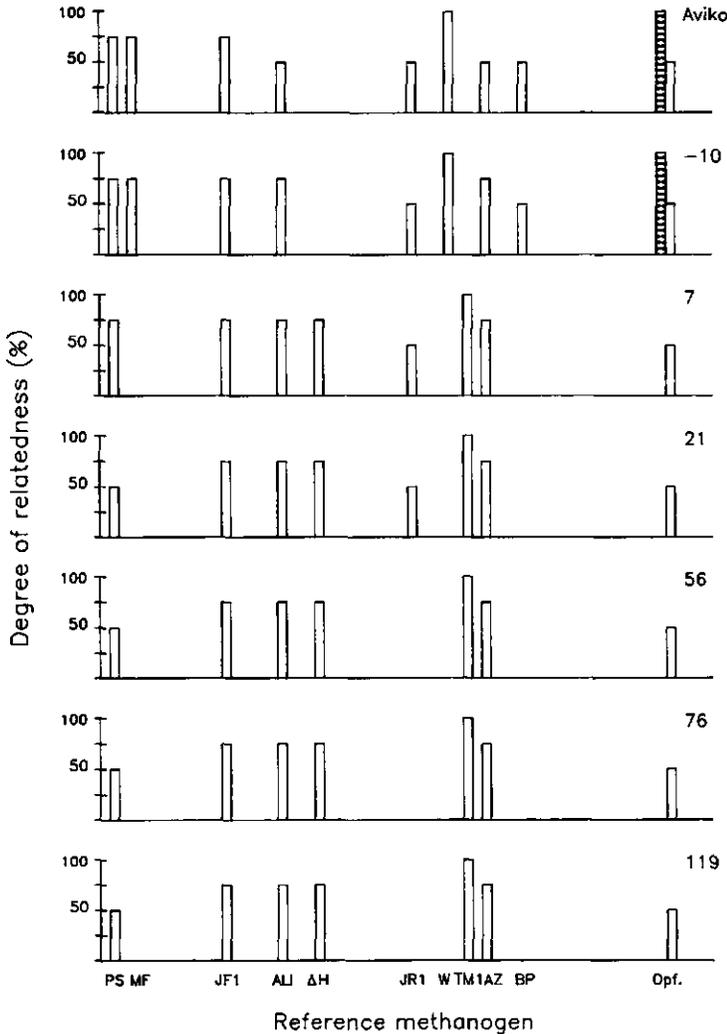
### 3.2.3 Results

#### Diversity of methanogenic bacteria before and after the temperature increase to 55°C

An increase in the process temperature of reactor R4 from 38 to 55°C resulted in a shift in the methanogenic subpopulations (Fig. 3.3). The immunologically detectable methanogenic subpopulations were morphologically the same but antigenically different from the reference organisms. However, the degree of antigenic relatedness of the methanogens closely related to *M. barkeri* W, *M. thermophila* TM1, and *M. soehngeni* Opfikon (shown by a hatched bar in Fig. 3.3) with the reference strains was 100%.

Reactor R4 was run for 31 days at 38°C before the temperature was shifted to 55°C. The pattern of methanogenic subpopulations in the granular inoculum and in sludge granules sampled at  $t=-10$  were the same despite the different substrate. Seven days after the temperature increase to 55°C, the methanogenic subpopulations related to *M. formicicum* MF and *M. mobile* BP and those antigenically indistinguishable from *M. barkeri* W and *M. soehngeni* Opfikon, respectively, were undetectable. Methanogens antigenically related to the thermophilic species *M. thermoautotrophicum* ΔH and *M. thermophila* TM1, which had been previously present as very minor subpopulations, increased in number. No other changes in the pattern of methanogenic subpopulations were observed during the following

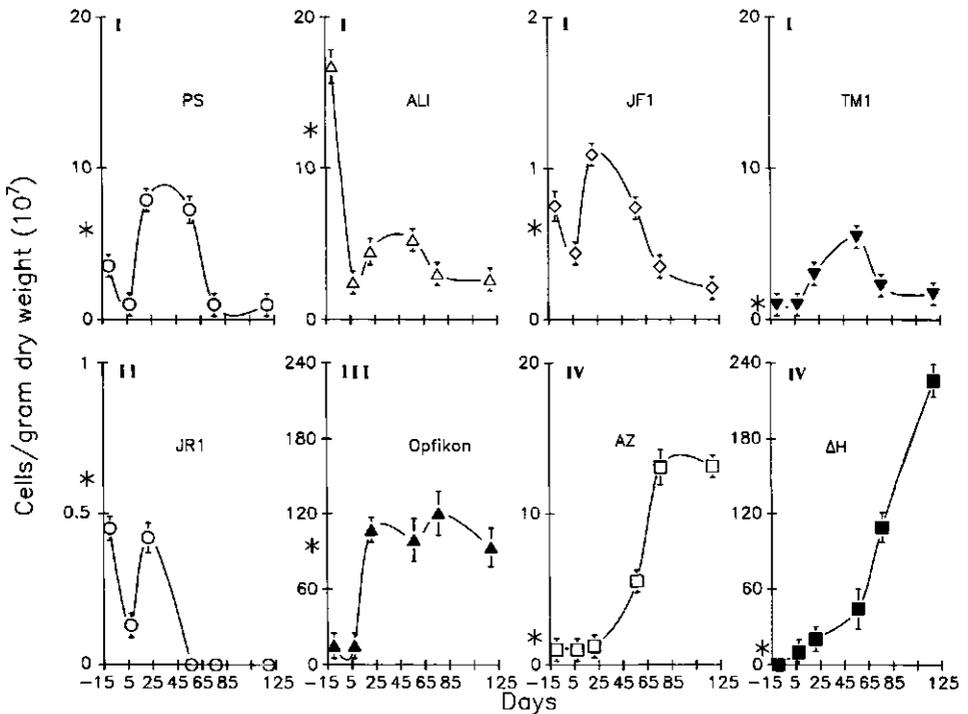
3 to 4 months the reactor was operated at 55°C, except for the subpopulation antigenically related to *M. cariaci* JR1, which was no longer detectable two months after the temperature increase.



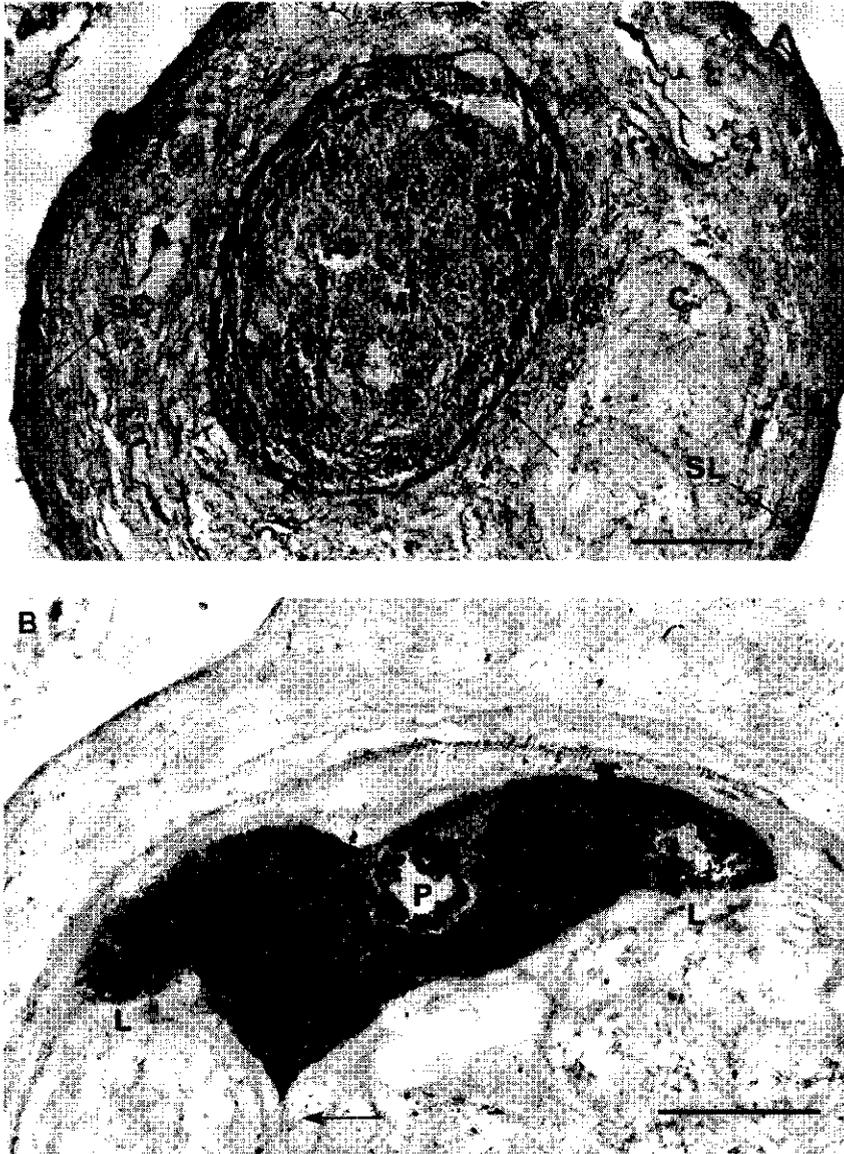
**Fig. 3.3** Pattern of methanogenic subpopulations in the Aviko granules of R4 at  $t = -10, 7, 21, 56, 76$  and  $119$  days as a result of the temperature shift from  $38$  to  $55^\circ\text{C}$ . The height of the bar measures the degree of relatedness to the reference methanogen indicated in the horizontal axis as follows: PS, No. 1; MF, No. 2; JF1, No. 7; ALI, No. 10;  $\Delta\text{H}$ , No. 12; JR1, No. 17; W, No. 19; TM1, No. 20; AZ, No. 21; BP, No. 23; and No. 30. Numbers refer to panel of reference methanogens (see Materials and Methods).

**Dynamics of methanogenic subpopulations**

The methanogenic subpopulations which were immunologically quantified displayed distinctive profiles following the temperature increase from 38 to 55°C (Fig. 3.4): I) an increase in cell concentration immediately after the fall caused by the temperature increase, followed by a short plateau and then a gradual decrease with lower levels persisting throughout the observation period; II) same as I) but instead of a plateau a subsequent rapid decrease to levels below detectability was observed; III) an increase in cell concentration immediately after the temperature change, reaching a plateau that persisted throughout the rest of the observation period; IV) a progressive increase in cell concentration after the temperature change, reflecting a more or less exponential growth. The highest levels were reached by the end of the observation period.



**Fig. 3.4** Time-course quantitative profiles of methanogenic subpopulations measured immunologically in granules from bioreactor R4 ( $\pm$  S.E.). The subpopulations were antigenically related to the following reference organisms: PS, No. 1; ALI, No. 10; JF1, No. 7; and TM1, No. 20 (profile I); JR1, No. 17 (profile II); Opfikon, No. 30 (profile III, pertaining to the subpopulation 50% related to Opfikon); and AZ, No. 21 and  $\Delta$ H, No. 12 (profile IV). Numbers refer to panel of reference methanogens (see Materials and Methods). The asterisk on the vertical axis indicates the concentration of each subpopulation in the inoculum.



**Fig. 3.5** A) Cross-section of a four-month thermophilic granule showing cortex -C- and medulla -M-, the thin denser layer separating them (broad arrow), the surface layer -SL- and two surface colonies -SC-. B) Large colony (dark) in between layers at the corticomedullary interface. Within this colony, *Methanosarcina* spec. form packets -P-, or a net of cellular cords -L-. Note the subdivision of the medulla into two lobes by a septum (arrow). Haematoxylin-eosin. Bars: 20  $\mu\text{m}$  (A), 5  $\mu\text{m}$  (B).

### Overall structure of the granules 4 months after the temperature increase

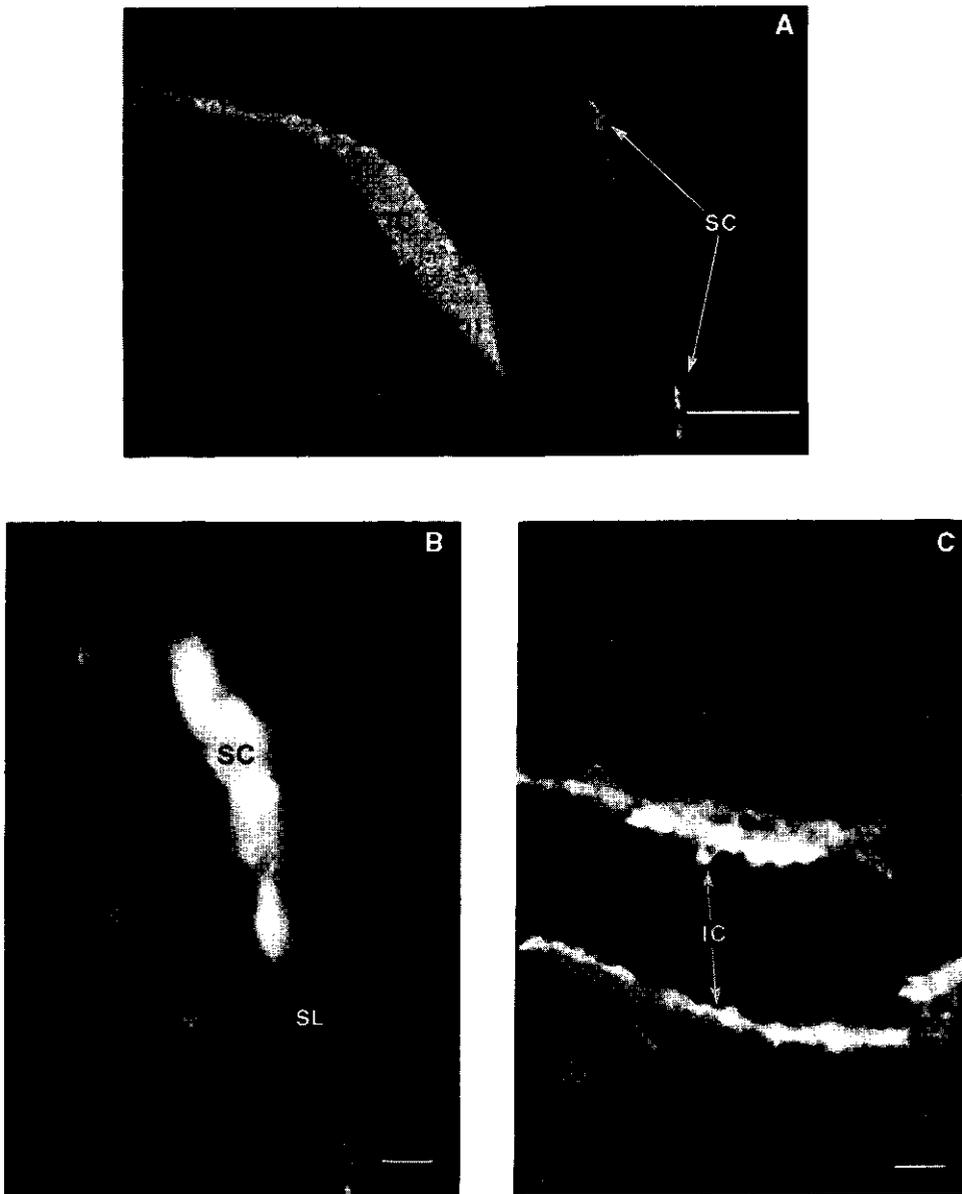
Fig. 3.5 shows the major features of the structure of the granule: a core or medulla surrounded by a wide peripheral zone or cortex. Thin, dense layers encircle the granule and the medulla. These features were observed in granules maintained under thermophilic as well as mesophilic conditions. Fig. 3.5a also shows two morphologic details, i.e. a spongy texture and microbial colonies sitting on the surface, typical of the granules grown at 55°C for four months. Note the lax texture of both medulla and cortex, with numerous empty irregular bubbles or elongated crevices. During the initial stages of the reactor start-up the mesophilic granules functioned as an effective carrier material for thermophilic organisms, which resulted in a fast recovery of methanogenesis. However, most of the organisms grew on the surface and in the interstices of the granules (Figs. 3.5 and 3.6). The inner part of the granules was dominated by a spongy texture. In all probability, this structure was the major cause for the 'mesophilic-thermophilic' granules finally falling apart (§ 3.1.3). Deterioration of these granules resulted in a wash-out of active thermophilic biomass and a decrease in the sludge bed volume in the UASB reactors.

### Topography of the newly formed methanogenic subpopulations in the sludge granules at 55°C

Immunohistochemical techniques were used to elucidate the spatial arrangements of the methanogenic colonies which were only present in the granules after the temperature increase. The methanogen antigenically related to *M. thermoautotrophicum*  $\Delta$ H formed two types of colonies in all the granules: one sitting on the surface of the granule and appearing as a flat layer and/or mounds; and a second appearing as elongated half-moons, between the granule's concentric layers (Fig. 3.6a). Both the outer and inner colonies did adhere to the adjacent granule's structural layer. If the outer layer was torn away, the colony went with it (Fig. 3.6b). If the granule was overstretched, inner colonies split in two with the halves sticking to each adjacent layer (Fig. 3.6c). This suggests a strong adherence of the bacterial colony to the surrounding layers.

The methanogen closely antigenically related to *M. thermophila* TM1 appeared in all granules as packets of globular aggregates, usually in the outer portion of the cortex, or as a net of cellular cords in the medulla and, more frequently, in the cortex. Packets and cords were found closely associated with colonies of the methanogen related to *M. thermoautotrophicum*  $\Delta$ H (see Fig. 3.5).

The methanogen only weakly antigenically related to the reference organism *M. soehngenii* Opfikon was found in bundles mostly occupying the interstices of the cortex of all the granules.



**Fig. 3.6** A) Cross-section of a four-month thermophilic granule showing an inner colony IC- and surface colonies -SC- characteristic of the methanogen antigenically related to *M. thermoautotrophicum*. B) The surface layer -SL- of the granule has been torn away from the cortex carrying with it a surface colony -SC-. C) Split inner colony -IC-; Indirect immunofluorescence with an antibody probe for the strain  $\Delta H$ ; Bars: 20  $\mu\text{m}$  (A); 2.5  $\mu\text{m}$  (B and C).

The mesophilic granules, and the granules maintained under thermophilic conditions for only one week, had a more compact texture than the thermophilic granules. The latter granules (see Figs. 3.5a and 3.5b) showed many elongated cavities. The newly formed methanogenic colonies described above were absent in the mesophilic granules. Instead, in these granules, colonies of cells closely related to *M. barkeri* W and *M. soehngenii* Opfikon were observed. Both strains are known to be typically mesophilic.

### 3.2.4 Discussion

The mesophilic granules functioned as an effective carrier material during the first months of the start-up. After this period, the granules disintegrated and the UASB process was limited by severe wash-out of active biomass (see also Chapter 3.1). This phenomenon could be attributed to the granular structure which was strongly influenced by the increase in temperature, and to structural limitations for microbial growth inside the granules. Immunohistochemical analyses of the granules after a period of 4 months at 55°C demonstrated a spongy matrix, in and on which newly formed thermophilic methanogens were located. The original granular compactness diminished considerably resulting in a collection of thermophilic methanogenic colonies without clear connections, except for the methanogens antigenically related to *M. thermophila* TM1 and *M. thermoautotrophicum* ΔH, which were sometimes found to be closely associated (Fig. 3.5). Our results tend to indicate that thermophilic start-up of UASB bioreactors using mesophilic granules as inoculum, under continuing thermophilic conditions, will not result in an enhancement of the granulation process leading to a compact microbial arrangement. However, it should be noted that this study was performed using a mixture of volatile fatty acids. Previous studies showed that the granulation process is enhanced if partially acidified or non-acidified feed is used (Wiegant and Lettinga, 1985; Hulshoff Pol, 1989; Vanderhaegen *et al.*, 1992). Furthermore, a thermophilic start-up at 55°C with the same inoculum, but now using a mixture of VFA and sucrose as substrate, led to a satisfactory granulation process (Chapter 6). A non-acidified fraction of the influent COD seems to be indispensable for the growth of thermophilic granular sludge of a good quality (see also Chapter 6). The crucial components could be the acidifying bacteria themselves or their products, such as polysaccharides, which are often found as a kind of sticky material in methanogenic granules (Grotenhuis *et al.*, 1991; Vanderhaegen *et al.*, 1992). Therefore, we think that the absence of compact methanogenic granules in the thermophilic reactors can not only be ascribed to the structural limitations for microbial growth but probably also to the lack of acidifying bacteria and their metabolites.

The adaptive behaviour of mesophilic granular sludge, with respect to temperature increase, is due to a shift in the methanogenic subpopulations inside the granules. Bacteria antigenically related to the typical mesophilic methanogens *M. cariaci*, *M. formicicum* MF, *M. mobile* P,

*M. barkeri* W and one of the *M. soehngeni* Opfikon immunotypes (MTSO-1) disappeared after the increase. In contrast, organisms antigenically related to methanogens, which are known to have an optimal growth temperature in the thermophilic range, e.g. *M. thermophila* and *M. thermoautotrophicum*  $\Delta$ H, increased in number. This was also the case for the other immunotype of *M. soehngeni* Opfikon (MTSO-2). Our results also reveal that the diversity of hydrogen-utilizing methanogens is more pronounced in sludge grown under mesophilic conditions than under thermophilic conditions, which is quantitatively dominated by the *M. thermoautotrophicum* immunotype.

Immunologic and immunohistochemical analysis of the sludges cultivated at 46 and 64°C showed comparative results with respect to the diversity (Chapter 3.1) and topography of the different methanogens (data not shown). The only difference was found in the subpopulation antigenically related to *M. thermoautotrophicum*, which was much less abundant at 46°C (Table 3.2). The high similarity of the results indicates the development of a thermophilic sludge bed with similar properties irrespective of the operation temperature. This is confirmed by the similar temperature characteristics of the sludges cultivated at 46, 55 and 64°C (Table 3.1, Chapter 4.1).

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## Chapter 4

### Temperature Susceptibility of Thermophilic Methanogenic Sludge

Temperature optima of thermophilic sludge cultivated at 46, 55 and 64°C were assessed. In Chapter 4.2 the role of sludge granulation on the temperature susceptibility is discussed.

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- (4.2) Van Lier, J.B., J.L. Sanz Martin and G. Lettinga (1995). Effect of temperature on the anaerobic thermophilic conversion of volatile fatty acids by dispersed and granular sludge. *Water Research*, Vol. 29, no 12.

## 4.1 Temperature Optima of Thermophilic Methanogenic Sludge: Implications for Reactor Start-up and Operation

### Abstract

The effect of temperature on the conversion rates of volatile fatty acids (VFA) by thermophilic methanogenic sludge grown under different conditions, was studied. Optimum temperatures for acetate degradation of sludges cultivated in serum bottles at 46, 55 and 64°C for 6 to 8 weeks were strongly dependent on the cultivation temperature. However, sludges obtained after a start-up period of 6 months in UASB reactors, fed with VFA mixtures at 46, 55 and 64°C, showed comparable temperature optima, irrespective of the temperature of cultivation. A high temperature susceptibility for methane production and propionate degradation and, to a lesser extent for butyrate degradation, was also found during the start-up of thermophilic UASB reactors. The reactors were started-up at 38°C with VFA mixtures. Thereafter, the process temperature was increased to 55°C in steps of 5°C. Each increment led to a sharp drop in the methane production rate. However, no severe deterioration of methanogenesis was observed if the increase in the process temperature was performed very slowly between 50 and 55°C. The results indicate that, with respect to the application of thermophilic high-rate systems, the sensitivity to temperature fluctuations will decrease in time. A high sensitivity is expected if the maximum microbial growth-rate is the predominant selection criterion for the thermophilic methanogens.

### 4.1.1 Introduction

Recent studies on thermophilic high-rate reactors demonstrated the kinetical advantages of anaerobic treatment at high temperatures. Compared with anaerobic sludge cultivated under mesophilic conditions, it was found that thermophilic sludge has a higher methanogenic activity. As a result, higher organic loading rates could be applied (Harris and Dague, 1992; Rintala and Lepistö, 1992; Schraa and Jewell, 1984; Souza *et al.*, 1992; Wiegant, 1986; Wiegant and De Man, 1986). On the other hand, several reports mention a higher instability of the anaerobic digestion process under thermophilic than under mesophilic conditions (Pohland and Bloodgood, 1963; Seif *et al.*, 1992; Soto *et al.*, 1992). Garber (1975) and Zinder *et al.* (1984b) found that an increase in the process temperature of only a few degrees, resulted in a complete irreversible deterioration of the thermophilic digestion process. A high sensitivity to a temperature increase would be disadvantageous for full scale applications where fluctuations in the process temperature cannot be prevented. In general, a temperature drop in thermophilic reactors will also lead to a decreased methanogenic

activity. This, however, is completely reversible (Schraa, 1983; Temper, 1983; Wiegant, 1986). In addition, Angelidaki and Ahring (1994) reported that a drop in temperature from 55°C to 40°C did not affect the process performance of thermophilic manure digesters. This phenomenon was attributed to a lower degree of ammonium inhibition at lower temperatures due to the lower concentration of unionized ammonia.

Thermophilic treatment of waste and waste water is applied in conventional mixed systems as well as in high-rate systems with high solids retention times. The applied process temperature ranges from 45 up to 75°C (Ahring, 1994; Zinder, 1986; Van Lier *et al.*, 1991). Because different reactor types are used and different process temperatures are applied, comparison of the results of the various studies is difficult. Differences in stability sometimes can be attributed to operational conditions rather than to characteristics of the thermophilic process.

In this chapter the temperature susceptibility of thermophilic methanogenic sludge is discussed which is cultivated under different conditions. The sludge types were characterized by assessing the temperature-response curves. The results show a high sensitivity to temperature changes when the sludge is adapted for a relatively short period to high temperatures. This is also found during the start-up of thermophilic UASB reactors when the methanogenic sludge is shifted from mesophilic to thermophilic conditions. However, if the increase of the process temperature is performed very slowly the stability of methanogenesis is much higher.

## 4.1.2 Materials and Methods

### Biomass

The thermophilic methanogenic sludge was cultivated either in serum bottles (1 litre) or in UASB reactors (5.75 litres) at 46°C, 55°C and 64°C. Serum bottles and UASB reactors were inoculated with Aviko-MGS (§ 2.2).

### Batch cultivation in serum bottles

The serum bottles were filled with 800 ml medium and 200 ml of Aviko-MGS. The standardized medium for batch experiments (§ 2.3) was completed with 3.37 g.l<sup>-1</sup> sodium acetate (NaAc) and 2.0 g.l<sup>-1</sup> NaHCO<sub>3</sub>. After closing the bottles and replacing the gas phase by N<sub>2</sub>-CO<sub>2</sub> (70:30; vol/vol), Na<sub>2</sub>S (1 ml.l<sup>-1</sup> from 1 M stock solution) was added to obtain complete anaerobic conditions. Hereafter, the bottles were incubated at 46, 55 and 64°C. After the first feed was completely degraded, 5 additional feedings were supplied so that any remaining acetate plus added acetate gave a total of 3.0 g acetate-COD.l<sup>-1</sup> at each feeding. A partially neutralized acetate stock solution (200 g acetate-COD.l<sup>-1</sup>, pH 4.8) was used to prevent a too high pH increase and sodium accumulation. The total adaptation period to the high temperatures was about 1.5-2 months. The sludge obtained was used to estimate the

maximum acetate-degrading activity at different temperatures.

#### **Start-up of thermophilic UASB reactors**

The start-up of the 3 UASB reactors, R2, R4 and R6, operated at 64, 55 and 46°C, respectively, is described in detail in Chapter 3.1. After 6 months of continuous operation sludge samples were taken from each reactor for assessment of the specific activity at various temperatures. To study the effect of temperature during transition from mesophilic to thermophilic conditions more closely, 2 other UASB reactors, R3 and R5, were started-up. In contrast to the reactors R2, R4 and R6, the temperature in R3 and R5 was shifted gradually from mesophilic to thermophilic conditions. Both these reactors were started at 38°C using Aviko-MGS as inoculum in the same amount of approximately 75 g volatile suspended solids (VSS) per reactor. The same VFA-mixture, acetate:propionate:butyrate = 3:1:1 (based on COD), was used as substrate. The experimental conditions and the medium of the reactors R3 and R5 were similar to that of the reactors R2, R4 and R6 (Chapter 3.1) After a short start-up period of one week the process temperatures of both reactors were increased to 45°C. To avoid substrate depletion, the organic loading rates were increased prior to the temperature increase. After one week at 45°C, the temperatures of both reactors were increased further to 50°C. Then, another temperature increase of 5°C was made in reactor R5, while reactor R3 was maintained at 50-51°C for 2 months. Thereafter, the temperature in the latter reactor was gradually increased to 55°C within a period of 3 weeks.

#### **Specific activity test for substrate conversion at various temperatures**

The temperature dependence of acetate conversion of the sludges cultivated in the 1 litre serum bottles was determined in triplicate using substrate depletion activity tests (§ 2.4). After completion of 6 acetate feedings at 46, 55 or 64°C, the sludge was distributed in serum bottles of 120 ml (5 ml wet sludge in each bottle). The specific activity was calculated from the linear decrease of the acetate concentration which was followed over a period of 11 days.

Acetate-, propionate- and butyrate-converting activities were separately assessed from the sludges cultivated in the thermophilic UASB reactors and from the inoculum, by using the same substrate-depletion activity tests in duplicate experiments (§ 2.4). At the beginning of each activity test the concentration of acetate, propionate or butyrate was raised to 3.0 g COD.l<sup>-1</sup>. The tests were performed over a period of 4 days.

#### **Analytical procedures**

Methods for VFA and VSS determinations are described in § 2.5.

### 4.1.3 Results

#### Thermophilic sludge grown in batch reactors at high substrate concentrations

The temperature dependence of the acetate-utilizing methanogenic activities of the sludges cultivated at 46, 55 and 64°C in the serum bottles are presented in Fig. 4.1. Due to the short adaptation times the acetate-utilizing activity is rather low. The amount of thermophilic methanogenic biomass after 6 feedings of 3 g acetate-COD. $\cdot l^{-1}$  would be 0.40-1.08 g biomass. $\cdot l^{-1}$ , if one assumes a growth yield of 0.022-0.060 g biomass. $\cdot g^{-1}$  acetate-COD utilized (Ahring and Westermann, 1985; Touzel *et al.*, 1985, Clarens and Moletta, 1990). The results show that the optimum temperature for acetate degradation is determined by the cultivation temperature. Three different temperature optima for the acetate-utilizing activity can be distinguished in the thermophilic range for the three types of sludge. The 46°C-sludge still exerts activity at temperatures as high as 55 and 60°C. Apparently, acetate-utilizing bacteria with a temperature optimum above the observed 'overall' optimum of 50°C are also present in the sludge. Also the sludge cultivated at 55°C demonstrated reasonable methanogenic activity at 10°C above its optimum. The sludge cultivated at 64°C exhibits more or less the same acetate-utilizing activity between 58 and 68°C, which might be caused by the presence of methanogens with different temperature optima. Above 70°C the specific activity declines sharply.

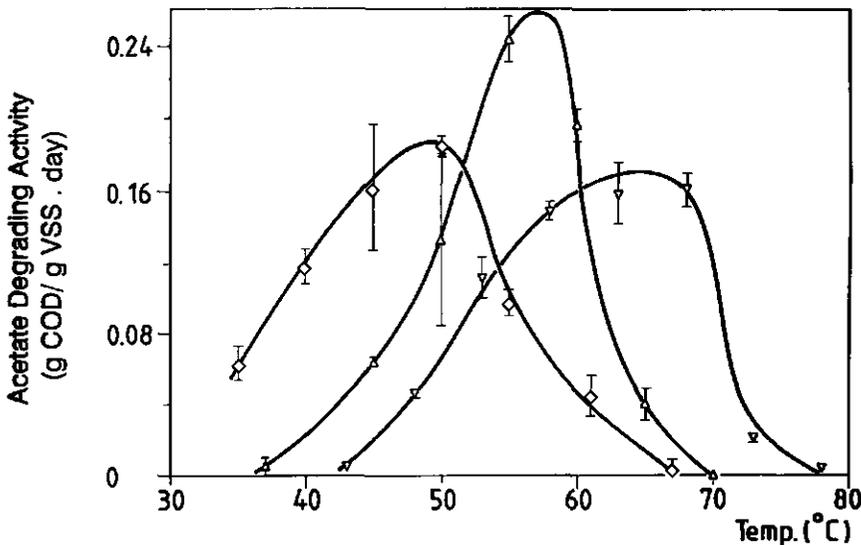


Fig. 4.1 Acetate-degrading activity (g Acetate-COD. $\cdot g^{-1}$  VSS. $\cdot day^{-1}$ ) at various temperatures of thermophilic sludge cultivated in serum bottles at ( $\diamond$ ) 46°C, ( $\Delta$ ) 55°C and ( $\nabla$ ) 64°C during a period of  $\pm$  6 weeks.

### Thermophilic sludge grown in UASB reactors at low substrate concentrations

The average concentrations of acetate, propionate and butyrate in the effluent of the UASB reactors, which were operated at 46, 55 and 64°C, are presented in Table 4.1.

**Table 4.1** Acetate, propionate and butyrate concentrations in the effluent of the UASB reactors operated for 6 months at 46, 55 and 64°C. Average values are calculated over the last three months of operation. In this period a more or less constant organic loading rate of 16-18 g VFA-COD.L<sup>-1</sup>.day<sup>-1</sup> was imposed to each UASB reactor. The influent concentrations were (g COD.L<sup>-1</sup>): acetate, 3.5; propionate, 1.2; butyrate, 1.2.

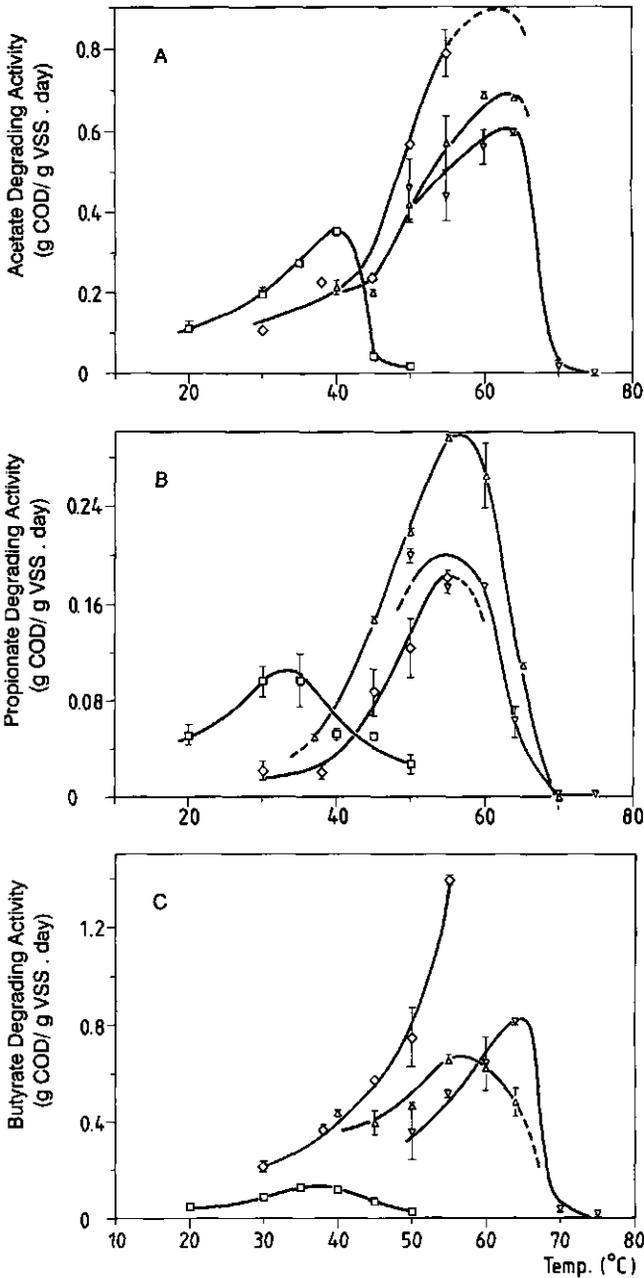
	T = 46°C (n=42)	T = 55°C (n=40)	T = 64°C (n=50)
Acetate	148 ± 105 <sup>a</sup>	118 ± 63	538 ± 296
Propionate	116 ± 83	177 ± 98	773 ± 233
Butyrate	3 ± 7	0 ± 1	10 ± 15

<sup>a</sup> Average values ± standard deviation; VFA concentrations in mg COD.L<sup>-1</sup>.

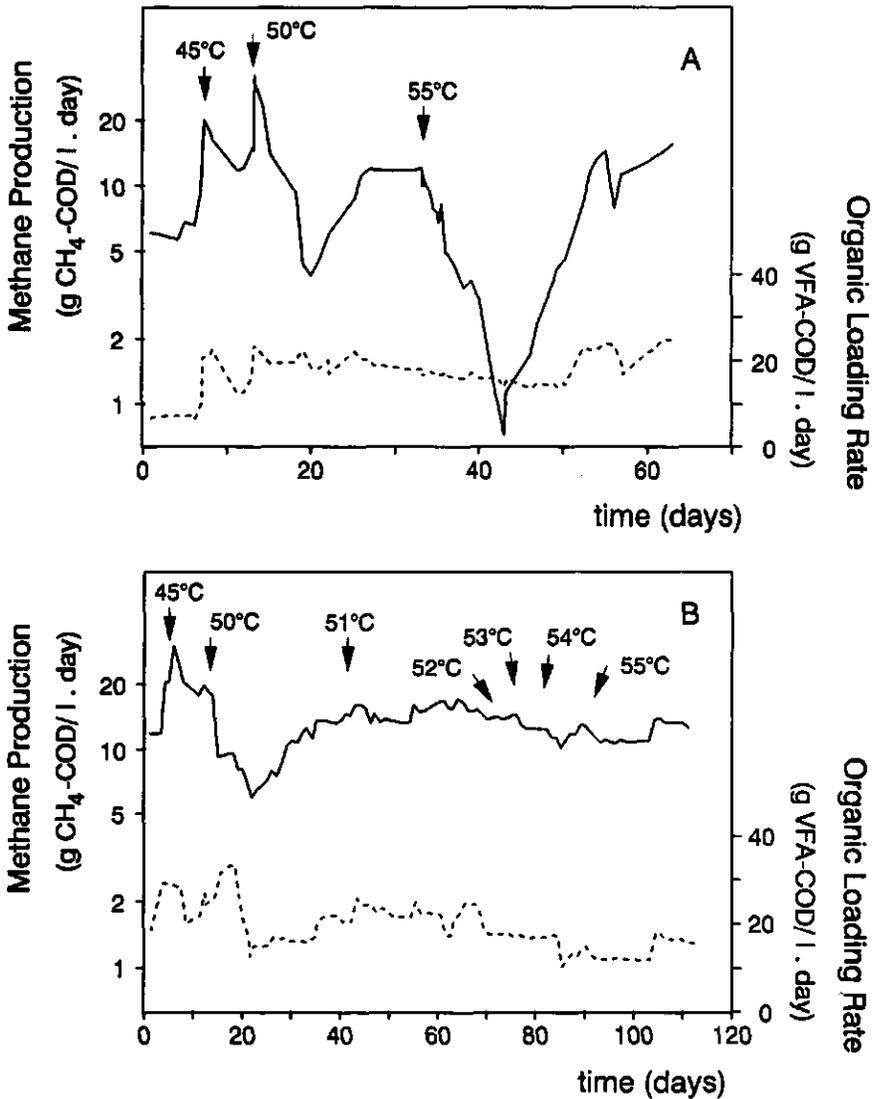
The influence of temperature on the maximum acetate-degrading capacity of the sludges cultivated at 46, 55 and 64°C in UASB reactors and of the mesophilic inoculum is depicted in Fig. 4.2a. Although the acetate-utilizing activity of the sludges cultivated at 46 and 55°C was not measured at temperatures as high as 70°C, the results still indicate clearly that all thermophilic sludges exerted their optimum temperature at 60-65°C. This temperature optimum differed significantly from that of the mesophilic inoculum. One single optimum, irrespective of the temperature of cultivation, was also found for the maximum propionate-converting activities. Thermophilic propionate oxidation exhibited an optimum at 55-60°C for the sludges cultivated at 46, 55 and 64°C (Fig. 4.2b). With respect to the butyrate degradation, such a clear single optimum for the different types of thermophilic sludge was not found (Fig. 4.2c).

### Start-up of thermophilic UASB reactors applying step-wise temperature increments

The reactors R5 and R3 were started at 38°C under almost identical conditions. The only difference was the imposed lower organic loading rate in reactor R5, what resulted in an initial higher removal efficiency (Figures 4.3, 4.4). After the start-up at 38°C, the temperature of reactor R5 was increased to 55°C in steps of about 5°C. Each temperature increase, except that from 38°C to 45°C, resulted in a severe drop in the methane production rate (Fig. 4.3a). To prevent a depletion of substrate in the sludge bed due to a possible higher methanogenic activity at higher temperatures, we increased the organic loading rate 1 day before the process temperature was set to 45°C. Therefore, the observed increase and subsequent decrease of the methane production rate occurring at that time was not only due to the temperature increase, but was also caused by the changes in organic loading rate.



**Fig. 4.2** A) Acetate-degrading activity ( $\text{g Acetate-COD} \cdot \text{g}^{-1} \text{VSS} \cdot \text{day}^{-1}$ ), B) propionate-degrading activity ( $\text{g Propionate-COD} \cdot \text{g}^{-1} \text{VSS} \cdot \text{day}^{-1}$ ) and C) butyrate-degrading activity ( $\text{g Butyrate-COD} \cdot \text{g}^{-1} \text{VSS} \cdot \text{day}^{-1}$ ) at various temperatures of thermophilic sludge cultivated in UASB reactors at ( $\diamond$ )  $46^\circ\text{C}$ , ( $\Delta$ )  $55^\circ\text{C}$  and ( $\nabla$ )  $64^\circ\text{C}$  during a period of 6 months. As a reference also the activities of the mesophilic inoculum ( $\square$ ) is depicted in the figures.



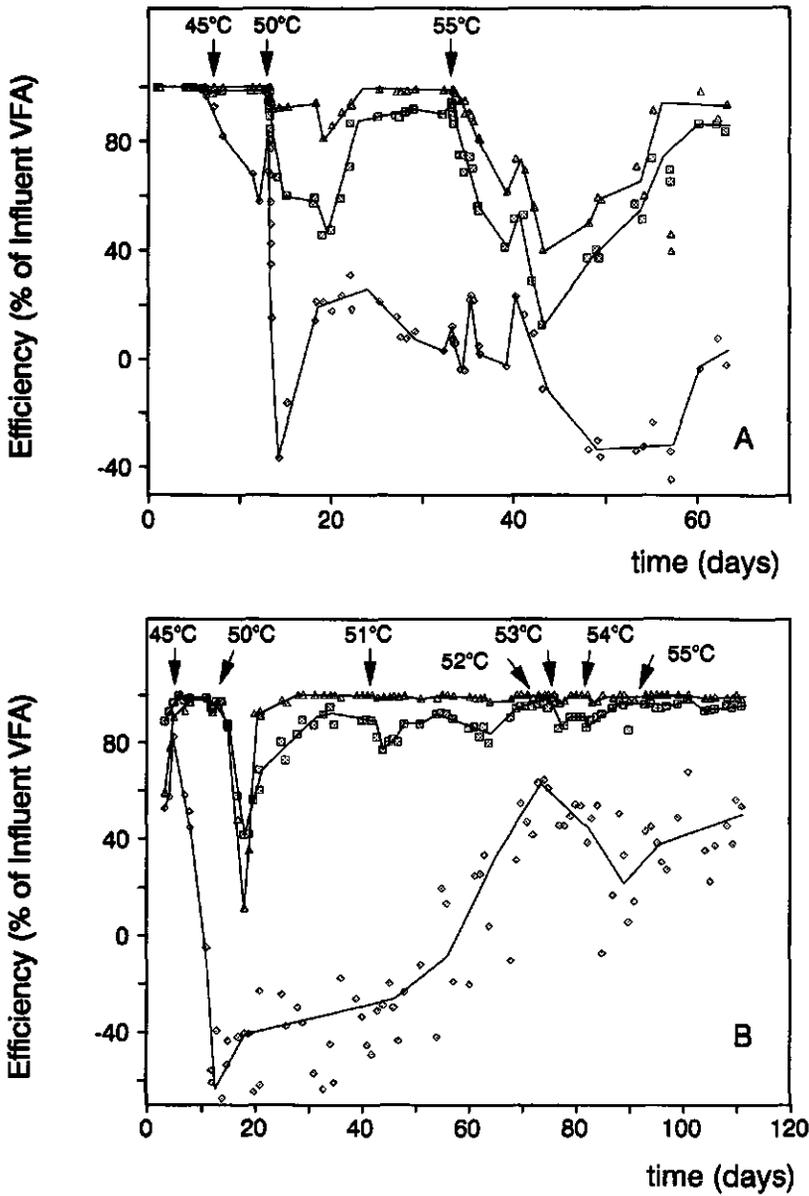
**Fig. 4.3** Course of the methane production rate ( $\text{g CH}_4\text{-COD}\cdot\text{l}^{-1}\text{ reactor}\cdot\text{day}^{-1}$ , —) in A) reactor R1 in which the process temperature was increased in steps from  $38^\circ\text{C}$  to  $45^\circ\text{C}$ ,  $50^\circ\text{C}$  and  $55^\circ\text{C}$  and B) reactor R2 in which the process temperature was increased in steps from  $38^\circ\text{C}$  to  $45^\circ\text{C}$ , and  $50^\circ\text{C}$ , followed by a gradual increase during two months to  $55^\circ\text{C}$ . Note: log-scale for methane formation. Times at which the temperature was increased are indicated by arrows. In the lower part the organic loading rate ( $\text{g VFA-COD}\cdot\text{l}^{-1}\text{ reactor}\cdot\text{day}^{-1}$ , - - -) is presented versus time.

Following the next temperature increase from 45°C to 50°C in reactor R5, the methane production rate increased temporarily to very high levels, i.e. about 26 g CH<sub>4</sub>-COD.l<sup>-1</sup> reactor.day<sup>-1</sup> at an organic loading rate of only 23 g VFA-COD.l<sup>-1</sup> reactor.day<sup>-1</sup> (Fig. 4.3a). This observed excess methane production, presumably, should be attributed to the digestion of mesophilic biomass still present at that time, because the system was exposed only for a relatively short period of time to 45°C. After the short peak production, the methane production rate dropped to a low level, and accordingly also the treatment efficiency dropped to rather low values (Fig. 4.4a). The subsequent slow recovery of the methane production rate indicates a shift in the methanogenic population.

An even much more severe decrease of the methane production rate occurred after the temperature was increased from 50°C to 55°C, indicating another shift in the methanogenic population. From the roughly exponential decrease and increase of the methane production rate at 50°C, we could estimate an apparent decay- and growth rate of 0.27 day<sup>-1</sup> and 0.16 day<sup>-1</sup>, respectively (Fig. 4.3a). At 55°C values of 0.23 day<sup>-1</sup> and 0.25 day<sup>-1</sup> were estimated for the apparent decay- and growth rate, respectively. The observed high temperature susceptibility of the sludge grown in UASB reactor R5 agrees with the results found for the serum bottles-cultivated sludges (Fig. 4.1).

In order to assess the need for applying a sufficiently long adaptation time we started UASB reactor R3 in which the process temperature was very slowly increased between 50 and 55°C. After the start-up at 38°C and operating the system for a short period at 45°C, the reactor R3 was run at 50-51°C for 2 months. The course of the methane production rate (Fig. 4.3b) doesn't show any clear peak after the reactor was set to 50°C, like we observed in reactor R5. Although this might have occurred, it was not measured, probably due to the only small amounts of data points which were taken during the temperature increase to 50°C. At day 40 the temperature was increased to 51°C. Further increments were performed from day 72 by a step of 1°C every 1 to 2 weeks (Fig. 4.3b). Methane production rates did not vary significantly over the temperature range of 51°C to 53°C. A small decrease in methane production rate was observed above 53°C. In contrast to reactor R5 (Fig. 4.3a), a sharp decrease in methane production was not observed after the temperature was set to 55°C at day 93. Very likely, a significant thermophilic population with an optimal temperature of 55°C or higher had developed during the 2 months at which reactor R3 was maintained at 50-51°C.

The effects of an increase in temperature on the removal efficiencies of the separate VFAs for reactor R5 and R3 are shown in Fig. 4.4a and 4.4b, respectively. From these results it is clear that the degradation of propionate is the most sensitive step during transition from mesophilic to thermophilic conditions. The temperature sensitivity of VFA degradation, and particularly that of propionate, was more pronounced at a high organic loading rate, i.e. substrate concentration. This is shown by the results obtained in reactor R5 and R3 during



**Fig. 4.4** Removal efficiency of acetate (□), propionate (◇) and butyrate (Δ) in A) reactor R5 and B) reactor R3. Efficiency is expressed as the percentage of the influent VFAs ( $100\% \times (\text{influent VFA} - \text{effluent VFA}) / (\text{influent VFA})$ ). For the calculation of acetate removal efficiency, the stoichiometric amount of acetate produced during the oxidation of propionate and butyrate was taken into account. Times at which the temperature was increased are indicated by arrows.

the temperature increase from 38 to 45°C and 45 to 50°C (Fig. 4.4a, 4.4b). The influence of the substrate concentration on the temperature sensitivity of substrate conversion is discussed in more detail in Chapter 4.2.

Propionate degradation deteriorated completely in both reactors. The removal efficiency dropped even to below zero because of a net propionate formation. Presumably, propionate was formed from acetate (Schink, 1984; Laanbroek *et al.*, 1982) and/or butyrate (Tholozan *et al.*, 1988). Alternative pathways for the degradation of propionate in a methanogenic sludge bed, especially under non-steady state conditions, has been observed by other researchers (Grotenhuis *et al.*, 1986). Consequently, the synthesis of propionate from unusual products such as acetate and butyrate can also be expected under stress-conditions such as a sudden increase in temperature.

#### 4.1.4 Discussion

The results obtained with the sludges adapted for a relatively short period to high temperatures in serum bottles, clearly demonstrate the presence of various temperature optima for acetate-utilizing methanogenesis in the thermophilic range. These optima depend on the temperature at which the sludge was cultivated. The prevalence of such temperature optima is of much minor importance after long adaptation times in continuous flow systems. In fact, we only found one optimum for acetate-utilizing methanogenesis for a thermophilic sludge cultivated in UASB reactors under the given conditions. The optimum of 60-65°C was independent of the temperature of operation.

The reason for this great difference in temperature susceptibility has to be attributed to the selection of specific thermophilic methanogens in the sludge bed. Several thermophilic acetate-utilizing methanogens with different optimum growth temperatures have been described (Table 4.2). For comparison, Table 4.2 includes some mesophilic acetate-utilizing methanogens as well. With respect to the differences in optimum growth temperatures, a selection of specific methanogens at a fixed process temperature is very likely. During the cultivation of thermophilic sludge in serum bottles all new organisms are retained in the system. Obviously, the bacteria with the highest growth rate will be predominant in the cultivated consortium. The temperature optima for methanogenesis found for the batch-cultivated sludges (Fig. 4.1), agree with the optimum growth temperatures for the different thermophilic acetate-utilizing methanogens described (Table 4.2). However, the various temperature optima found for the sludges cultivated in serum bottles contradicts to the single temperature optimum found for the sludges cultivated in UASB reactors. Other selection criteria than the specific growth rate are of importance during the development of a thermophilic methanogenic consortium in a UASB reactor (see also § 3.1.4).

**Table 4.2** Temperature optima and growth kinetic parameters of several acetate-utilizing methanogenic cultures

Acetate-utilizing methanogens	T <sub>opt</sub> (°C)	T <sub>max</sub> (°C)	μ <sub>max</sub> (h <sup>-1</sup> )	K <sub>s</sub> (Ac) (mg COD.l <sup>-1</sup> )	Ref.
<i>Methanosarcina barkeri</i>	35-40	n.r. <sup>a</sup>	0.023	320	1
<i>Methanosarcina thermophila</i>	50	55-60 <sup>b</sup>	0.058	288	2, 3
<i>Methanosarcina</i> CALS-1	55-58	60	0.058	n.r.	4
<i>Methanosarcina</i> MP	55	60	n.r.	n.r.	5
<i>Methanosarcina</i> MSTA-1	55	65	0.053	685	6
<i>Methanosarcina</i> CHTI 55	57	63	0.085	614	7
<i>Methanotherix soehngeni</i>	37	45-50 <sup>b</sup>	0.0085	45	8
<i>Methanotherix concilii</i>	35-40	40-45 <sup>b</sup>	0.029	77	9
<i>Methanosaeta</i> sp. P <sub>T</sub>	55	65-70 <sup>b</sup>	0.020	n.r.	10
TAM	60	70	0.012	51	11
<i>Methanotherix</i> sp. CALS-1	60	65-70 <sup>b</sup>	0.028	< 64	12
<i>Methanotherix thermoacetophila</i>	65	70	n.r.	n.r.	13
Acetate oxidizing co-culture	60	n.r.	0.019	n.r.	14

<sup>a</sup> n.r. = not reported,

<sup>b</sup> no growth observed at highest temperature of given range,

Ref: 1, Smith and Mah (1978); 2, Zinder and Mah (1979); 3, Zinder *et al.* (1985); 4, Zinder *et al.* (1984a); 5, Ollivier *et al.* (1984); 6, Clarens and Moletta (1990); 7, Touzel *et al.* (1985); 8, Huser *et al.* (1982); 9, Patel (1984); 10, Kamagata and Mikami (1991); 11, Ahring and Westermann (1985); 12, Zinder *et al.* (1987); 13, Nozhevnikova and Chudina (1984); 14, Zinder and Koch (1984).

Kinetic and attachment properties of thermophilic methanogens rather than optimal growth temperatures might have determined the bacterial composition of the sludge bed. During the 6 months of operation, the reactor effluent had very low acetate concentrations (Table 4.1), except for the reactor operated at 64°C. The methanogens with the highest growth rate all belong to the genus *Methanosarcina* (Table 4.2). However, the substrate affinity of *Methanosarcina* species, is relatively low compared with that of *Methanotherix*, "*Methanosaeta*" (Patel and Sprott, 1990), species. Very likely, this can be attributed to the difference in K<sub>m</sub> value for acetate of the acetate activating enzymes in both methanogens (Jetten *et al.*, 1990). Therefore, *Methanotherix* will be selected at low substrate concentrations, irrespective of its lower maximum specific growth rate at lower temperatures. In addition, *Methanotherix* may have better adherence properties than *Methanosarcina* because of its long filamentous shape and its rather uncharged surface at neutral pH (Grotenhuis *et al.*, 1992). Granular anaerobic sludge from UASB reactors is mostly dominated by bacteria of the genus *Methanotherix*, both in the mesophilic (De Zeeuw, 1984; Grotenhuis *et al.*, 1991)

and the thermophilic range (Chapter 3.1; Wiegant and De Man, 1986; Uemura and Harada, 1993). Apparently, *Methanosarcina* cannot be retained as a predominant bacterium in UASB reactors operated at low effluent substrate concentrations. In contrast to cultivation in batch reactors in which all bacteria are retained, *Methanosarcina* species are outcompeted, and washed-out from continuous-flow systems. The latter explains the phenomena found in our experiments. In addition, a shift in the methanogenic population from a *Methanosarcina*- to a *Methanotherix*-dominated consortium was observed during the anaerobic digestion of municipal refuse at 58°C (Zinder *et al.*, 1984b). This was also attributed to better kinetic properties of the latter organism at low substrate concentrations.

Recently, however, Ahring (1991) reported the development of sludge granules in thermophilic UASB reactors in which *Methanosarcina* species were found to be the only acetate-utilizing methanogens. A possible reason for this great difference in sludge development could be the source of inoculum as explained in the introduction (§ 1.5.2). In the latter experiments the UASB reactors were inoculated with the digested residue of large scale thermophilic biogas plants treating a mixture of manure and industrial waste (Ahring, 1991). This seed sludge was also predominated by *Methanosarcina* species. Due to the extremely large number of *Methanosarcina* bacteria and the negligible number of *Methanotherix*-like bacteria, development of granules predominated by *Methanotherix* species is very difficult, if possible at all. Nonetheless, the *Methanosarcina*-aggregates formed well settleable granular sludge at loading rates of 10-16 g VFA-COD.l<sup>-1</sup> reactor.day<sup>-1</sup> (Ahring, 1991; Ahring *et al.*, 1993; Schmidt and Ahring, 1993). The stability of bacterial aggregates and the selection of *Methanotherix* species might also be influenced by the applied superficial liquid and biogas loading rate (Wiegant and De Man, 1986). For our UASB reactors, operated at 46, 55 and 64°C, a total superficial loading rate of about 2.1 m.day<sup>-1</sup> was calculated. Another reason for the large differences in bacterial composition of the sludge granules might be a difference in morphology of the *Methanosarcina* species. Single cells of these organisms are washed out more easily than large clumps, packets or lamina (Schmidt *et al.*, 1992). Production of single cells is induced by high bivalent cation concentrations (Ahring, 1991; Schmidt *et al.*, 1992). However, in our UASB reactors the Mg<sup>++</sup> and Ca<sup>++</sup> concentrations were very low; about 15 and 35 mg.l<sup>-1</sup>, respectively.

Until now almost all of the *Methanotherix*-type aceticlastic methanogens described, have an optimum growth temperature between 60 and 65°C (Table 4.2). This might result in a temperature optimum at 60-65°C for acetate conversion of methanogenic sludge from thermophilic UASB reactors, despite the lower temperature of operation. Besides a cleavage of acetate into carbon-dioxide and methane, a syntrophic oxidation of acetate may also occur in the sludge bed; see also § 1.4. The aceticlastic reaction is performed by methanogens of the genus *Methanosarcina* and *Methanotherix*, but the latter is a two-step reaction performed by a homo-acetogen and a hydrogen-consuming methanogen (Weber *et al.*, 1984; Zinder and Koch, 1984). The affinity for acetate of this syntrophic association is similar to that of

*Methanotrix* species. This alternative pathway for acetate degradation might become important, particularly under thermophilic conditions at low substrate concentrations (Petersen and Ahring, 1991). According to the recent results of Ahring *et al.* (1993) and Uemura and Harada (1993), a considerable fraction of the thermophilic biomass in anaerobic sludge granules may consist of syntrophic acetate oxidizing consortia. However, so far, little information is available regarding the characteristics and the kinetics of such consortium and until now only one culture is described which exhibits an optimum growth temperature of 60°C (Zinder and Koch, 1984), similar to that of *Methanotrix* species. The sludges used in our experiments and which were cultivated in the UASB reactors at 46, 55 and 64°C were dominated by *Methanotrix*-like rods as evidenced by the fingerprinting analyses described in Chapter 3.1. However, it should be noted that the immunological methods for bacterial identification as described in Chapter 3 are exclusively for methanogens. It is therefore impossible to recognize an eventual fraction of homo-acetogenic bacteria. Moreover, the high abundance of the hydrogen-consuming methanogen *Methanobacterium thermoautotrophicum*  $\Delta H$  in the cultivated sludges, particularly at 55°C and 64°C, may indicate the occurrence of such acetate oxidizing consortium. Whether or not thermophilic acetate oxidizers play a role in the sludge from our UASB reactors, yet remains unclear.

For the thermophilic sludges we also found only one optimum temperature for the conversion of propionate (Fig. 4.2b). The optimum of 55-60°C corresponds to the optimal growth temperature of a thermophilic propionate-oxidizing enrichment culture, recently described by Stams *et al.* (1992). Up to now no other thermophilic propionate-oxidizing cultures have been described. The results found in this study explain why in our previous experiments propionate was barely degraded at 64°C (Fig. 3.2c). But at 55°C stable propionate oxidation is also difficult to achieve, as illustrated by Fig. 4.4a and 4.4b.

With respect to the temperature optima for butyrate oxidation, the results were not very clear. Between 40 and 50°C no optimum was found but different optima were found for the sludges cultivated at 55 and 64°C. To our knowledge temperature optima for thermophilic butyrate degradation have not been reported thus far. However, good growth of thermophilic butyrate oxidizers was observed at 55°C (Henson and Smith, 1985) as well as at 60°C (Ahring and Westermann, 1987).

If the maximum growth rate is the predominant selection criterion, different temperature optima in the thermophilic range can be expected. This might be a problem during the start-up of thermophilic UASB reactors, particularly if the process temperature is increased stepwise from mesophilic to thermophilic conditions. Once a mesophilic methanogenic sludge bed is exposed to high temperatures, a considerable part of the mesophilic methanogenic activity will be lost (Van Lier *et al.*, 1990; Speece and Kem, 1970). This will be followed by a recovery of the methanogenic activity due to an increase of thermophilic methanogens with an optimum growth temperature near the newly installed process temperature (Chapter

3.2). In reactor R5 we found a drop in methanogenesis after each temperature increase, followed by a recovery (Fig. 4.3a). However, long adaptation times resulted in an increase of the maximum applicable temperature (Fig. 4.3b), probably caused by a gradual shift in the methanogenic population.

Different temperature optima and/or a higher temperature susceptibility are also to be expected in conventional thermophilic CSTR systems without sludge retention. In these systems the composition of the thermophilic biomass is determined by the highest growth rate of the different bacteria involved in the digestion process. A drastic shift in bacterial populations was observed after a temperature increase of a thermophilic CSTR from 58°C to 64°C (Zinder *et al.*, 1984b). Garber *et al.* (1975) reported a drop in the methanogenic activity of a sewage-sludge digestion plant after the temperature was increased from the subthermophilic range (46-50°C) to above 52°C.

The temperature susceptibility of thermophilic anaerobic sludge depends to a great extent on the mode of operation, the type bioreactor and the duration of the adaptation time to a high temperature. Whenever the maximum specific growth rate is the predominant selection criterion for the methanogenic consortium, a higher sensitivity to temperature changes can be expected. This will be the case during reactor start-up and also during the operation of conventional completely mixed systems. In order to keep the rate of methanogenesis at a high level if a reactor is shifted from mesophilic to thermophilic conditions, temperature increments should be imposed very slowly, i.e. 1°C every 1 to 2 weeks. For the application of thermophilic wastewater treatment, the use of high-rate systems with a high solids-retention time is preferred over the CSTR-type systems.

## 4.2 Effect of Temperature on the Anaerobic Thermophilic Conversion of Volatile Fatty Acids by Dispersed and Granular Sludge

### Abstract

The effect of temperature on the rate of VFA conversion by thermophilic methanogenic sludge, cultivated in high-rate reactors at 55°C, was studied using both batch activity tests and continuous-flow experiments. The temperature dependence of acetate conversion in the range between 37°C-70°C could be described by an Arrhenius derived model when dispersed sludge with a low specific activity was used. For this sludge the optimum acetate conversion rate was found at 65°C. The maximum acetate-utilization rate was not affected by temperature in the range between 50°C to 65°C when granular sludge with a high specific methanogenic activity was used. Crushing the granules led to a 2 to 3 fold increase in the maximum activity at 60-65°C, indicating that the conversion rate was very likely limited by the diffusion rate of acetate into the granules. Similar results were obtained with butyrate as the substrate. The temperature dependence of the crushed granules was similar to that of the less active dispersed sludge. In contrast, the thermophilic propionate oxidation rate was highest with the intact granular sludge, while a similar temperature dependence was found for both the granular and dispersed sludges. The affinity for VFA increased with decreasing temperature. This phenomenon was most pronounced for the granular sludge. The thermophilic treatment of a VFA-mixture in a UASB reactor appeared to be only slightly affected by temperature when moderate loading rates were applied, i.e. 20 g COD. $l^{-1}$ .day $^{-1}$ . However, temperature had a strong effect applying loading rates of 40-90 g COD. $l^{-1}$ .day $^{-1}$  accompanied with high effluent VFA concentrations. The results reveal a high thermostability of the thermophilic wastewater treatment process in the range 45-60°C if high-rate reactors with a granular sludge bed are used.

### 4.2.1 Introduction

Temperature has a distinct effect on the growth rate and maximum substrate conversion rate of all bacteria. Between the minimum and optimum growth temperature of a specific bacterium the effect of temperature on the growth and activity generally can be described using the Arrhenius equation (Pavlostathis and Giraldo-Gomez, 1991). This accounts also for anaerobic bacteria from both the mesophilic and thermophilic temperature range. However, in anaerobic bioreactors the temperature dependence of the methanogenic sludge is not always following such Arrhenius type of relation, which obviously might be due to the presence of a mixed methanogenic flora. Also, there seems to be a difference in temperature

susceptibility between methanogenic sludge from the mesophilic and from the thermophilic range. Research on thermophilic CSTR-type reactors revealed a relatively high temperature sensitivity of these systems, as reviewed by Zinder (1986, 1990). Consequently, the temperature ranges, in which these reactor systems safely could be operated, are relatively narrow. Based on these literature results, general statements are sometimes formulated expressing that thermophilic anaerobic treatment is very sensitive to temperature changes. However, it should be noted that, so far, very limited research has been carried out on the stability of thermophilic high-rate systems with immobilized biomass. In addition, CSTR-type reactors are much more sensitive to environmental changes in any temperature range (Wheatley, 1990). Results described in Chapter 4.1 reveal that thermophilic high-rate systems like UASB reactors, may be operated in a wide temperature range without inactivating the methanogenic sludge. In the cultivated biomass, an acetate-degrading consortium with a temperature optimum at 60-65°C was predominant, irrespective of the applied reactor temperature in the range between 46-64°C. It was hypothesized that adherence and kinetic properties are more important selection criteria for the thermophiles than their maximum specific growth rate (Chapter 4.1). For large scale applications, it is of utmost importance that the treatment system can tolerate moderate temperature fluctuations. Therefore, sludge bed reactors offer good prospects for the application of thermophilic wastewaters treatment.

While the activity of anaerobic sludge is affected strongly by temperature (Pavlostathis and Giraldo-Gomez, 1991), the effect of temperature on substrate diffusivity is only marginal (Perry and Green, 1984). Therefore, immobilization of bacteria in biofilms and/or granules, may enhance the thermostability of the high-rate process due to the fact that the maximum conversion rate will most likely be determined by diffusion limitation of the substrate (Smith, 1981; Lens *et al.*, 1993; Pavlostathis and Giraldo-Gomez, 1991). Obviously, such effect will manifest particularly when the methanogenic sludge is characterized by a high specific activity. Therefore, mass transfer limitation probably plays a major role in the thermostability of thermophilic high-rate reactors with immobilized biomass. In the present study, we investigated the thermostability of the thermophilic conversion process by using both dispersed and granular methanogenic sludge.

## 4.2.2 Materials and Methods

### **Biomass**

Substrate-depletion activity tests were performed using two types of thermophilic granular sludge (TGS) harvested from USSB reactors which were operated at 55°C (see also Chapter 6). TGS-1 was grown for 6 months in a USSB reactor inoculated with digested organic fraction of municipal solid waste (OFMSW, § 2.2) and fed with a sucrose-VFA mixture. This mixture consisted of sucrose:acetate:propionate:butyrate = 3:1:1:1, based on COD. Despite the feeding with the partially acidified substrate, which is found to enhance growth

of thermophilic granular sludge (Chapter 6, Uemura and Harada, 1993; Wiegant and Lettinga, 1985), the particle size of TGS-1 was still very small ( $< 1$  mm) after 6 months of operation. The biomass concentration used in the activity-test-vials with TGS-1 was 2-2.5 g VSS. $l^{-1}$ . The sludge sample TGS-2 was taken from a USSB reactor which was started with partially 'crushed' Aviko-MGS (§ 2.2) and fed with the same sucrose-VFA mixture for a period of almost 2 years. In contrast to TGS-1, TGS-2 sludge consisted of large granular aggregates (2-4 mm). In order to examine the possible role of the granules, part of the TGS-2 sample was crushed prior to the activity tests. The granules were ground manually by squeezing a plastic bag filled with the sludge sample, effluent from the USSB reactor, and  $N_2$ -gas. The biomass concentration in the activity tests ranged from 0.5-0.8 and 0.9-1.5 g VSS. $l^{-1}$  for crushed and intact TGS-2, respectively. When propionate was used as a substrate, the biomass concentration used in the serum bottles was 2-3 g VSS. $l^{-1}$  for both the crushed and the intact TGS-2.

The UASB reactor was inoculated with thermophilic granular sludge at a concentration of 30 g VSS. $l^{-1}$ . The granular sludge, referred to as TGS-3, was cultivated in our laboratory in a 5.75 l UASB 'breeding' reactor at 55°C. The 'breeding' reactor (Fig. 2.1) was fed with a sucrose:acetate:propionate:butyrate mixture of 1:4:2:4, based on COD, for a period of approximately 9 months. In this period the reactor was operated with an organic loading rate (OLR) of about 30 g COD. $l^{-1} \cdot day^{-1}$ , and a hydraulic retention time (HRT) of 8-9 hours. Effluent VFA concentrations were below 0.5 g COD. $l^{-1}$ . The 'breeding' reactor was inoculated with Aviko-MGS (§ 2.2).

#### Assessment of $A_{max}$ and $K_m$ .

The  $A_{max}$  and apparent  $K_m$  values of TGS-1 and TGS-2 were both assessed using substrate-depletion activity tests in 120-ml serum bottles (§ 2.4).  $A_{max}$  was calculated from the linear decrease in substrate concentration, by using linear regression and the exact amount of biomass. Apparent  $K_m$  values were estimated from the substrate depletion curves, by using a Michaelis-Menten derived equation and a nonlinear regression routine for parameter estimation, based on the algorithm developed by Nelder and Mead (1965). The algorithm is available in the Matlab software package (Math works Inc., Natick, Massachusetts, USA). The substrate conversion rate depends on the biomass concentration and the specific activity of the biomass according to:

$$\frac{dS}{dt} = -A \cdot X \quad (4.1)$$

where:

$$A = A_{max} \cdot \frac{S}{K_m + S} \quad (4.2)$$

Integration of the combined equations (1) and (2) gives:

$$K_m \cdot \ln \left( \frac{S}{S_0} \right) + S - S_0 = -A_{\max} \cdot X \cdot t \quad (4.3)$$

A similar equation was successfully used by Wu *et al.* (1993) for estimating the substrate affinity of mesophilic sludge. Equation 4.3 was also used to estimate  $A_{\max}$  from the depletion curves. Obviously, when a high  $K_m$  occurs, the latter approach gives a much higher  $A_{\max}$  than the linear regression method. It should be noted that under such conditions the  $A_{\max}$  calculated with equation 4.3 reflects a theoretical value which never will be reached with the existing sludge structure and the applied substrate concentrations. Therefore, results were interpreted using  $A_{\max}$  calculated with the linear regression method. Activity tests were performed in triplicate when acetate was used as the substrate, and in duplicate when propionate and butyrate were used. The initial substrate concentration in each test was 3.0 g COD.l<sup>-1</sup>. During the tests, serum bottles were incubated in temperature controlled water baths on a Gerhardt RO 202 rotating shaker (Bonn, Germany) at 50 rpm. Temperatures were in the range between 35°C and 75°C.

The temperature dependence of the maximum acetate conversion rate of TGS-1 was fitted by using an Arrhenius derived equation which describes the effect of temperature on the net microbial activity by recognizing the occurrence of a process of biosynthesis and a process of microbial decay (Pavlostathis and Giraldo-Gomez, 1991):

$$A_{\max} = k_1 \cdot e^{a_1 (T-x_T)} - k_2 \cdot e^{a_2 (T-x_T)} \quad (4.4)$$

In the lower temperature range, up to 55/60°C, equation 4.4 describes an exponential increase of the conversion rate with increasing temperature. Beyond the temperature optimum of the thermophilic methanogenic consortium, substrate conversion is limited by the high decay rate in the second term of the equation. For  $T < 333$  K (60°C), the second term of equation 4.4 is negligible and the constants  $k_1$  and  $a_1$  can be calculated using linear regression of the semi-logarithmic plot. The kinetic constants  $k_2$  and  $a_2$  in the second term, as well as the temperature correction factor,  $x_T$ , in both terms, are calculated using the non-linear regression routine for parameter estimation.

The methanogenic activity of TGS-3 was assessed at 45°C and 55°C using the "head-space method" (§ 2.4). With this method the methanogenic activity is assessed at a more or less constant substrate level. The activities were measured at 10 different acetate concentrations in the range of 0-6 g acetate-COD.l<sup>-1</sup> at pH 7.0-7.4. Apparent  $K_m$  values were calculated from the substrate dependent methane production rates, by using modified Haldane kinetics as described in detail in Chapter 6.2.

### Continuous flow experiments in UASB reactor

The same experimental set up for the 5.75-l UASB reactor was used as described in Chapter 3.1. The reactor (Fig. 2.1) was fed with a VFA mixture consisting of acetate:propionate:butyrate = 3:1:1, on a COD basis. After adding the seed sludge (TGS-3), the reactor was operated for 1-1.5 months in order to achieve a stable performance at moderate loading conditions, i.e. 20 g COD.l<sup>-1</sup>.day<sup>-1</sup>. The HRT was set at 8 hours and remained constant throughout the study. Thereafter, the temperature experiments were started, which consisted of a stepwise decrease of the temperature from 55 to 35°C. In this period the effluent VFA concentrations remained low. Next, a stepwise increase of the temperature to 55°C was performed at high effluent VFA concentrations. In order to obtain these concentrations, the OLR was increased prior to an increase in the temperature.

### Analysis

Methods for VFA, CH<sub>4</sub> and all other determinations are described in § 2.5.

## 4.2.3 Results

### Batch activity tests

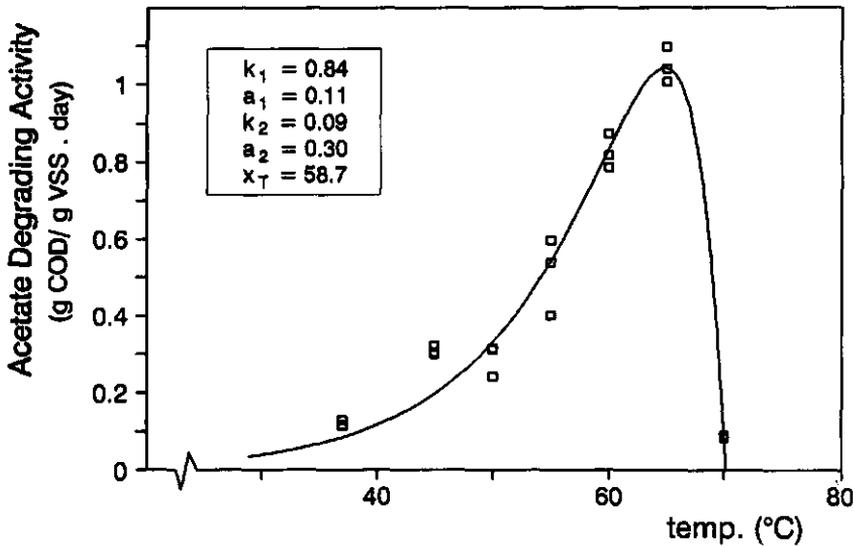
The maximum acetate utilization rate of TGS-1 at various temperatures could be fitted using equation 4.4. Results show a temperature optimum at 65°C (Fig. 4.5), which is in agreement with our previous results (Chapter 4.1). The specific methanogenic activity of TGS-1 was rather low. A large fraction of the sludge consisted of non-viable organic biomass such as wood particles originating from the seed material.

**Table 4.3**  $A_{max}$  and apparent  $K_m$  of acetate conversion by TGS-1 and TGS-3

Sludge	Temperature	$A_{max}$ (g COD.g <sup>-1</sup> VSS.day <sup>-1</sup> )	$K_m$ (g COD.l <sup>-1</sup> )
TGS-1 <sup>a</sup>	45°C	0.31 ± 0.01	0.13 ± 0.01
	55°C	0.51 ± 0.08	0.54 ± 0.07
TGS-3 <sup>b</sup>	45°C	2.26	0.7
	55°C	2.71	1.5

<sup>a</sup>  $A_{max}$  and  $K_m$  estimated from acetate depletion curves

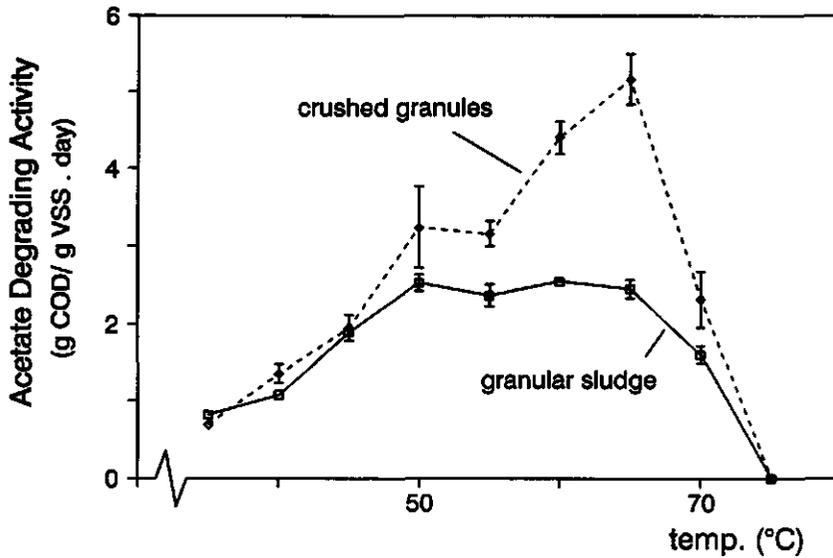
<sup>b</sup>  $A_{max}$  and  $K_m$  estimated from the maximum CH<sub>4</sub> production rates at various acetate concentrations in the range 0-6 g COD.l<sup>-1</sup>.



**Fig. 4.5** Maximum acetate-utilizing methanogenic activity at various temperatures of TGS-1 grown for 6 months on a sucrose-VFA mixture. Solid line is computed using equation 4.4.

In addition to  $A_{max}$  also apparent  $K_m$  increased with increasing temperature (Table 4.3). Apparently, the low activity at low temperatures was compensated by a high substrate affinity.

A maximum acetate-utilization rate of approximately  $2.5 \text{ g COD} \cdot \text{g VSS}^{-1} \cdot \text{day}^{-1}$  was found for the intact TGS-2 granules. Surprisingly, the maximum rate remained unaffected by temperature in the range between  $50^\circ\text{C}$  to  $65^\circ\text{C}$  (Fig. 4.6). Crushing these thermophilic granules led to a 2 to 3-fold increase in the methanogenic activity at  $65^\circ\text{C}$ . This was accompanied by a high temperature dependency, similar to that of TGS-1. The course of the acetate depletion curves was clearly influenced by the size of the microbial aggregates, particularly at high temperatures. For the intact TGS-2 granules apparent  $K_m$  values reached rather extreme levels at  $55\text{--}65^\circ\text{C}$  (Table 4.4). Moreover, using the latter sludge the threshold value for acetate was approximately  $100 \text{ mg acetate-COD}$  at  $65^\circ\text{C}$ , while rapid and complete acetate removal occurred in experiments with the crushed granules at  $65^\circ\text{C}$  (results not shown). Apparent  $K_m$  values of crushed TGS-2 were much lower. Using latter sludge no clear correlation between the acetate affinity and the temperature was observed. A minimum for the apparent  $K_m$  was found at  $50^\circ\text{C}$  while ' $K_m$  optima' were observed at both  $40^\circ\text{C}$  and  $60^\circ\text{C}$  (Table 4.4).



**Fig. 4.6** Maximum acetate-utilizing methanogenic activity at various temperatures of intact ( $\square$ ) and crushed ( $\diamond$ ) TGS-2.

A distinct difference between intact and crushed TGS-2 was also found when butyrate was used as the substrate. As was observed with acetate, crushing of the TGS-2 granules resulted in a 2 to 3-fold increase of the maximum conversion rate (Fig. 4.7). However, in contrast to acetate, the affinity for butyrate increased with increasing temperature for the crushed sludge; while in the case of the intact granules, apparent  $K_m$  was highest at 55 and 60 °C (Table 4.4). On the other hand, compared to crushed TGS-2, we observed a shorter lag-phase preceding butyrate conversion with the intact granules (Table 4.5). This difference was most pronounced at 35 and 40°C. At temperatures higher than 45°C the lag-phase was always less than 1.5 days for both crushed and intact TGS-2. Such striking difference in lag-phase was not found for the other substrates. Contrary to acetate and butyrate, the highest conversion rate of propionate was obtained with the intact granules. The difference of  $A_{max}$  between intact and crushed TGS-2 was most pronounced at 50-60°C (Fig. 4.8). Moreover, apparent  $K_m$  for propionate oxidation was more or less similar for both the crushed and the intact TGS-2 granules (Table 4.4).

**Table 4.4** Apparent  $K_m$  of thermophilic granular sludge (TGS-2) calculated using equation (3). Values of the intact granules as well as those of the crushed granules are presented.

Temperature	Apparent $K_m$ values in g COD.l <sup>-1</sup>					
	Acetate		Propionate		Butyrate	
	granular	crushed	granular	crushed	granular	crushed
35°C	0.25 ± 0.10	0.24 ± 0.08	N.C. <sup>a</sup>	N.C.	0.18 ± 0.01	0.44 ± 0.05
40°C	0.59 ± 0.14	0.60 ± 0.09	0.11 ± 0.03	0.18 ± 0.00	0.57 ± 0.02	0.25 ± 0.01
45°C	0.25 ± 0.08	0.34 ± 0.02	0.13 ± 0.03	0.09 ± 0.02	0.19 ± 0.00	0.29 ± 0.07
50°C	0.20 ± 0.06	0.24 ± 0.02	0.07 ± 0.01	0.09 ± 0.03	0.10 ± 0.04	neg. <sup>b</sup>
55°C	1.81 ± 0.11	0.87 ± 0.05	0.28 ± 0.09	0.13 ± 0.02	1.84 ± 0.03	0.03 ± N.C.
60°C	8.2 ± 2.6	1.23 ± 0.17	0.32 ± 0.05	0.22 ± 0.02	0.70 ± 0.10	0.02 ± 0.01
65°C	13.6 ± 4.7	0.63 ± 0.22	-	-	N.C.	neg.

<sup>a</sup> N.C., Not considered because of too few data.

<sup>b</sup> neg., negative values

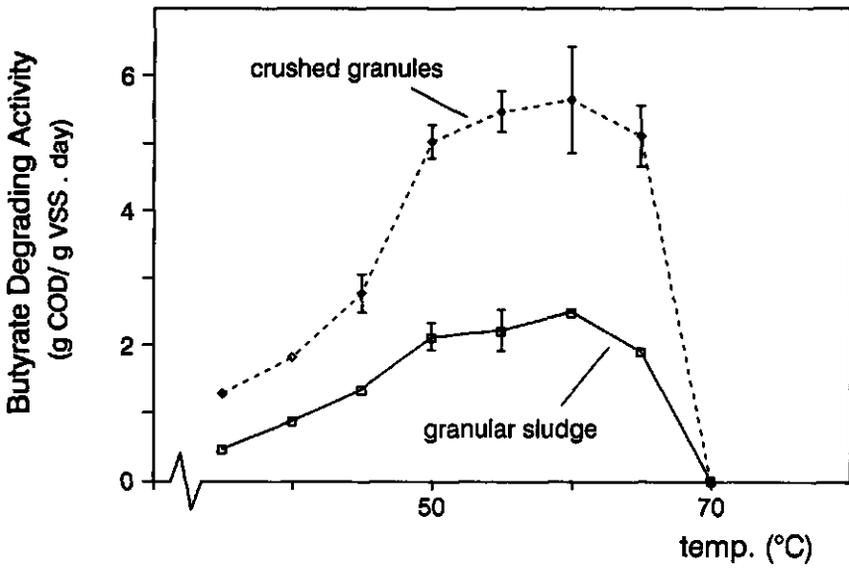


Fig. 4.7 Maximum butyrate-utilizing activity at various temperatures of intact (□) and crushed (◇) TGS-2.

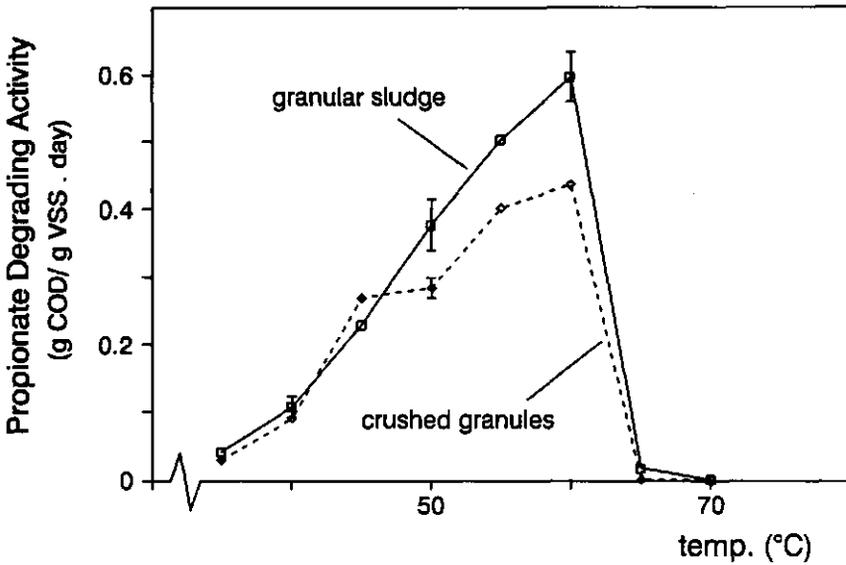


Fig. 4.8 Maximum propionate-utilizing activity at various temperatures of intact (□) and crushed (◇) TGS-2.

$A_{\max}$  values depicted in Figures 4.5-4.8 were calculated using linear regression and represent the maximum conversion rates at the actual substrate concentrations (up to 3 g COD.l<sup>-1</sup>), neglecting the effect of a high apparent  $K_m$ . With very high substrate conversion rates, i.e. at high temperatures using intact TGS-2 as inoculum and acetate or butyrate as the substrate, only a limited number of useful data were available to perform linear regression. Due to the very high apparent  $K_m$  prevailing under the latter conditions, distinctly higher  $A_{\max}$  values were calculated with equation 4.3 (results not shown). The substrate concentrations obviously were too low to estimate absolute  $A_{\max}$  using linear regression. However, for practical applications temperature effects at VFA concentrations higher than 3.0 g COD.l<sup>-1</sup> are of less importance, because generally, VFA concentrations in reactor effluents are much lower.

**Table 4.5** 'Lag phase' period<sup>a</sup> of TGS-2 preceding the maximum butyrate-degrading activity

Temperature (°C)	Crushed sludge	Intact granules
35	7.2	3.0
40	3.5	2.1
45	2.0	1.1
50	1.3	0.8
55	1.1	0.5
60	0.8	0.5
65	1.5	1.1

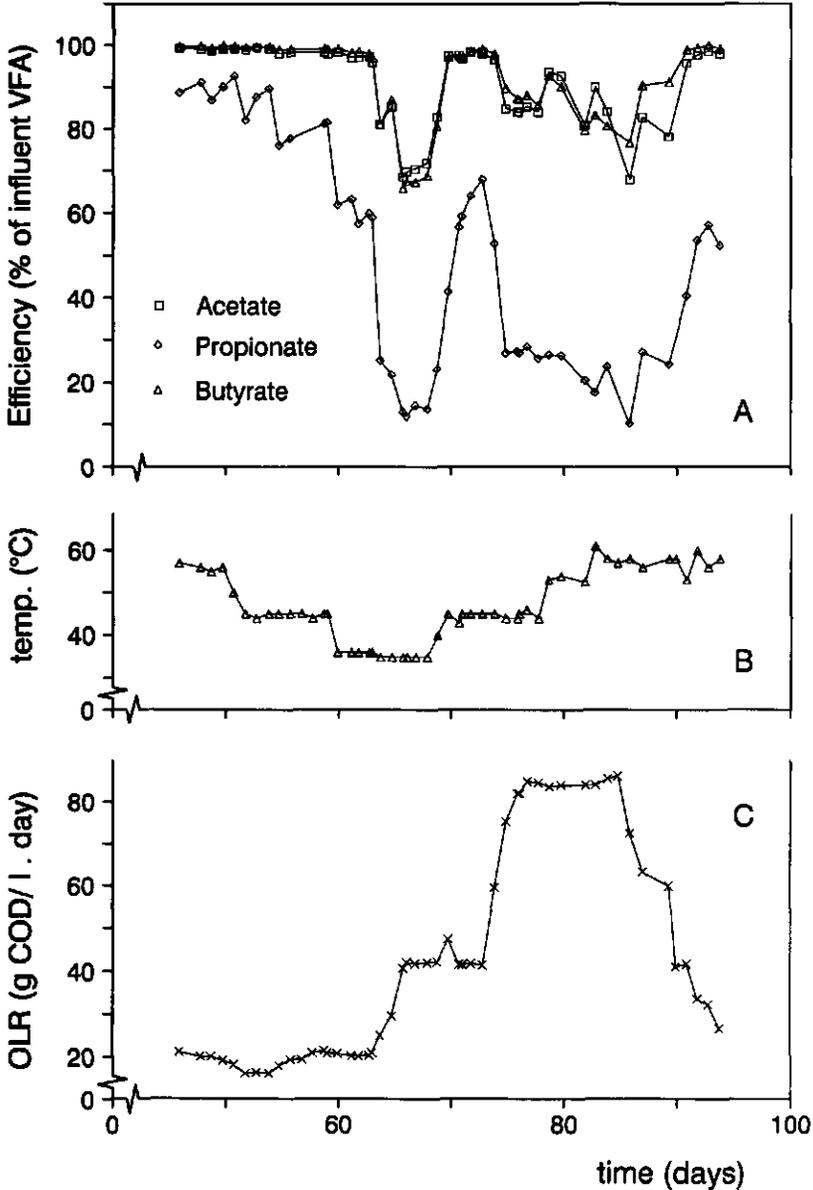
<sup>a</sup> 'Lag phase' period in days

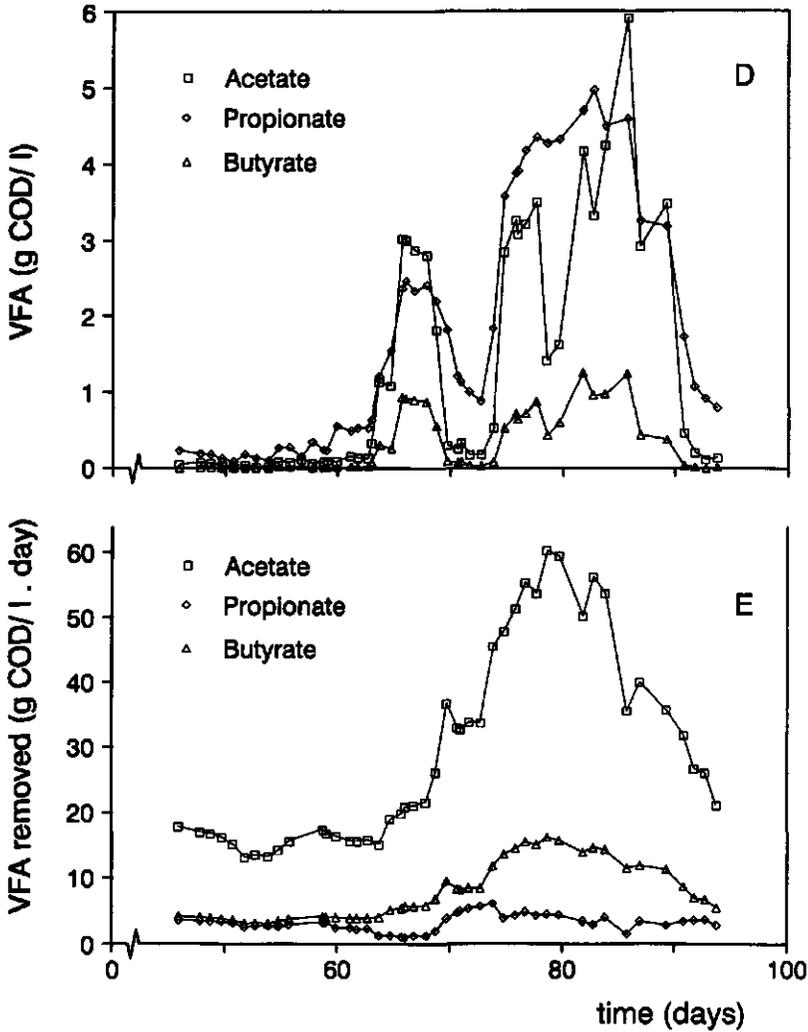
### Continuous flow experiments

The effects of the temperature on the performance of a thermophilic UASB reactor was studied at effluent substrate concentrations < 500 mg VFA-COD.l<sup>-1</sup> as well as > 5000 mg VFA-COD.l<sup>-1</sup>. Due to the large differences in apparent  $K_m$  for acetate at 45°C and 55°C (Table 4.3), the phenomenon of temperature compensation was to be expected for the TGS-3 sludge, especially at low substrate concentrations. At the start of the experimental period, a moderate OLR was applied of approximately 20 g COD.l<sup>-1</sup>.day<sup>-1</sup> at an operating temperature of 55°C (Fig. 4.9). At day 50/51 the operation temperature was lowered from 55°C to 45°C, followed by a subsequent decrease to 35°C at day 59 while the OLR remained unchanged. From the results it is clear that under these moderate to low loading conditions, the digestion process is hardly affected by a temperature drop except for propionate for which the removal efficiency gradually decreased from 90% to less than 60%. The specific propionate-utilizing activity of the sludge was apparently very low since no complete propionate removal was observed at 55°C. For the calculation of acetate removal efficiency, the stoichiometric amount of acetate produced during the oxidation of propionate and butyrate was taken into

account.

Apparently, the available methanogenic capacity of thermophilic sludge was almost entirely used at 35°C, because a relatively small increase in the OLR from 20 to 25 g COD.l<sup>-1</sup>.day<sup>-1</sup> at day 64 resulted in a significant drop in the removal efficiency of all fatty acids (Fig. 4.9a). The OLR was increased subsequently to 43 g COD.l<sup>-1</sup>.day<sup>-1</sup> at day 65/66 which led to a further deterioration of the removal efficiencies resulting in high concentrations of VFA in the effluent (Fig. 4.9d).





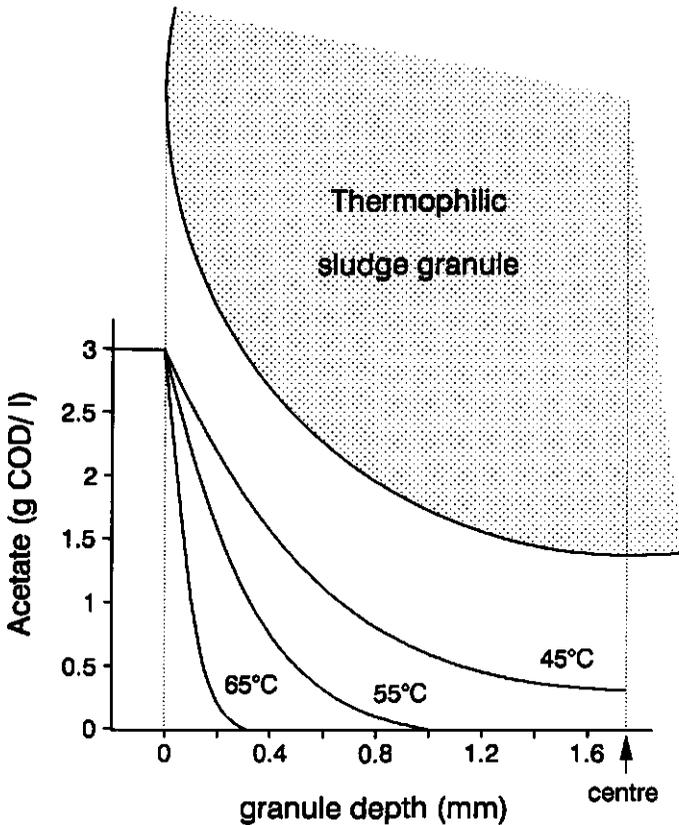
**Fig. 4.9** Performance of the UASB reactor under fluctuating temperature and loading conditions. A) VFA removal efficiency (relative to the influent concentration); B) temperature ( $^{\circ}\text{C}$ ); C) volumetric organic loading rate in  $\text{g COD}\cdot\text{l}^{-1}\cdot\text{day}^{-1}$ ; D) effluent VFA concentration ( $\text{g COD}\cdot\text{l}^{-1}$ ); E) VFA removal, absolute values in  $\text{g COD}\cdot\text{day}^{-1}$ .

Nonetheless, we did observe an increase in the absolute amount of acetate and butyrate converted. The opposite was found for propionate (Fig. 4.9e). The deterioration of propionate degradation might be explained by the presence of a too high concentration of acetate and/or hydrogen, as both are inhibitory to the propionate conversion process (see also Chapter 5). However, a remarkable recovery was achieved after the process temperature was shifted from 35 to 45°C on day 69, which can be explained by the increase in the propionate-degrading activity at elevated temperatures. In order to assess the effects of a subsequent increase of temperature to 50/55°C at VFA concentrations higher than 5000 mg COD.l<sup>-1</sup>, the OLR was increased to 85 g COD.l<sup>-1</sup>.day<sup>-1</sup>. With the exception of propionate conversion, again an improvement of the conversion rates for the VFAs was observed after the temperature was increased on day 78. However, it was impossible to maintain the performance of the system under the prevailing conditions due to heavy sludge wash-out caused by the high biogas production. Apparently, the loading limits of this single stage UASB reactor were reached and recovery of the performance was only possible after a sharp reduction of the OLR (Fig. 4.9). In Chapter 6 we demonstrate that a compartmentalized upflow reactor (Fig. 2.2) is much more feasible for thermophilic treatment under such extreme conditions since the produced biogas is withdrawn at various heights along the reactor.

#### 4.2.4 Discussion

Temperature has a strong effect on the maximum VFA conversion rates of thermophilic methanogenic sludge. The susceptibility for temperature fluctuations decreases considerably if the biomass is immobilized in granules. In all probability, this phenomenon can be attributed to substrate limitation due to diffusion resistance (Smith, 1981). If the substrate conversion rate in the outer layers of the granule is higher than the mass transport rate to the inner layers, only part of the granule will contribute to the conversion process. The actual penetration depth of the substrate into the granules depends on various factors such as: i) substrate concentration in the bulk liquid phase; ii) size and shape of the granules; iii) density and distribution of the active biomass in the granules; and iv) temperature which determines the maximum activity of the biomass. Fig. 4.10 shows possible acetate concentration profiles in a thermophilic sludge granule (diameter  $\approx$  3.5 mm) at various temperatures. For sake of convenience, a stagnant layer surrounding the granule was neglected. The maximum specific conversion rate of the viable biomass is highest at 65°C, which results in a limited diffusion depth of acetate into the granules (Fig. 4.10). Due to a lower maximum conversion rate at lower temperatures, acetate penetrates deeper into the granule at 55°C, meanwhile the overall specific activity of the sludge granule remains unaffected. At 45°C the entire granule becomes exposed to substrate. Consequently, the overall specific activity is affected immediately by a further change in temperature. Crushing the granule structure will enhance the overall mass transfer by decreasing the distance required for diffusion. This subsequently results in a

higher temperature sensitivity. The above explanation is in agreement with the results found for TGS-2 (Fig. 4.6). Compared to the intact granules, the maximum specific activity assessed at the range 50-65°C is significantly higher for the crushed TGS-2. Also, the temperature susceptibility found for crushed TGS-2 was similar to that of TGS-1, which consists of small particles and was characterized by a low specific activity (Fig. 4.5, 4.6). Mass transport limitations were also observed in granular sludge cultivated under mesophilic conditions (Alphenaar *et al.*, 1993; Kato, 1994; Morvai *et al.*, 1992). Obviously, this phenomenon is of particular importance in granular sludges with a very high specific activity.



**Fig. 4.10** Possible acetate concentration profiles in a thermophilic sludge granule at 45°C, 55°C and 65°C.

Generally, well adapted thermophilic granular sludge is characterized by a high  $A_{\max}$  and a diameter of 1-3 mm (Chapter 6, Souza *et al.*, 1992; Uemura and Harada, 1993; Wiegant and De Man, 1986). Therefore, under thermophilic conditions, mass transport limitations may even occur at substrate concentrations as high as 3.0 g acetate-COD.l<sup>-1</sup>, as was observed for the intact TGS-2 granules (Fig. 4.6).  $A_{\max}$  increased considerably after crushing, meanwhile apparent  $K_m$  decreased. Apparently, during cultivation in the USSB reactor, there was enough substrate in the inner part of the granules to prevent starvation of the methanogens. Our results are in conflict with the results found by Alphenaar *et al.* (1993) who did not find any increase of the specific activity upon crushing the granule structure. Latter was explained by an inactive zone in the core of these granules (Alphenaar *et al.*, 1993).

The effect of temperature on the intrinsic substrate affinity of dispersed thermophilic methanogens remains unclear. The results of the present investigations don't allow us to draw any conclusions about the question whether the intrinsic kinetic characteristics of the thermophilic methanogens contribute to the temperature compensation effect. Since bacterial aggregates were clearly present in the sludge with the smallest particle size, i.e. TGS-1 and crushed TGS-2, mass transfer limitation cannot be excluded for these sludges. In contrast to our present results, other researchers observed a drop in the substrate affinity with decreasing temperature (Lawrence and McCarty, 1969; Lin *et al.*, 1987). Although, for pure cultures of the mesophilic methanogen *Methanosarcina barkeri* a compensation for temperature inactivation was found as a result of an improved substrate affinity (Westermann *et al.*, 1989). In addition, Clarens and Moletta (1990) found a drop in the threshold acetate concentration when the incubation temperature of a thermophilic *Methanosarcina* culture decreased from 55°C to 30°C. An increase in the substrate affinity with decreasing temperature was recently also found for nitrifying bacteria by Wijffels *et al.* (1994). They observed that the effect was enhanced considerably upon immobilizing the cells in  $\kappa$ -carrageenan.

Remarkably, our results don't show a clear trend with respect to the effects of temperature on the acetate affinity for the crushed granules (Table 4.4). The occurrence of 2 ' $K_m$  optima' might be explained by a possible participation of different types of microorganisms in the degradation of acetate. Acetate is converted either through a direct splitting of the acetate molecule by acetoclastic methanogens or by a two-step reaction where acetate is first oxidized to  $H_2/CO_2$  by acetogens, followed by a reduction of  $CO_2$  to  $CH_4$  by hydrogenotrophic methanogens; see also § 1.4. According to Petersen and Ahring (1991) acetate oxidation might become significant at low substrate concentrations under thermophilic conditions. Both conditions are met in sludge granules from thermophilic high-rate reactors. Moreover, it was demonstrated recently, that a large fraction of the anaerobic biomass in thermophilic sludge granules consists of such syntrophic acetate oxidizing consortia (Ahring *et al.*, 1993; Uemura and Harada, 1993). The participation of various trophic groups in the degradation of a single substrate hampers an unambiguous mathematical interpretation. Also with respect to butyrate

it is not clear whether the presence of small sludge aggregates and/or the presence of various butyrate-degrading subpopulations (see also Chapter 4.1) have influenced the temperature dependence of butyrate conversion (Fig. 4.7). Nevertheless, a distinct increase of the butyrate conversion rate upon crushing the granule structure was found. In contrast to our results, Schmidt and Ahring (1993) found a decrease in the butyrate utilization rate of about 20% after disrupting the granule structure of thermophilic sludge. In the latter study the granules were disintegrated with a tissue homogenizer. The observed decrease in activity was attributed to the disruption of syntrophic butyrate-degrading consortia (Schmidt and Ahring, 1993). It should be noted that in our study the crushed granules still consisted of small aggregates. Probably, the syntrophic butyrate-degrading associations were much less affected by the disintegration method we used. Also, the butyrate-degrading activity of intact TGS-2 was 2 to 3 times higher than the activity of the sludge used in the study of Schmidt and Ahring (1993). A very steep gradient of the substrate concentration along the granule depth is to be expected in granules with a high specific activity. Apparently, crushing the granule structure brings about a dual effect. On one hand it decreases the specific butyrate-degrading activity due to disruption of the syntrophic associations. On the other hand, it may lead to a higher sludge activity due to an enhanced mass transfer rate. Obviously, for the TGS-2 granules the latter was of more importance. Crushing the granule structure negatively affected butyrate conversion at low temperatures (35-40°C), since the lag-phase period preceding the period of maximum activity is significantly longer (Table 4.5). The reason for this prolonged lag-phase is not yet clear. However, assuming a doubling time of about 2 days for mesophilic butyrate oxidizers (McInerney *et al.*, 1981), it might indicate growth of different butyrate degraders at these temperatures. Therefore, the calculated activities at 35 and 40°C shown in Fig. 4.7 may present an over-estimation.

In contrast to acetate and butyrate degradation, a relatively high temperature sensitivity was found for the degradation of propionate (Fig. 4.8). Likely, substrate diffusion limitation is much less pronounced for propionate oxidation. This might be attributed to the rather low specific propionate-degrading activity of TGS-2, which may result in a complete penetration of propionate into the granules. Apparently, the conditions prevailing in the reactor where TGS-2 was grown (Chapter 6) were not optimal for the cultivation of a dense propionate-oxidizing consortium. Similar to butyrate, an effective degradation of propionate requires also a tight and balanced association of acetogenic and methanogenic bacteria (Mucha *et al.*, 1988; Schmidt and Ahring, 1993; Stams *et al.*, 1992). For thermodynamic reasons the need for such an association is even higher for propionate oxidation than for butyrate conversion. Probably due to the relatively low specific propionate-degrading activity and the necessity of a tight syntrophic consortium, the activity of the sludge decreased significantly upon crushing the granule structure.

The formation of thick granular sludge as well as low effluent substrate concentrations are two factors expected to enhance mass transfer limitation. This subsequently results in a lower specific sludge activity while on the other hand it improves the thermostability of the overall

## 5 Effects of Acetate, Propionate and Butyrate on the Thermophilic Anaerobic Degradation of Propionate by Methanogenic Sludge and Defined Cultures

### Abstract

The effects of acetate, propionate, and butyrate on the anaerobic thermophilic conversion of propionate by methanogenic sludge and by enriched propionate-oxidizing bacteria in syntrophy with *Methanobacterium thermoautotrophicum*  $\Delta H$  were studied. The methanogenic sludge was cultivated in a UASB reactor fed with 3.9 g COD. $l^{-1}$  propionate ( $\approx 35$  mM) as the sole substrate for a period of 80 days. Propionate degradation was shown to be severely inhibited by addition of 3.2 g acetate-COD. $l^{-1}$  ( $\approx 50$  mM) to the influent of the UASB reactor. The inhibitory effect remained even when the acetate concentration in the effluent was below the level of detection. Recovery of propionate oxidation occurred only when acetate was omitted from the influent medium.

Propionate degradation by the methanogenic sludge in the UASB reactor was not affected by the addition of an equimolar concentration (35 mM  $\approx 5.6$  g COD. $l^{-1}$ ) of butyrate to the influent. However, butyrate had a strong inhibitory effect on the growth of the propionate-oxidizing enrichment culture. In that case, the conversion of propionate was almost completely inhibited at a butyrate concentration of 1.6 g COD. $l^{-1}$  (10 mM). By addition of a butyrate-oxidizing enrichment culture the inhibitory effect was abolished, and propionate oxidation was even stimulated. All experiments were conducted at pH 7.0-7.7. The thermophilic syntrophic culture showed a similar sensitivity to acetate and propionate as mesophilic cultures described in the literature. Additions of butyrate or acetate to the propionate medium had no effect on the hydrogen partial pressure in the biogas of a UASB reactor, nor was the hydrogen partial pressure in propionate-degrading cultures affected by the two acids. Our results indicate that the level of hydrogen in the biogas of thermophilic anaerobic reactors is not the appropriate control parameter for propionate degradation. High concentrations of NaCl, sodium acetate, or sodium propionate (100 mM) did not affect the growth of *M. thermoautotrophicum*  $\Delta H$  on hydrogen.

### 5.1 Introduction

Acetate, propionate, and butyrate are intermediate products in the anaerobic bioconversion of organic matter to methane and carbon dioxide (Boone, 1982; McInerney, 1988; Zehnder, 1978). Under balanced methanogenic conditions propionate and butyrate are further oxidized to acetate, hydrogen and bicarbonate, the main precursors of methanogenesis (Table 5.1). For

thermodynamic reasons propionate and butyrate can be degraded only when acetate and especially hydrogen are effectively eliminated by the methanogens (Ahning and Westermann, 1987, 1988; Boone and Bryant, 1980; Boone and Xun, 1987; Henson and Smith, 1985; McInerney *et al.*, 1979; Mucha *et al.*, 1988; Stams *et al.*, 1992). At elevated temperatures the thermodynamics for the acetogenic conversions are somewhat more favourable (Table 5.1). Nevertheless, propionate often is the first and main VFA which accumulates during thermophilic anaerobic treatment of waste and wastewater, as already mentioned in Chapter 3.1 (Lin *et al.*, 1986; Wiegant *et al.*, 1986). According to Wiegant *et al.* (1985), the contribution of propionate to the total amount of VFA in the effluent of UASB reactors is much higher under thermophilic conditions than under mesophilic conditions. The reason for the high sensitivity of propionate oxidation in thermophilic methanogenic systems is not clear yet.

**Table 5.1** Acetogenic and methanogenic reactions involved in propionate oxidation and the Gibbs free-energy changes<sup>a</sup>

Reaction	$\Delta G^{\circ}$ (kJ.mol <sup>-1</sup> ) at 25°C	$\Delta G^{\circ}$ (kJ.mol <sup>-1</sup> ) at 55°C
$\text{HCO}_3^- + 4\text{H}_2 + \text{H}^+ \rightarrow \text{CH}_4 + 3\text{H}_2\text{O}$	-135.6	-122.5
Acetate <sup>-</sup> + H <sub>2</sub> O → HCO <sub>3</sub> <sup>-</sup> + CH <sub>4</sub>	-31.0	-34.7
Propionate <sup>-</sup> + 3H <sub>2</sub> O → HCO <sub>3</sub> <sup>-</sup> + Acetate <sup>-</sup> + H <sup>+</sup> + 3H <sub>2</sub>	+76.1	+62.3
Butyrate <sup>-</sup> + 2H <sub>2</sub> O → 2 Acetate <sup>-</sup> + H <sup>+</sup> + 2H <sub>2</sub>	+48.1	+37.9

<sup>a</sup> Energy changes were calculated by using the van 't Hoff equation, standard enthalpy values of compounds (Chang, 1977), and Gibbs free energy changes at 25°C (Thauer *et al.*, 1977).

Recently we have described the enrichment of thermophilic, spore-forming, propionate-oxidizing bacteria in syntrophy with *Methanobacterium thermoautotrophicum*  $\Delta\text{H}$  or *Methanobacterium thermoformicum* Z245 (Stams *et al.*, 1992). Growth of the propionate-oxidizing bacteria was possible only after inoculation into a hydrogen-pregrown culture of *M. thermoautotrophicum*  $\Delta\text{H}$  cells which were embedded in FeS precipitates. In that study, results were obtained which show that the decay rate of *M. thermoautotrophicum*  $\Delta\text{H}$  under unfed conditions is extremely high. Therefore, starvation of hydrogenotrophic methanogens might play an important role in the sensitivity of propionate oxidation in thermophilic methanogenic ecosystems. Growth of the propionate-degrading enrichment was stimulated additionally by the presence of acetoclastic methanogens. Studies on propionate conversion under mesophilic conditions have demonstrated that the anaerobic oxidation of propionate is influenced by both the acetate and propionate concentrations (Fukuzaki *et al.*, 1990; Gorris *et al.*, 1989; Lin *et al.*, 1986; Mawson *et al.*, 1991; Nanba *et al.*, 1983). Furthermore, deterioration of propionate degradation is accompanied by increased concentrations of acetate

both in the mesophilic range and the thermophilic range (Chapter 3.1; Lin *et al.*, 1986; Wiegant *et al.*, 1985). In this chapter we describe the effects of acetate, propionate and butyrate on the thermophilic oxidation of propionate in continuous-flow methanogenic sludge bed systems and in defined propionate-oxidizing cultures.

## 5.2 Materials and Methods

### Inoculum

The UASB reactor was seeded with Aviko-MGS (§ 2.2) in the amount of 22.6 g VSS. $l^{-1}$  of reactor volume, which corresponds to a total volume of 2 litres of wet sludge.

### Media

The UASB reactor was fed with a concentrated stock solution which contained (g. $l^{-1}$ ): sodium propionate, 67.5;  $NH_4Cl$ , 3.7;  $MgSO_4 \cdot 7H_2O$ , 1.5;  $NaH_2PO_4 \cdot 2H_2O$ , 13.8;  $K_2HPO_4$ , 10.6,  $CaCl_2 \cdot 2H_2O$ , 0.2; yeast extract, 0.3. The pH of the feed stock was 6.5 which resulted in a reactor pH of 7.0-7.7. To each 1 litre of stock solution 13.3 ml of a trace element solution (§ 2.3) was added. Before it was fed to the UASB reactor, the stock solution was diluted 20 times with hot tap water. Sodium acetate and sodium butyrate were added during short time intervals in the ratio described below. Defined culture studies were performed by using the same medium and culture conditions as described previously (Stams *et al.*, 1992).

### Conversion to COD equivalents

In this chapter all VFA concentrations and ratios are expressed in mol. $l^{-1}$ . The chemical oxygen demand of 1 mol. $l^{-1}$  acetate, propionate and butyrate equals 64, 112, and 160 g  $O_2$ . $l^{-1}$ , respectively.

### UASB Reactor

The continuous flow experiments were performed at 55°C in 5.75 l UASB reactors (§ 2.1). The reactor was operated for more than 80 days with propionate as the sole carbon source with a hydraulic retention time of 8 h. Day 70 was defined as zero time for the butyrate inhibition experiment. Before the experiment was started, the propionate load was raised to a level of 100 mmol propionate. $l^{-1}$  reactor volume. $day^{-1}$ , corresponding to 11.2 kg COD. $m^{-3}$  reactor volume. $day^{-1}$ . After 10 days of acclimatization butyrate was added as the second carbon source in the influent at a molar ratio of propionate to butyrate of 1:0.4; the propionate concentration was kept at 35 mM. Two weeks after the first addition, the supply of butyrate was increased to a molar ratio of propionate to butyrate of 1:1 for 8 days. Thereafter, butyrate was left out of the medium and the reactor was operated for two months with propionate as the sole substrate. The end of this period was defined as zero time for the acetate inhibition experiment. Acetate was added to the influent for a period of 2 weeks at

a molar ratio of propionate to acetate of 1:1.7 at a propionate concentration of 27 mM. This procedure was repeated after a 3-week period during which the system was fed only propionate as the carbon source. Then, the supply of acetate was increased to a molar ratio of propionate to acetate of 1:2.6 for about 1 week; then the reactor was operated again with propionate as the sole carbon source for 15 days.

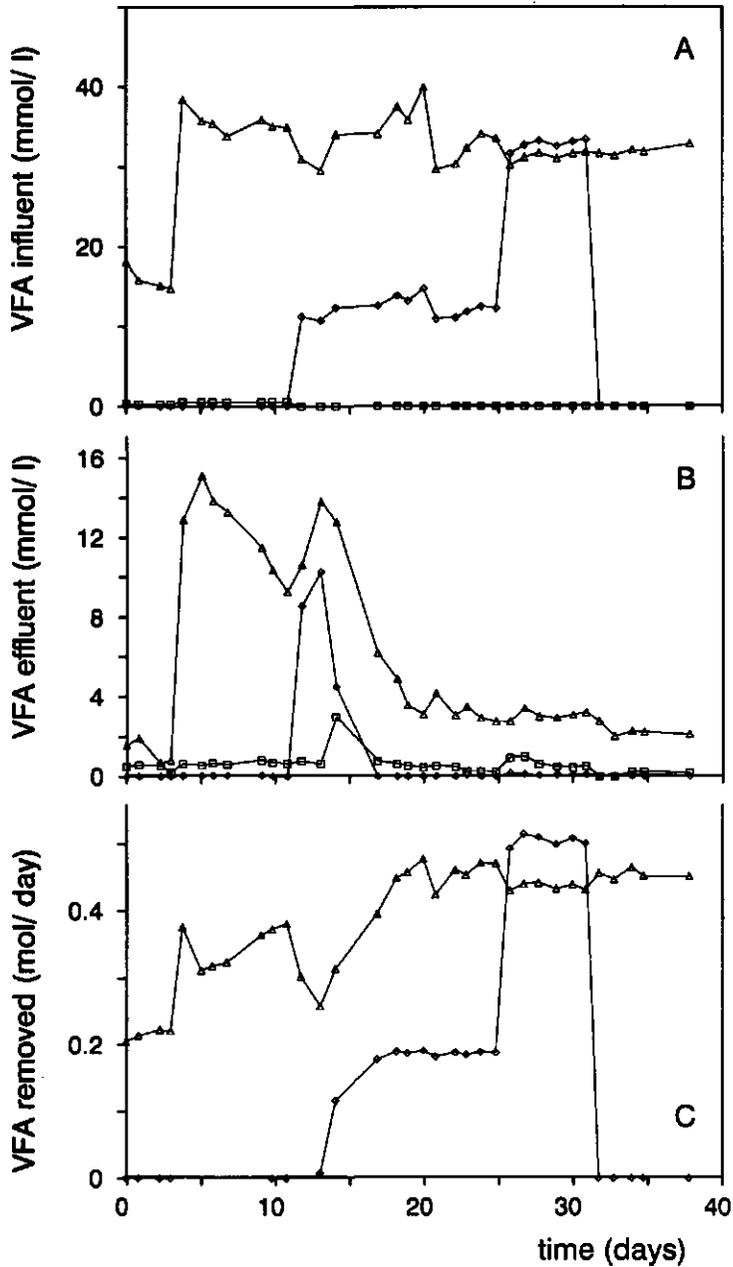
### Defined cultures

In a previous paper we described the enrichment of thermophilic, spore-forming, propionate-oxidizing bacteria from thermophilic (55°C) methanogenic sludge, by repeated transfer in hydrogen-pregrown cultures of *M. thermoautotrophicum*  $\Delta$ H (Stams *et al.*, 1992). This culture did not contain acetoclastic methanogens. In the present study the highly enriched propionate-oxidizing culture was used to assess the effects of VFAs on the thermophilic propionate conversion. In all experiments the propionate-oxidizing culture was inoculated into hydrogen-pregrown cultures of *M. thermoautotrophicum*  $\Delta$ H by using the same medium and culture conditions described previously (Stams *et al.*, 1992). To investigate the effect of acetate, propionate, or butyrate on growth of the hydrogenotrophic methanogens, *M. thermoautotrophicum*  $\Delta$ H was pregrown either in the presence or in the absence of the fatty acid salt. In the latter experiment the fatty acid salt was added after growth of *M. thermoautotrophicum*  $\Delta$ H on  $H_2/CO_2$ . In both experiments the final step was inoculation of 10% of the dense propionate-oxidizing culture into the media. This was defined as zero time for the experiment. However, no differences were found between the two different experiments. Therefore, the results of these experiments are presented as duplicate results (see Fig. 5.3 and 5.4).

In one experiment we investigated the effect of butyrate-oxidizing bacteria on propionate conversion. Butyrate-oxidizing acetogens were obtained by enriching thermophilic butyrate-degrading bacteria by repeated transfer (10%) in hydrogen-pregrown cultures of *M. thermoautotrophicum*  $\Delta$ H with 20 mM butyrate as the sole carbon source. *M. thermoautotrophicum*  $\Delta$ H appeared to be the most abundant or even the only syntrophic partner of the butyrate oxidizers. Inocula of 2% of the dense propionate-oxidizing culture and 1% of the butyrate-oxidizing culture were added together with  $FeCl_2$  (final concentration, 1 mM) to the hydrogen-pregrown culture of *M. thermoautotrophicum*  $\Delta$ H. Prior to inoculation the gas phase was changed to 172 kPa of  $N_2-CO_2$  (80:20; vol/vol). Propionate (20 mM) and butyrate (0 to 50 mM) were added to the bottles. All incubations were carried out without shaking at 55°C.

### Analysis

The VFA, Hydrogen and gas composition were determined by gas chromatography (§ 2.5). Regarding the defined culture experiments, determination of the gas composition as well as VFA analysis were done as described by Stams *et al.* (1992).



**Fig. 5.1** Effect of butyrate additions to the influent of a UASB reactor which was started up with propionate as the sole substrate. The figure shows acetate ( $\square$ ), propionate ( $\Delta$ ), and butyrate ( $\diamond$ ) concentrations in **A**) the influent and **B**) the effluent of the reactor as well as **C**) the absolute amount of removed butyrate and propionate in  $\text{mol}\cdot\text{day}^{-1}$ .

### Kinetic analysis

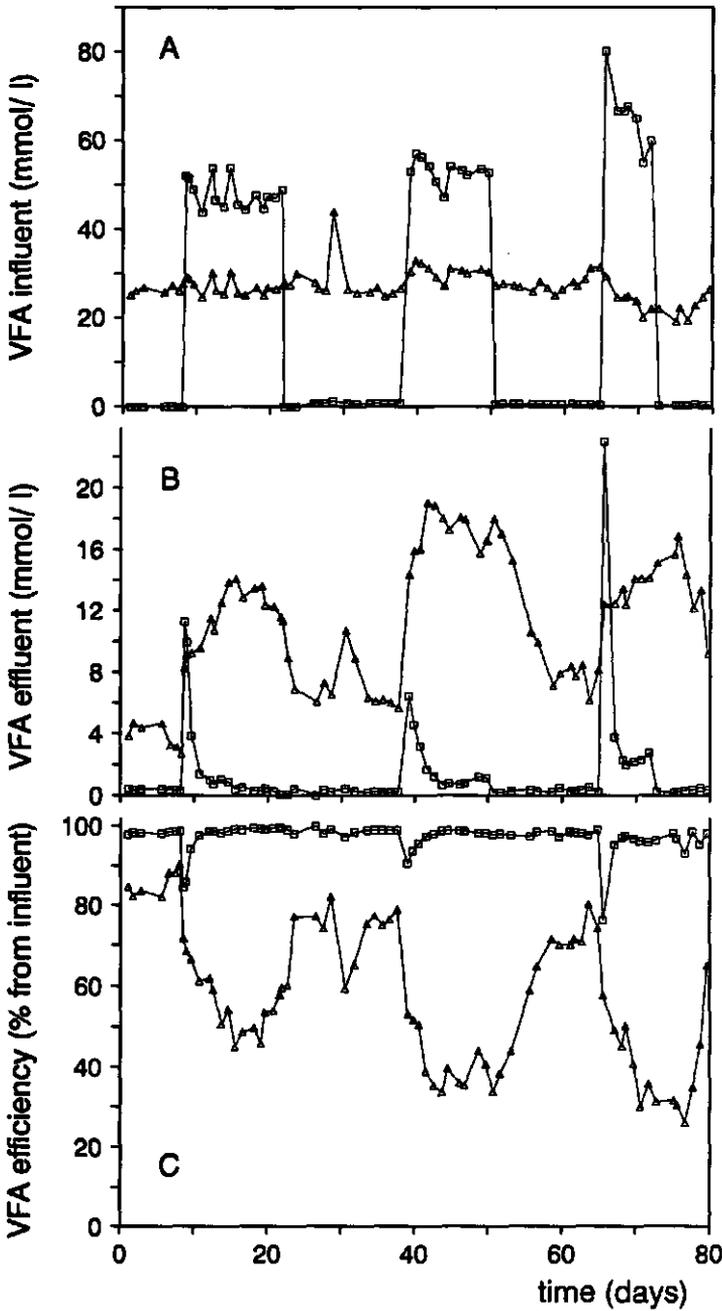
Kinetic constants were estimated by using the 'Nelder' nonlinear regression routine (Nelder and Mead, 1965) for parameter estimation. Data were fitted to a noncompetitive product inhibition model as described by other workers (Fukuzaki *et al.* 1990; Nanba *et al.*, 1983).

## 5.3 Results

### Effects of VFAs on propionate conversion by thermophilic methanogenic sludge in a UASB reactor

The effects of the butyrate addition on propionate conversion are shown in Fig. 5.1. Propionate degradation was affected by butyrate only during the first 3 days of exposure. During this period a very fast increase in the butyrate-degrading activity by the methanogenic consortium was measured. After 3 days, butyrate removal was almost complete, and no inhibitory effects of this acid were observed. Even after the butyrate concentration was raised to 35 mM, the propionate conversion rate remained unaffected. The hydrogen partial pressure in the gas phase of the reactor remained at 20 Pa throughout the whole experimental period, except during the first day of butyrate addition (day 13) when the hydrogen partial pressure temporarily increased to 1,000 Pa; it dropped to its initial level of 20 Pa the next day. At day 34 of the inhibition experiment, butyrate was omitted from the medium and the reactor was operated again with propionate as the sole substrate. In this period substrate inhibition was observed after a sudden increase in the propionate concentration from 32 to 52.5 mM (data not shown). This resulted in a decrease in the propionate removal rate of 20% and a concomitant decrease in the effluent pH from 7.7 to 7.3. According to the results obtained in the defined-culture studies (Table 5.2), this slight drop in pH should not be detrimental for the propionate conversion. However, an influent pH of 6.5 for the feed stock solution resulted in an undissociated propionic acid concentration of 1.38 mM at the inlet point of the reactor. Previous studies on mesophilic propionate degradation revealed a considerable inhibition at this concentration (Fukuzaki *et al.*, 1990). Stable reactor performance was obtained after the propionate concentration in the influent was decreased to 27 mM.

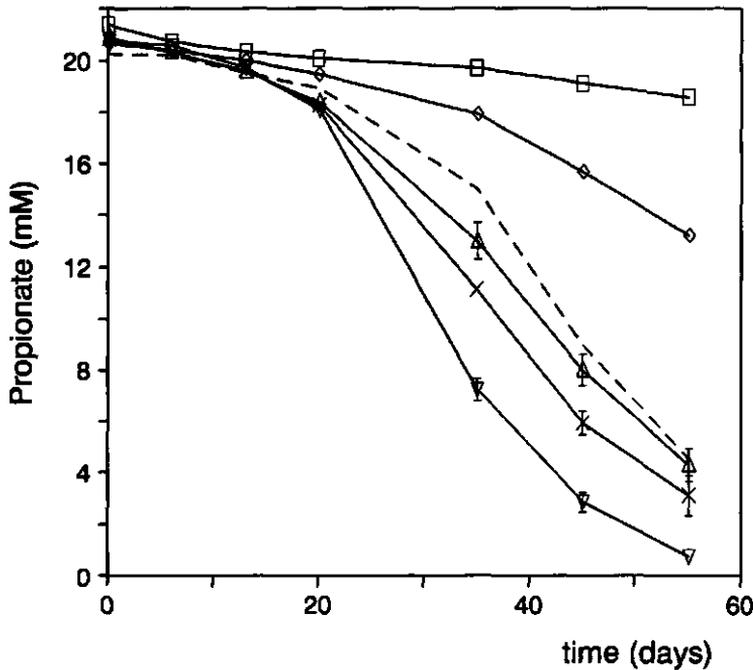
One week after zero time of the acetate inhibition experiment, acetate was added to the propionate medium at a molar ratio of propionate to acetate of 1:1.7 for a period of 2 weeks (Fig. 5.2). This addition was repeated during days 38 to 51 and then during days 65 to 73. In the latter period a higher concentration of acetate was used. During all of the acetate exposures the propionate concentration remained at about 27 mM in the influent. Fig. 5.2 shows the effect of acetate addition on propionate conversion by the methanogenic sludge bed. The propionate peak at day 29 (Fig. 5.2a) was caused by a disturbance in the supply of substrate. In contrast to the butyrate additions, acetate apparently initiated an increase in the propionate concentration in the effluent of the UASB reactor, which persisted until the



**Fig. 5.2** Effect of acetate additions to the influent of a UASB reactor degrading propionate as the sole substrate. The figure shows acetate ( $\square$ ) and propionate ( $\Delta$ ) concentrations in A) the influent and B) the effluent of the reactor as well as C) the relative amount of removed acetate and propionate, expressed as the % of the influent amount.

supply of acetate was eliminated. This inhibitory effect of acetate remained, and even increased, despite the very low acetate concentrations in the effluent. The propionate conversion capacity dropped with 40-50% during the acetate additions (Fig. 5.2c). Because of the great differences between the absolute amounts of acetate and propionate which were removed per day, we expressed the amounts of daily acetate and propionate removal in Fig. 5.2c as percentages of the influent amount.

**Effects of VFAs on propionate degradation by a defined propionate-degrading culture.** The inhibition effect of lower fatty acids on the growth of thermophilic propionate oxidizers in syntrophy with *M. thermoautotrophicum*  $\Delta H$  was investigated by using a highly enriched propionate-oxidizing culture which was unable to degrade acetate (Stams *et al.*, 1992).



**Fig. 5.3** Propionate degradation by the thermophilic propionate-oxidizing enrichment culture at various acetate concentrations, 0 mM (▽), 5 mM (x), 20 mM (Δ), 50 mM (◇), and 100 mM (□). Cultures were inoculated (10%) in cultures of *M. thermoautotrophicum*  $\Delta H$  pregrown on hydrogen. As a reference the effect of 100 mM NaCl (----) is also plotted.

**Table 5.2** Growth rate of propionate oxidizers at different initial concentrations of propionate. Rates were calculated from the exponential increase of acetate during the first 25 days of incubation

Total Propionate (mM)	Undissociated <sup>a</sup> Propionic acid (mM)	$\mu$ (day <sup>-1</sup> ) <sup>b</sup>	$\mu$ (day <sup>-1</sup> ) <sup>c</sup>
5	0.04	0.053	0.054
10	0.08	0.092	0.103
25	0.21	0.085	0.096
50	0.42	0.098	0.071
100	0.84	0.084	0.072

<sup>a</sup> Calculated by  $pK_a = 4.92$ ; 55°C (Sillen and Martell, 1964), pH 7

<sup>b,c</sup> Propionate oxidizers were inoculated in cultures of *M. thermoautotrophicum*  $\Delta H$  pregrown on hydrogen, either in the presence<sup>b</sup> or absence<sup>c</sup> of propionate

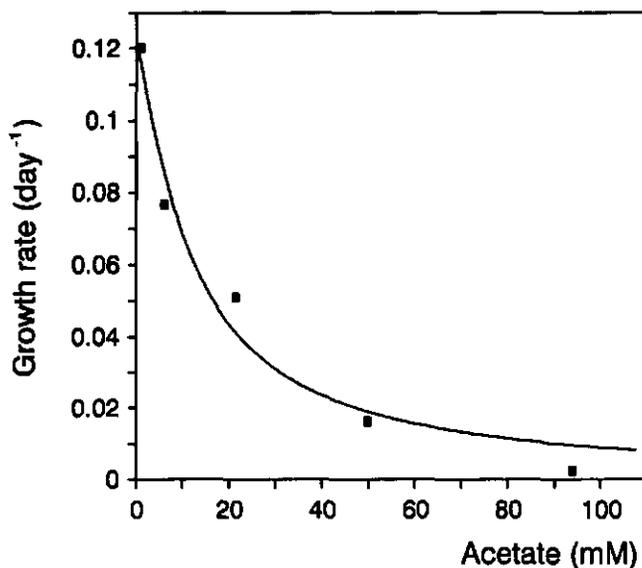
The addition of inorganic salts or fatty acid salts had only little effect on *M. thermoautotrophicum*  $\Delta H$  itself. Comparable growth rates of *M. thermoautotrophicum*  $\Delta H$  were measured with hydrogen in media to which 0, 50 or 100 mM KCl, NaCl, potassium acetate, sodium acetate or sodium propionate was added (data not shown). Furthermore, no differences in the rates of propionate conversion were found irrespective of whether the methanogens were pregrown in the presence or absence of the fatty acid salts (Fig. 5.3 and 5.5). Therefore, we attribute the effects of fatty acids on the propionate-oxidizing enrichment culture to inhibition of the acetogens.

As depicted in Fig. 5.3, propionate oxidation was severely inhibited at acetate concentrations higher than 20 mM. This inhibition effect can be attributed to acetate, as 100 mM NaCl had a less severe inhibitory effect (Fig. 5.3). Inhibition of propionate oxidation by acetate can be described by using the following noncompetitive inhibition equation used by other workers (Fukuzaki *et al.* 1990; Nanba *et al.*, 1983):

$$\mu = \frac{\mu_{\max}}{1 + (Ac/K_{i,n})^n} \quad (5.1)$$

where  $\mu$  is the specific growth rate of the acetogens;  $\mu_{\max}$  is the maximum specific growth rate; Ac is the acetate concentration;  $K_{i,n}$  is the noncompetitive inhibition constant; and  $n$  is the exponent of inhibition. The kinetic constant  $\mu_{\max}$  and  $\mu$  were calculated from the exponential decrease in the propionate concentration. Fig. 5.4 shows a plot of the growth rate of the propionate-oxidizing coculture versus acetate concentration. The solid line was computed statistically by using the commonly used nonlinear absolute least-square criterion as the error function. Computed values for  $K_{i,n}$  and  $n$  are 12.4 mM acetate and 1.20, respectively. The  $K_{i,n}$  value equals 78  $\mu M$  of undissociated acetic acid, as calculated by using

a pKa of 4.80 at 55°C (Sillen and Martell, 1964). Because of the heterogeneity of our data, application of the alternative relative least-square criterion is more appropriate (Sáez and Rittman, 1992). When the latter error function was used, the computed constants were somewhat higher: 18.4 mM acetate (116  $\mu$ M undissociated acetic acid) for  $K_{i,n}$  and 2.27 for  $n$ .

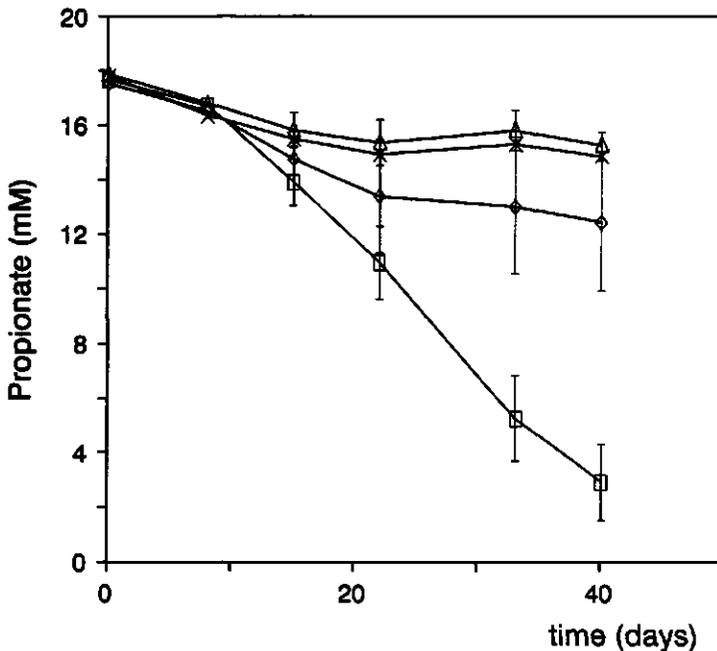


**Fig. 5.4** Growth inhibition of the propionate-oxidizing enrichment culture by acetate. Cultures were fed with 20 mM propionate at pH  $7.0 \pm 0.1$ . Growth rates were calculated from the propionate conversion rates after a lag phase of 20 days.

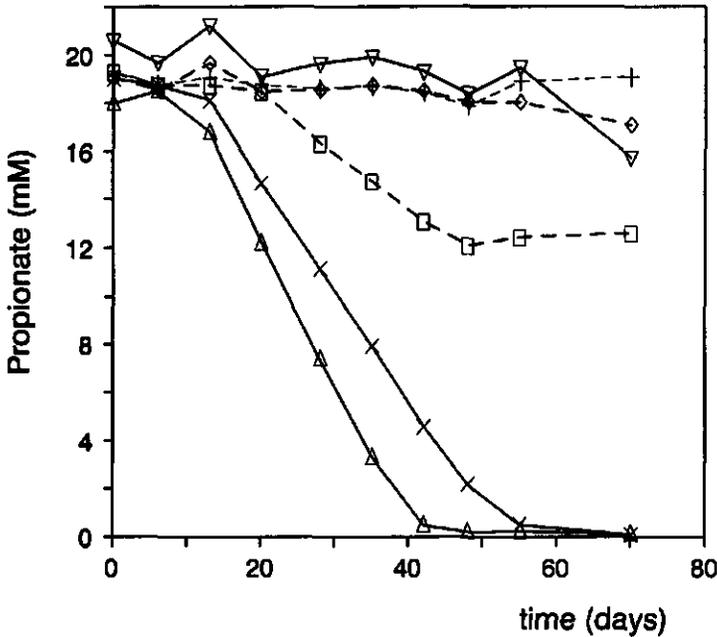
The effect of propionate was studied by adding propionate concentrations up to 100 mM to the medium. Table 5.2 summarizes the calculated growth rates of the propionate oxidizers at the various initial concentrations of total propionate, as well as undissociated propionic acid. Surprisingly, propionate conversion rates were in the same range, irrespective of the propionate concentration. The growth rates were calculated by using the exponential increase in the acetate concentration during the first 3 to 4 weeks of incubation. Thereafter, inhibition by acetate may have influenced the propionate degradation rate, especially at high initial propionate concentrations.

Fig. 5.5 shows that propionate conversion is very severely inhibited by butyrate. Although the number of useful data was limited, computer analyses in which the equation given above was used were carried out to estimate the following butyrate inhibition constants: 1.7 mM butyrate (13  $\mu$ M undissociated butyric acid, calculated by using a pKa of 4.90 at 55°C (Sillen and Martell, 1964)) for  $K_{i,n}$  and 2.65 for  $n$ . Addition of butyrate oxidizers to the culture

abolishes the inhibition effect caused by butyrate itself. Instead, the acetate and hydrogen formed from butyrate influence propionate conversion. In order to assess the effect of the butyrate oxidizers, a *M. thermoautotrophicum*  $\Delta H$  culture pregrown on hydrogen in the presence of 20 mM propionate was inoculated with the propionate-oxidizing enrichment culture (2% of a stock culture) and supplied with butyrate (0, 10, and 50 mM). Some of the bottles were also inoculated with a butyrate-oxidizing enrichment culture. Fig. 5.6. shows a plot of propionate concentration versus incubation time at the various butyrate concentrations with and without the butyrate-oxidizing acetogens. The culture with only propionate oxidizers was unable to degrade propionate completely because of the small inoculum used (2%), in contrast to the other experiments in which inocula were larger (10%). This phenomenon is due to the high starvation rate of *M. thermoautotrophicum*  $\Delta H$  (Stams *et al.*, 1992). As expected, no degradation occurred after addition of 10 mM butyrate to the medium. However, when the butyrate-oxidizing acetogens were also supplied to that medium, propionate was completely converted. Moreover, even a stimulatory effect by the butyrate-oxidizing enrichment was observed when no butyrate was added to the medium.



**Fig. 5.5** Propionate degradation by the thermophilic propionate-oxidizing enrichment culture at various butyrate concentrations, 0 mM (□), 5 mM (◇), 10 mM (△), and 20 mM (×). Cultures were inoculated (10%) in cultures of *M. thermoautotrophicum*  $\Delta H$  pregrown on hydrogen.



**Fig. 5.6** Effect of a butyrate-oxidizing enrichment culture on the thermophilic oxidation of propionate at various butyrate concentrations, 0 mM (□, Δ), 10 mM (+, x), and 50 mM (◇, ▽). Propionate-oxidizing cultures were inoculated (2%) in hydrogen-pregrown cultures of *M. thermoautotrophicum* ΔH and supplied with propionate and butyrate (—), whereas others were also inoculated (1%) with a butyrate-oxidizing enrichment culture (---).

In the experiments in which 10 mM or 50 mM butyrate was added to medium, butyrate was completely converted within 1 week. Degradation of 50 mM butyrate resulted in a build-up of acetate to a concentration of almost 100 mM, which is too high to allow propionate conversion. In none of the bottles was acetate converted within the experimental period. The butyrate-oxidizing enrichment culture was also tested on its ability to degrade propionate. Hydrogen-pregrown cultures of *M. thermoautotrophicum* ΔH were inoculated with the butyrate-oxidizing acetogens at various butyrate concentrations with and without 20 mM propionate. In these tests, propionate was not degraded in any of the bottles, and butyrate degradation was not affected by a propionate concentration of 20 mM (data not shown).

## 5.4 Discussion

Thermophilic anaerobic oxidation of propionate is inhibited by VFAs in such a manner that the extent of inhibition is dependent on the concentration and on the medium pH. Deterioration of propionate degradation can be attributed to an inhibitory effect on the propionate-oxidizing acetogens and/or on the hydrogen-consuming methanogens. Growth of the methanogens on hydrogen was not affected by moderately high fatty acid concentrations. Therefore, we conclude that the observed effects can be attributed to inhibition of the propionate-oxidizing acetogens. Fukuzaki *et al.* (1990) demonstrated that the degree of inhibition was strongly dependent on the concentration of the undissociated form of the fatty acids. The neutral form of these acids diffuses more easily across the bacterial membrane, causing a drop in intracellular pH. The increased diffusivity might be further enhanced by the interference of the apolar chain with the bacterial membranes, which results in a higher permeability of these membranes. As in toxicity of alcohols, which is correlated with chain length (Dürre *et al.*, 1988), one might expect a higher degree of inhibition with longer-chain-length fatty acids. In our experiments growth of thermophilic propionate-oxidizing bacteria was most severely inhibited by butyrate. Strong inhibition even occurred at low concentrations. However, compared with acetate, high concentrations of propionate affected the growth of the thermophilic propionate-oxidizing acetogens only slightly (Table 2). This may have been the net result of inhibitory and stimulatory effects. Substrate inhibition probably is also caused by toxicity of the undissociated form of propionic acid (Fukuzaki *et al.* 1990; Nanba *et al.*, 1983) and its effect on the bacterial membranes. At high propionate concentrations the high amount of sodium may also contribute to inhibition. On the other hand, a high propionate concentration, and thus a low acetate/propionate ratio, lowers the Gibbs free energy changes for propionate conversion (Table 5.1). A very low ratio enhances the growth of the acetogenic bacteria. The effects of propionate on propionate oxidation rates were similar to those observed by Fukuzaki *et al.* (1990) with mesophilic propionate-acclimatized sludge. This indicates similar susceptibilities of mesophilic and thermophilic propionate-oxidizing consortia.

Addition of butyrate-oxidizing acetogens to the media eliminated the inhibitory effect of butyrate and even stimulated propionate conversion. Stimulation of the growth of the propionate degraders in the presence of the butyrate-oxidizing acetogens might be caused by excretion of essential growth factors by the butyrate oxidizers. However, it is more likely that the effect can be attributed to an increase in the number of hydrogenotrophic methanogens. The butyrate oxidizers were also enriched in hydrogen-pregrown cultures of *M. thermoautotrophicum*  $\Delta H$ , and therefore, methanogens were inoculated in small numbers together with the acetogens. Furthermore, growth of hydrogenotrophic methanogens also occurs as a result of butyrate degradation to acetate and hydrogen. These and previous results (Stams *et al.*, 1992) show that the number of viable hydrogenotrophic methanogens is very important for a rapid and complete conversion of propionate. In the UASB experiments

adverse effects of butyrate on the propionate conversion were observed only during the first 3 days after the first addition of butyrate to the substrate.

Growth of the propionate-oxidizing enrichment culture was affected by acetate to a considerable degree (Fig. 5.4). This was also the case, although to a lesser extent, for the methanogenic consortia in the UASB reactor. In the latter system exposure to high acetate concentrations is possible only in certain compartments of the sludge. Addition of acetate to the influent of the UASB reactor immediately resulted in a sharp increase of the propionate concentration in the effluent, which was accompanied by a high acetate concentration (Fig. 5.2b). The acetate conversion capacity of the sludge bed increased during the period when acetate was added to the influent. However, despite that, propionate degradation decreased further even when almost complete acetate removal was achieved by the methanogenic consortium. On the basis of the substrate conversion kinetics equation given above, we might expect a recovery of propionate degradation whenever the acetate concentration in the sludge bed drops. The reason that such an effect was not found is not clear. One explanation might be that the number of hydrogenotrophic methanogens in the sludge bed had decreased, resulting in a lower hydrogen removal efficiency. However, also in that case, recovery of propionate removal might be expected because of the high growth rate of the thermophilic methanogens (Balch *et al.*, 1979). Inhibition of propionate conversion by extremely low concentrations of acetate in a mesophilic methanogenic fluidized bed reactor was described by Gorris *et al.* (1989). However, it is not clear whether in that study a causal connection exists between the observed effects.

Hydrogen, an important intermediate of thermophilic propionate conversion, has two effects. For thermodynamic reasons a high level of hydrogen directly inhibits the oxidation of propionate to acetate and carbon dioxide (Table 5.1). On the other hand, a high concentration of hydrogen stimulates the growth of hydrogenotrophic methanogens. The latter are essential for the development of a stable and dense propionate-oxidizing subpopulation in which the acetogens are in close proximity to the methanogens. Results obtained here indicate that propionate oxidation is stimulated by other hydrogen-producing conversions of nontoxic intermediates (i.e., butyrate). In a previous study on thermophilic propionate conversion conducted at our department, it was proposed that a two-step digestion process should be applied whenever propionate is a major compound and hydrogen production is inevitable during the thermophilic treatment of wastewater (Wiegant *et al.*, 1986). In the second reactor of this two-stage system propionate would be removed. Indeed significantly better results were obtained. The overall better performance was attributed to a decreased hydrogen partial pressure in the second reactor, which for thermodynamic reasons stimulates propionate oxidation. Our present results show that in addition to a low hydrogen concentration, a low level of acetate seems to be indispensable for effective degradation of propionate. It should be noted that both conditions can be achieved easily in the above mentioned digestion process consisting of two or more steps. In this study we found a severe inhibition of propionate

oxidation without any increase of the hydrogen partial pressure in the biogas. Therefore, the suitability of hydrogen as an overall control parameter for anaerobic digestion (Whitmore *et al.*, 1987) has to be reconsidered, a conclusion which was recently also drawn by other workers (Kidby and Nedwell, 1991).

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## Chapter 6

### Prospects of compartmentalized reactor systems for high-rate thermophilic anaerobic wastewater treatment

The process stability of thermophilic anaerobic wastewater treatment is distinctly enhanced by using compartmentalized reactor systems. The segregated development of specific types of thermophilic sludge in the various compartments of the plug-flow reactor is discussed.

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- (6.1) Van Lier, J.B., F. Boersma, M.M.W.H. Debets, and G. Lettinga (1994). High Rate thermophilic anaerobic wastewater treatment in compartmentalized upflow reactors. *Wat. Sci. Technol.*, 30-12: 251-261.
- (6.2) Van Lier J.B., Groeneveld N., and Lettinga G. (1995). Development of thermophilic methanogenic sludge in compartmentalized upflow reactors. *Biotechnol. and Bioeng.*, submitted.

## 6.1 Start-up and Performance of Thermophilic Upflow Staged Sludge Bed (USSB) Reactors

### Abstract

Thermophilic anaerobic treatment of acidified and partially acidified wastewater was studied, by using upflow staged sludge bed (USSB) reactors. These upflow reactors consist of various compartments, each of which equipped with a gas-solid separator. This novel approach for thermophilic wastewater treatment led to a reduction, or even complete elimination, of major biological and physical limitations of conventional thermophilic high-rate processes. The main achievements of the plug-flow reactor were i) very low concentrations of volatile fatty acids (VFA) in the effluent; ii) a high degree of sludge retention; and iii) stable reactor performance. The start-up of the reactors was done with crushed mesophilic granular sludge and with digested organic fraction of municipal solid waste as inoculum. Mixtures of VFA and sucrose-VFA were used as feed. An excellent operation performance was achieved within 1 month, and sludge granulation of the thermophilic biomass was clearly visible after 1-1.5 months of operation with the sucrose-VFA feed. Within 2 to 3 months, the organic loading rate could be increased up to 100 g sucrose-VFA COD.l<sup>-1</sup>.day<sup>-1</sup> with COD removal efficiencies higher than 90%. The hydraulic retention time was about 2-2.5 h. The wash-out of thermophilic biomass remained low, despite the applied extreme biogas load of 40-50 l.l<sup>-1</sup> reactor.day<sup>-1</sup>. Sucrose was found to be an essential component for thermophilic sludge granulation because in the reactors fed with solely a VFA mixture, little if any new granules were formed. Nonetheless, also in latter reactors a satisfactory biomass hold up prevailed despite the rather dispersed nature of the sludge. The advantage of using compartmentalized reactors was clearly demonstrated under the extreme loading conditions. A typical characteristic sequence in the degradation of the partially acidified substrate was found. In the first compartment sucrose was converted, followed by the conversion of butyrate and acetate in the next compartments. As usual, propionate was the most difficult intermediate to degrade, but in the last compartments also this fatty acid was degraded almost completely.

### 6.1.1 Introduction

Thermophilic anaerobic digestion offers an attractive alternative for the treatment of medium and high strength wastewaters, and especially for those wastewaters which are discharged at high temperatures, such as wastewaters from many food processing industries (alcohol distillery, canning factory), and pulp and paper factories (Lettinga *et al.*, 1991); see also Table 1.5. At high temperatures reaction rates proceed much faster what, in principle, may

lead to much higher loading potentials of thermophilic reactors in comparison to mesophilic reactors (see also Chapter 1). So far, full scale applications of thermophilic treatment are mainly restricted to completely mixed reactors, with or without sludge recirculation. Results with these reactors show that indeed higher loading rates can be applied than with mesophilic mixed systems (see also § 1.5.1), but the overall performance is less satisfactory than the well defined high-rate reactors at the mesophilic temperature range. A drawback of thermophilic anaerobic processes is the high concentration of VFAs which are generally found in effluents of these reactors (see also § 1.1). Results from the previous chapter (Chapter 5) show that thermophilic anaerobic sludge is sensitive to an accumulation of intermediate products, such as hydrogen, acetate and propionate. This sensitivity of thermophilic biomass might be the reason why the fatty acid concentration of thermophilic reactors is still high. Lab-scale studies on the feasibility of thermophilic treatment in UASB reactors showed high effluent VFA concentrations which were accompanied with a severe sludge wash-out when (partially) unacidified wastewater was treated at high loading rates (Wiegant *et al.*, 1985). Both, high VFA concentrations and severe sludge wash-out, may be overcome by a well designed reactor concept. We investigated the start-up of compartmentalized thermophilic anaerobic upflow reactors for the treatment of acidified and partially acidified wastewater. Application of a plug-flow system, in which the various stages of the degradation process are separated, prevents accumulation of intermediates. In addition, various gas-solid separators were installed by which a better retention of viable biomass is to be expected under high loading conditions and low hydraulic retention times.

## 6.1.2 Material and Methods

### Reactor concept

A schematic representation of the Upflow Staged Sludge Bed (USSB) reactor and the experimental set-up is presented in Fig. 2.2 (§ 2.1). Four reactors, RI-RIV, were placed in a waterbath at a temperature of 55°C which was controlled by a thermostat (Haake D1-1, FRG). The reactors were fed with either a VFA mixture or with a sucrose-VFA mixture (Table 6.1). The adjustments in the medium composition of reactor RII and RIV were necessary, due to the higher cell yield of acidifying biomass and to prevent excessive pH drops when VFA accumulates in the first compartments of this reactor. The concentrated stock solutions were diluted 14-18 times with hot tap water (55°C) containing 30 mg Ca<sup>++</sup>.l<sup>-1</sup>, and then led into the reactor. For the reactors fed with the sucrose-VFA mixture (RII and RIV) the dilution factor was decreased to 8-12 during the course of start-up. No effluent recycle was applied. The concentrated sucrose-VFA solutions were stored at 4°C which prevented acidification of the feed stock. Peristaltic pumps (Watson Marlow 202 and 503, Falmouth, Cornwall, UK) were used.

### Seed material

Reactors RI and RII were inoculated with digested OFMSW (§ 2.2). Reactors RIII and RIV were seeded with partially crushed Aviko-MGS (§ 2.2). The MGS was crushed using a magnetic stirrer for an overnight period. Then, the sludge was distributed over 1 l serum bottles and further disrupted by passing the biomass through a 0.5 mm syringe needle. Approximately 3.5 l of the crushed granules, equivalent to about 200 g volatile suspended solids (VSS), was added to each USSB reactor, which sufficed to evenly fill all the compartments.

Table 6.1 Composition of the concentrated feedstock solutions.

Compound	Reactor:	RI <sup>a</sup> , RIII <sup>b</sup>	RIV <sup>a</sup> , RIV <sup>b</sup>
	Feed:	VFA-mixture	sucrose-VFA mixture
		(g.l <sup>-1</sup> )	(g.l <sup>-1</sup> )
acetic acid <sup>c</sup>		55	15
propionic acid <sup>c</sup>		13	10.6
butyric acid <sup>c</sup>		10.8	8.8
Sucrose		-	48
	total COD:	100	100
NH <sub>4</sub> Cl		2.0	5.6
MgSO <sub>4</sub> ·7H <sub>2</sub> O		2.8	2.8
NaH <sub>2</sub> PO <sub>4</sub> ·2H <sub>2</sub> O		1.7	1.7
K <sub>2</sub> HPO <sub>4</sub>		2.0	2.0
CaCl <sub>2</sub> ·2H <sub>2</sub> O		0.2	0.2
NaHCO <sub>3</sub>		19	40
yeast extract		0.3	0.3
trace elements	see § 2.3	7.0 (ml.l <sup>-1</sup> )	7.0 (ml.l <sup>-1</sup> )
pH		6.2-6.5	7.8-8.0

<sup>a</sup> inoculated with digested OFMSW

<sup>b</sup> inoculated with partially crushed Aviko-MGS

<sup>c</sup> neutralized with NaOH

### Analyses

Methods for VFA, VSS, COD, Ethanol, Methanol, Lactate, Formate, Biogas composition, and Hydrogen analyses are described in § 2.5.

### 6.1.3 Results

#### Start-up

Each reactor was daily analyzed for pH, COD, VFA, TSS, and VSS concentration in the effluent during the start-up period. Also, the biogas production was daily measured and its composition was weekly determined. Reactors were started at a HRT of about 35 h and an organic loading rate (OLR) of  $5 \text{ g COD} \cdot \text{l}^{-1} \cdot \text{day}^{-1}$ . Within a period of 2 to 3-months, the HRT was gradually reduced to approximately 2 h and the OLR was concomitantly increased up to  $100 \text{ g COD} \cdot \text{l}^{-1} \cdot \text{day}^{-1}$ . The OLR was only increased after the acetate concentration in the effluent had decreased to below  $100 \text{ mg COD} \cdot \text{l}^{-1}$ . This procedure was followed to stimulate propionate degradation which is strongly affected by the concentration of acetate (Chapter 5). A more or less exponential increase in the OLR could be imposed to the system at an almost unaffected high COD removal efficiency. During the first 1 to 2 months of the start-up, the treatment efficiency was limited due to a poor degradation of propionate. Thereafter, also propionate was sufficiently degraded. In all reactors the COD removal rate increased more or less linearly with the increase in the applied OLR (Fig. 6.1). However, loading potentials and process stability of the reactors treating the sucrose-VFA mixture were considerably higher than the reactors fed with the VFA substrate.

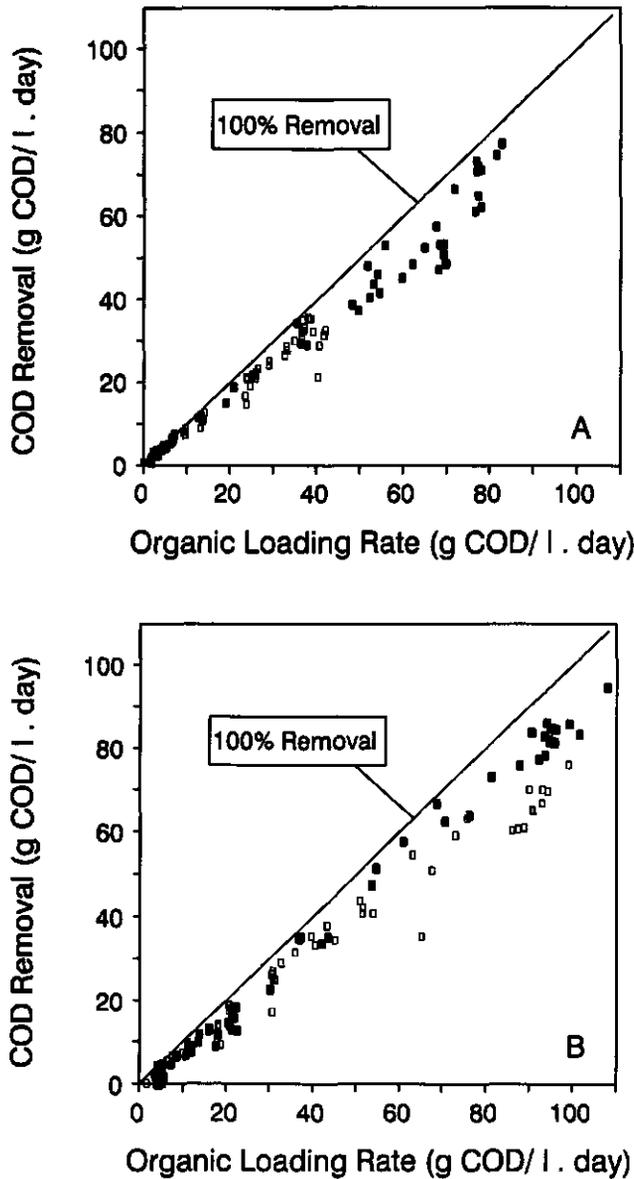
Because the liquid-gas interface in each compartment could be separately adjusted, there were only minor problems with respect to clogging of the gas outlet. Nevertheless, in the reactors treating the sucrose-VFA mixture under high loading conditions, foaming problems occurred in the lower compartments. These problems could be solved by adding an anti-foam agent (Mogul DX-236, Mijdrecht, The Netherlands), which doesn't affect the biological activity of the sludge. Clogging or deliberately closing of one of the lower or intermediate compartments directly resulted in a build-up of VFA in the effluent. Foaming problems resulted in an overloading and a retarded increase of the methane production rate in reactor RII between day 50-70 (Fig. 6.2a).

The reactors RIII and RIV were operated for about one year under moderately high to high loading conditions ( $50\text{-}80 \text{ g COD} \cdot \text{l}^{-1} \cdot \text{day}^{-1}$ ). In this period the performance of the reactors was remarkably stable, despite the variations in load and temperature which sometimes occurred as a result of disturbance in feed supply and temperature control.

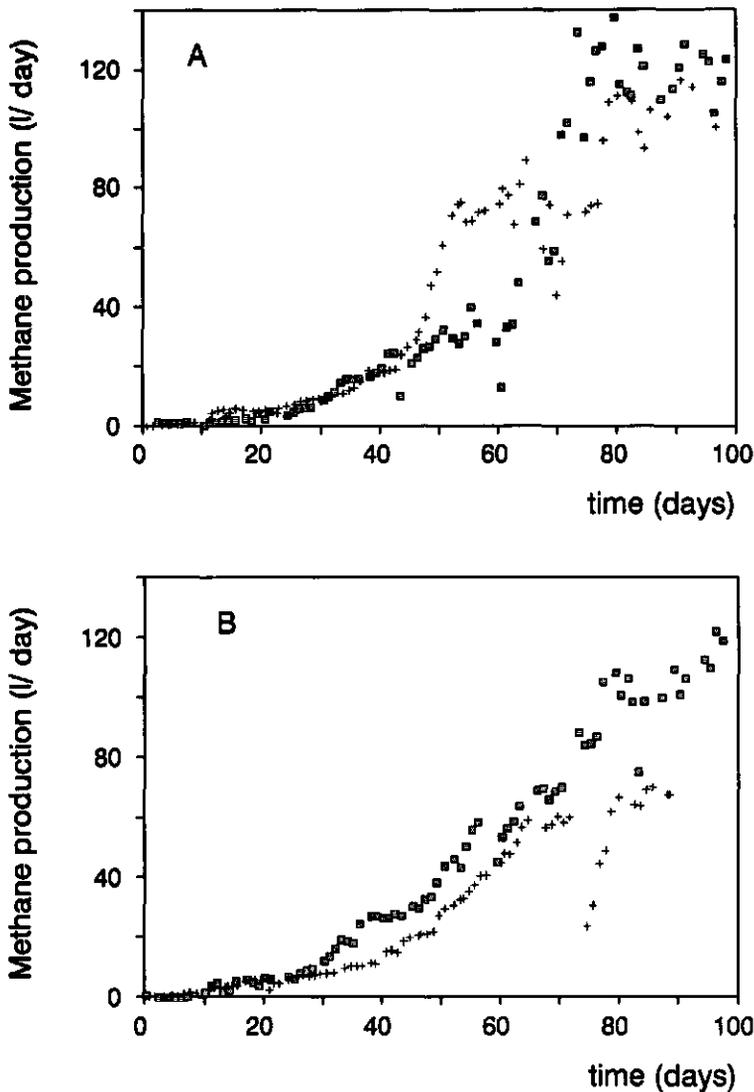
#### Influence of seed material and substrate composition

A faster start and a more rapid increase of thermophilic methanogenesis was observed in the reactor seeded with digested OFMSW and fed with the sucrose-VFA mixture (Fig. 6.2a). This finding is in accordance with our previous results showing that the initial thermophilic activity of digested OFMSW is higher than that of MGS (Van Lier *et al.* 1993). However, using the VFA mixture as feed, the increase in the methane production rate was slightly higher in the reactor inoculated with Aviko-MGS. In the case of VFA-feed the use of MGS

might be more profitable because of the initially tight adherence of the thermophilic organisms to the mesophilic granules (Chapter 3.2; Ohtsuki *et al.*, 1992). In the presence of unacidified substrate the latter property is apparently of minor importance.



**Fig. 6.1** COD removal rate versus applied organic loading rate of A) reactors RI and RII, inoculated with OFMSW, and B) reactors RIII and RIV, inoculated with MGS. Reactors were fed with VFA (□) or Sucrose-VFA (■).



**Fig. 6.2** Increase of the  $\text{CH}_4$  production rate in reactors fed with A) Sucrose-VFA, and B) solely VFA. Reactors were inoculated with OFMSW (+) or MGS ( $\square$ ).

An important difference between the reactors treating the sucrose-VFA mixture (RII and RIV) and the VFA mixture (RI and RIII), was the development of granular sludge, irrespective of the seed material used. A clear granulation of the biomass hardly proceeded in the reactors fed with VFA only. In contrast, in reactors RII and RIV methanogenic granules appeared within 1-1.5 months. Due to the occurrence of biomass granulation the

relative wash-out of viable sludge was less while higher loading rates could be imposed on the system. The difference in capacity to retain viable sludge in the reactor can be deduced from the increase in the methane production rate which was more or less exponential for reactor RII and RIV, whereas it was linear in the reactors treating the VFA mixture (Fig 6.2).

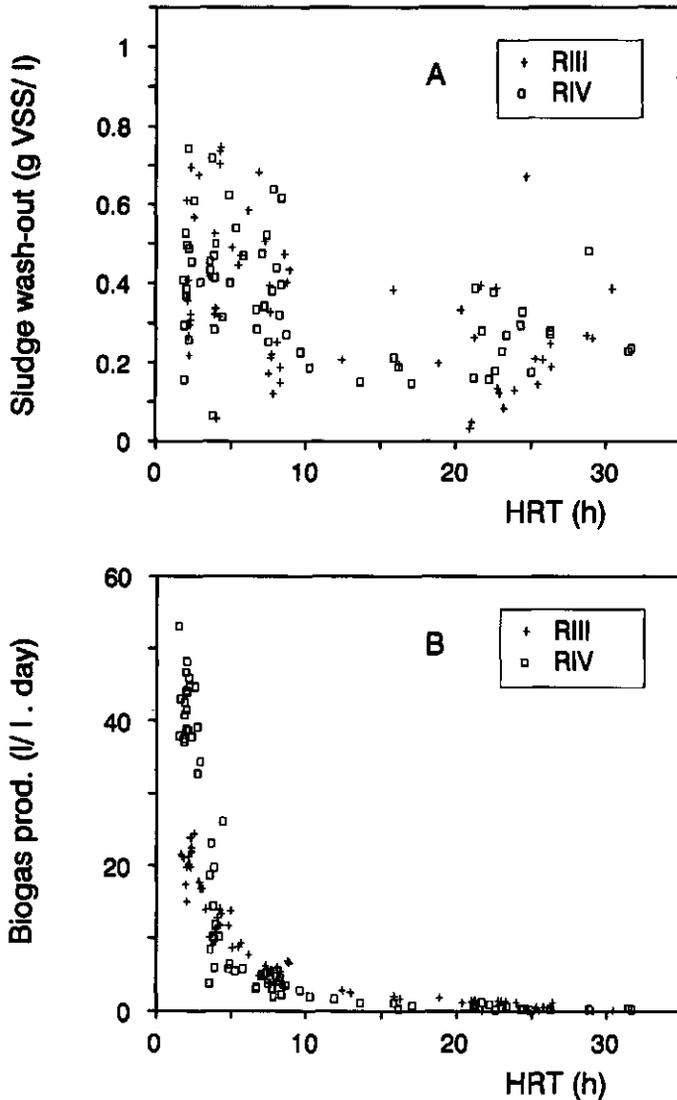


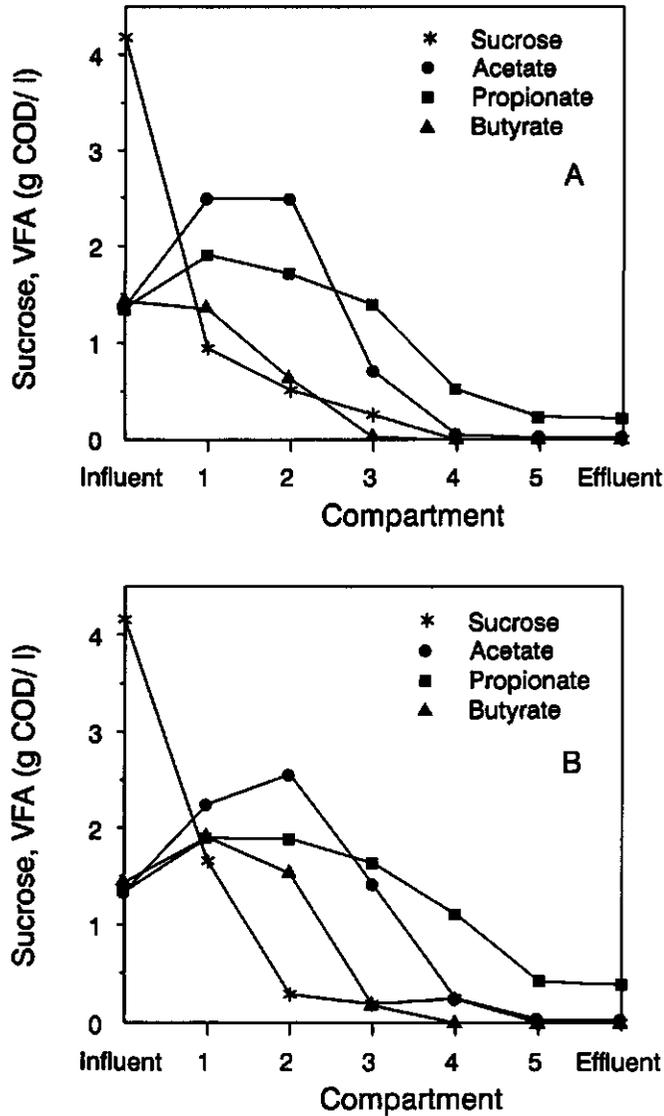
Fig. 6.3 A) Sludge wash-out and B) biogas production rate of the reactors inoculated with MGS, both as a function of HRT.

The biomass wash-out per litre of effluent was the same in all reactors. However, the total sludge production was considerably higher in the reactors RII and RIV, treating the sucrose-VFA mixture. In initial VSS and TSS determinations, the suspended bacteria were not included. However, based on COD analyses of effluents and centrifugated effluents, it appeared that wash-out of suspended bacteria was only slightly higher in the reactors RI and RIII fed with VFA only. The very effective retention of biomass is illustrated in Fig. 6.3, which shows that the wash out of solids per litre of effluent hardly increased with decreasing HRT down to 2 h, even when this was accompanied with extreme biogas loading conditions of 40-50  $l.l^{-1}.day^{-1}$ .

#### **Analyses of the different compartments before and after an increase of the OLR**

All compartments of each reactor were periodically sampled and analyzed for pH, VFA, COD, biogas flow and biogas composition in order to study the degradation pattern of the substrate over the height of the reactor. The obtained results with VFA or sucrose-VFA as feed were comparable, irrespective of the seed material used. Figs. 6.4, 6.5, and 6.6 show the effect of a sudden increase in the OLR from 48 to 73  $g.l^{-1}.day^{-1}$  and a concomitant decrease of the HRT from 4 to 2.7 h, on the degradation pattern of the sucrose-VFA mixture over the height of reactor RII. The results show a typical sequence in the degradation of the partially acidified substrate (Fig. 6.4). In the first compartment sucrose was converted, followed in the next compartments by conversion and removal of butyrate and acetate. Propionate was only degraded in the last compartments.

The sudden increase of the OLR hardly affected the COD removal efficiency and the effluent COD concentration, although propionate concentrations temporarily increased from 200 to 400  $mg\ COD.l^{-1}$ . It decreased to its original level within 1-2 days. The fact that the thermophilic process is limited by the conversion of propionate is clearly shown by Fig. 6.4b. As a result of the increase in the OLR the degradation of the various compounds is shifted to higher regions in the reactor. Apparently, the capacity of the system to degrade propionate is affected first upon increasing of the OLR. An accumulation of other soluble intermediates was not observed, neither during stable digestion conditions, nor after an increase in the OLR. Very low concentrations of formate (30  $mg\ COD.l^{-1}$ ) and lactate (75  $mg\ COD.l^{-1}$ ) were only found in the first compartment at an OLR of 73  $g.l^{-1}.day^{-1}$ . Valeriate and iso-butyrate were present in the first compartments of the reactor at concentrations of 100-250  $mg\ COD.l^{-1}$ . Concentrations of ethanol and methanol were always below the detection level.



**Fig. 6.4** Degradation pattern of the partial acidified substrate in the various compartments of reactor RII, A) before and B) after the OLR increase.

The production and composition of the biogas is strongly influenced by an increase in OLR. The overall biogas production increased from 28 to about 40  $l.l^{-1}.day^{-1}$  when the OLR was increased from 48 to 73  $g.l^{-1}.day^{-1}$ . The amounts of  $CH_4$  and  $CO_2$  produced in each compartment are shown in Fig. 6.5. The methane production rate increased in all

compartments due to the OLR increase, except in the first compartment where the higher acidification rate caused a decrease in the methane production rate by 50%. Most of the COD was removed in the third and fourth compartment. The last compartment only contributed significantly at high OLRs. An increase in the hydrogen partial pressure only was observed in the first compartment after the OLR increase (Table 6.2). The other compartments maintained a low hydrogen partial pressure.

Table 6.2. Hydrogen partial pressure in reactor RII\*

Compartment	pH <sub>2</sub> (Pa)	
	OLR: 48 g.f <sup>-1</sup> .day <sup>-1</sup>	OLR: 73 g.f <sup>-1</sup> .day <sup>-1</sup>
1 bottom	163	2230
2	62	57
3	23	24
4	26	27
5 top	1	16

\* measured in the various compartments before and after the OLR increase.

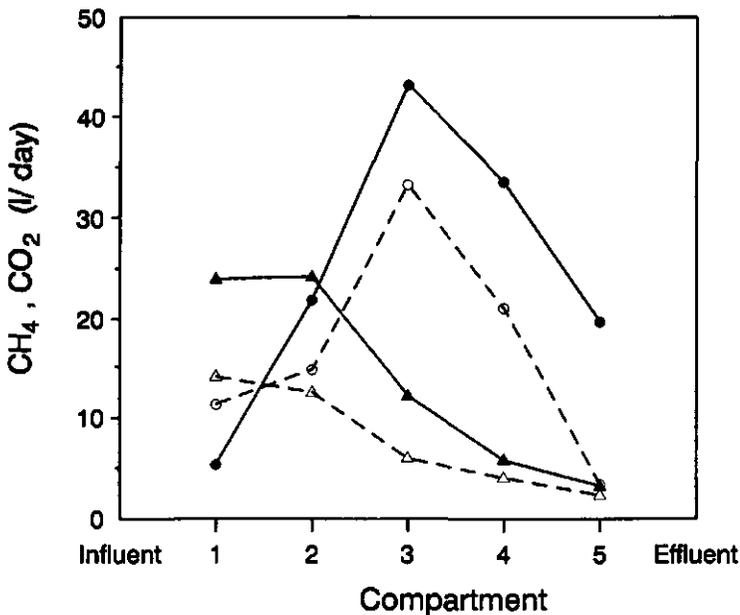


Fig. 6.5 Specific CH<sub>4</sub> (●, ○) and CO<sub>2</sub> (▲, △) production rate over the height of reactor RII, before (open symbols) and after (closed symbols) the OLR increase.

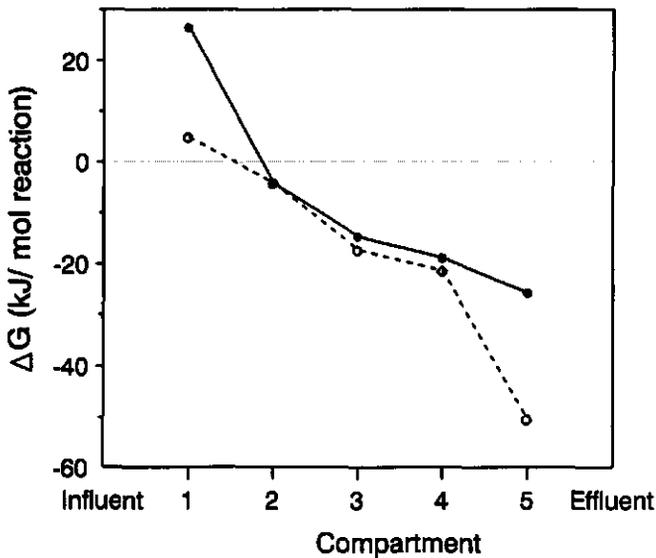
### 6.1.4 Discussion

In the introduction of this chapter we hypothesized that concentrations of VFA and suspended biomass in the effluent will be low, when a staged process is applied for high-rate thermophilic wastewater treatment. Results of the above experiments reveal that, by using compartmentalized upflow reactors, indeed a very efficient high-rate thermophilic digestion process can be achieved. The high efficiency and stability of the thermophilic USSB reactors can be attributed to the establishment of optimal conditions for the degradation of all specific compounds in the various reactor compartments. In the course of the start-up, a segregation of the different degradation steps spontaneously proceeds along the height of the plug-flow reactor. The staging phenomenon at the same time indicates that the thermophilic digestion process can become limited by a retarded degradation of specific intermediates, probably due to high concentrations of acetate and hydrogen as discussed in Chapter 5.

As already mentioned in Chapter 1, a typical feature of thermophilic reactors is the frequently occurrence of high concentrations of VFAs in the effluent, which particularly accounts for propionate (Chapter 3.1; Wiegant *et al.*, 1986). This may be attributed to inhibitory effects of various intermediates produced in the degradation process as illustrated by Figs. 6.4 and 6.6 (see also Chapter 6.2). A staged anaerobic conversion process promotes the development of specific sludge types in each compartment, dependent on the conditions imposed. When treating a more complex substrate the acidifying stage of digestion is localized in the first compartment and as a result, the specific methanogenic activity in this compartment remains relatively low. Consequently, in the subsequent compartments, sludge will be cultivated with a high specific acetogenic and methanogenic activity. Results to be presented in Chapter 6.2, will show that a high digestion performance only can be maintained by withdrawing excess sludge production from the bottom of the reactor, because otherwise, the voluminous acidifying sludge would push the extremely active acetogenic and methanogenic consortia out of the system.

In contrast to earlier reports dealing with experiments with compartmentalized anaerobic processes under mesophilic conditions (Grobicky and Stuckey, 1991), formate only was present in the first compartment in low amounts (30 mg COD. $l^{-1}$ ). The imposed increase in the OLR from 48 to 73 g COD. $l^{-1}$ .day $^{-1}$ , resulted in a 10-fold increase in the H<sub>2</sub> concentration in this compartment (Table 6.2), while the formate concentration remained at the same level. Apparently, under thermophilic conditions, the reducing equivalents are channelled through H<sub>2</sub> as an intermediate rather than through formate. Recent results of Schmidt and Ahring (1993) support this hypothesis. Despite the fact that for thermodynamical reasons a thermophilic digester can accommodate a relatively high hydrogen partial pressure, hydrogen still might play an important role in the degradation of specific VFAs such as propionate (Wiegant *et al.*, 1986). Propionate oxidation is thermodynamically the most difficult reaction in the whole digestion process. Thermodynamic calculations show that

conditions for propionate oxidation are unfavourable in the first compartments (Fig. 6.6). For growth of a syntrophic propionate-oxidizing consortium a Gibbs free-energy change of at least 17-20 kJ.mol<sup>-1</sup> is required (Schink, 1992). This condition is only met in the upper compartments. The Gibbs free-energy changes for propionate oxidation in each of the compartments were calculated according to the method presented by Thauer *et al.* (1977), by using the local pH, temperature (55°C), and the measured concentrations of acetate, propionate and hydrogen. The bicarbonate concentrations were calculated from the partial CO<sub>2</sub> pressure, by using Henry's Law. It should be noted that our  $\Delta G$  computations were made by using the liquid-bulk concentrations (Fig. 6.6). However, the validity of these calculations for the actual situation in the bacterial aggregates is questionable, because here concentrations are much lower.



**Fig. 6.6** Gibbs free-energy change of the propionate oxidation reaction in the various compartments of reactor RII, before (●) and after (○) the OLR increase.

Propionate degradation also was restricted to the higher compartments of the reactors fed with the VFA mixture, despite the very low H<sub>2</sub> partial pressures prevailing in the lower compartments (1 - 17 Pa) of these reactors. These observations suggest that the propionate oxidizers and/or methanogens also are inhibited by other compounds, probably by acetate (see also Chapter 5). In well mixed thermophilic sludge (bed) systems, such as contact processes with or without sludge recycling, and/or conventional UASB reactors, it is very

difficult to maintain low concentrations of these intermediate compounds. Due to the detrimental effects of both hydrogen and acetate on the thermophilic mineralization process, therefore, in these systems, high OLRs cannot be accommodated for prolonged periods without an increase in the effluent VFA concentrations. In the proposed compartmentalized reactor concept, hydrogen is effectively removed from the system, because the produced biogas is released from each separate compartment. In this way, in the last compartments both acetate concentration and hydrogen partial pressure can be maintained at a very low level. Consequently, low effluent VFA concentrations can be maintained in a staged process even under very high loading conditions.

Wiegant (1986) found that in single stage UASB reactors, the OLR potentials can become limited by the occurrence of severe wash-out of viable biomass. The UASB studies described in Chapter 3.1 were limited by the same phenomenon. The results presented in this chapter reveal that the retention of biomass in a staged process is very satisfactory. The improved sludge retention in a staged reactor can be attributed to the low biogas load on the final gas-liquid separator which creates optimal settling conditions at top of the reactor. In addition, due to the very low effluent VFA concentrations, the biogas production in the final compartment is very small. Even after prolonged periods of time under extreme loading conditions, we could not detect any significant wash-out of sludge particles. Furthermore, the effluent VSS concentration hardly increased with decreasing HRT (Fig. 6.3a). An improved sludge retention also was found in an anaerobic baffled reactor (ABR). However, despite the compartmentalized set up of the ABR, heavy sludge wash out occurred during hydraulic shock loads, imposing a HRT of 1 h (Grobicki and Stuckey, 1991). The start-up of our reactors proceeded rapidly, i.e. within 1 to 2 months, although, differences were observed with respect to biomass retention and development of granular sludge depending on the type of feed. In contrast to the results of Ohtsuki *et al.* (1992) and Wiegant and De Man (1986), sludge granulation hardly proceeded in the reactors fed with the VFA mixture. As a result, the wash-out of methanogenic bacteria was higher in that case and consequently, the loading potentials considerably lower. Apparently, the presence of a fraction of non-acidified substrate is indispensable for a rapid and satisfactory cultivation of thermophilic granules. This observation is in accordance with that of Wiegant and Lettinga (1985) and more recently of Uemura and Harada (1993). The required amount of non-acidified substrate is not clear yet. According to recent results, obtained by Alphenaar (1994) under mesophilic conditions, it seems to depend on the sludge loading rate. He found that under moderately high to high loading conditions ( $0.7 - 1.0 \text{ g COD} \cdot \text{g}^{-1} \text{ VSS} \cdot \text{day}^{-1}$ ), promotion of granular growth occurred by addition of sucrose up to 30% of the influent COD. Beyond this value a deterioration occurred (Alphenaar, 1994). Apparently, the acidifying bacteria and/or one or more of their excretion products are essential in the granulation process, particularly at high temperatures, see also § 1.5.3. Interestingly, Schmidt and Ahring (1994) found less extracellular polymers in thermophilic granular sludge compared to mesophilic granules, which probably can be due to the high mineralization rate under thermophilic conditions

(Soto *et al.*, 1992). A lack of extracellular polymers might explain the formation of dispersed biomass in the reactors fed with the completely acidified substrate, i.e. reactor RI and RIII. Despite the fact that granulation hardly occurred in these reactors, the process performance remained relatively stable, even under high loading conditions. This particularly accounts for reactor RIII, which was started with partially crushed MGS as seed material. The OLR could be increased to high values with high COD removal efficiencies (Fig. 6.1b). The results demonstrate clearly that by using a compartmentalized reactor, equipped with interim biogas withdrawal facilities, very high OLRs can be accommodated under thermophilic conditions, even when sludge granulation doesn't proceed satisfactorily.

## 6.2 Development of Thermophilic Methanogenic Sludge in Compartmentalized Upflow Reactors

### Abstract

The characteristics and development of thermophilic anaerobic sludge in upflow staged sludge bed (USSB) reactors were studied. The compartmentalized reactors were inoculated with partially crushed mesophilic granular sludge and then fed with either a mixture of volatile fatty acids (VFA) or a mixture of sucrose and VFA. The staged degradation of the soluble substrate in the various compartments led to a clear segregation of specific types of biomass along the height of the reactor, particularly in reactors fed with the sucrose-VFA mixture. Both the biological as well as the physical properties of the cultivated sludge were affected by the fraction of non-acidified substrate. The sludge in the first compartment of the reactor treating the sucrose-VFA mixture was whitish and fluffy, most likely resulting from the development of acidifying bacteria. Sludge granules which developed in the top part of this reactor possessed the highest acetogenic and methanogenic activity and the highest granule strength as well. The experiments also revealed that the conversion of the sucrose-VFA mixture into methane gradually deteriorated at prolonged operation at high organic loading rates (50-100 g COD.l<sup>-1</sup>.day<sup>-1</sup>). A stable reactor long-term performance can only be achieved by preserving the sludge segregation along the height of the reactor. In the reactor fed solely with the VFA mixture little formation of granular sludge occurred. In this reactor, large differences in sludge characteristics were also observed along the reactor height. Li<sup>+</sup>-tracer experiments indicated that the hydraulic regime in the USSB reactor is best characterized by a dynamic plug-flow pattern. The formation of granular sludge was found to influence the liquid flow pattern.

### 6.2.1 Introduction

In the previous section of this chapter (Chapter 6.1) the stability of high-rate thermophilic treatment systems was enhanced, by applying a staged digestion process in a reactor denominated as the Upflow Staged Sludge Bed (USSB) reactor. In USSB reactors, a mixture of sucrose and VFA could be efficiently treated by 90-95% under extreme organic loadings of 50-100 g COD.l<sup>-1</sup>.day<sup>-1</sup>. An important advantage of the compartmentalized reactor set-up is the possibility to release the produced biogas from various heights along the reactor. This results in a very low superficial biogas load in the final compartment and, therefore, to optimal settling conditions of the sludge. The applied reactor system could accommodate volumetric biogas loads up to 40-50 m<sup>3</sup> biogas.m<sup>-3</sup> reactor.day<sup>-1</sup> without any significant

wash-out of biomass (Chapter 6.1).

The main achievement of the USSB reactor is the low concentration of VFA in the effluent. This is attributed to the very low concentrations of intermediate compounds such as acetate and hydrogen in the upper compartments. As already discussed in Chapter 5, both these intermediates inhibit acetogenic reactions, like propionate conversion, at relatively low concentrations. Obviously, the staged degradation of organic substrate results in a segregated development of specific biomass along the reactor height. The development of thermophilic anaerobic sludge in the various compartments of the USSB reactor is described in more detail in this Chapter. The sludge from the compartments was characterized with respect to some physical-chemical properties, the specific substrate conversion rates, and the digestibility.

## 6.2.2 Materials and Methods

### Compartmentalized upflow reactor

Continuous flow experiments were started using two 5.1 litre USSB reactors, R-III and R-IV (Fig. 2.2). A detailed description of the experimental set up and the start-up procedure is presented in Chapter 6.1. During the first months of operation, reactor R-III was fed with a VFA mixture consisting of acetate, propionate, and butyrate in a COD ratio 3:1:1. After 78 days of continuous operation the VFA mixture was changed to a COD ratio of 2:1:2. Reactor R-IV was started up with a sucrose, acetate, propionate, butyrate mixture in a COD ratio of 3:1:1:1. After 10 months of operation the amount of sucrose was lowered to 10% of total COD as described below. The concentrations of basal nutrients and COD in the concentrated feed stock solution of both reactors are given in Table 6.1. Reactor performance data of both reactors are presented in Table 6.3 and Table 6.4.

### Biomass

The reactors were seeded with partially crushed Aviko-MGS (see also Chapter 6.1).

### Hydrodynamic flow characteristics

The flow characteristics of the USSB-reactor were studied using LiCl as tracer (Bolle *et al.*, 1986; Grobicki and Stuckey, 1992). At zero time of these tracer experiments a pulse of LiCl was added to the influent of the reactor, corresponding to an average reactor concentration of 2 mg Li<sup>+</sup>.l<sup>-1</sup>. Periodically, 10 ml of effluent samples were collected during 4-5 hydraulic retention times, by using an automatic sampling device, giving a total number of 100 samples. The tracer experiments were performed under various operation conditions (Table 6.5). The results were interpreted using a "tanks-in-series" model based on the work of Levenspiel (1974). The model was applied and described by Grobicki and Stuckey (1992) for characterizing the hydrodynamics of the anaerobic baffled reactor (ABR). From the measured data a C-curve was generated in which  $E_0$ , the normalized tracer concentration (equation 6.1)

is plotted versus  $\Theta$ , the normalized time (equation 6.2):

$$E_{\theta} = \frac{C_t}{C_0} \quad (6.1)$$

$$\theta = \frac{\text{time (exp.)}}{\text{HRT}} \quad (6.2)$$

where  $C_t$  = effluent tracer concentration at time  $t$ ,  $C_0$  = total amount of tracer per volume of reactor liquid,  $\text{time}(\text{exp.})$  = experimental time ( $t$ ), and  $\text{HRT}$  = hydraulic retention time (= volume reactor/total flow). The mathematical equations to calculate the mean (equation 6.4) and the variance (equation 6.5) of the obtained C-curve as well as the percentage dead space (equation 6.6), and the theoretical number of completely mixed compartments (equation 6.7) in the reactor, are given in an appendix. In comparison to the approach of the above mentioned authors all calculations were based on the amount of  $\text{Li}^+$  recovered within a 2 HRT-period (Levenspiel, 1974; Grobicki and Stuckey, 1992). The experiments were performed in singular except the tracer experiment performed during the start-up which was done in duplicate.

### Granule strength

The granule strength was determined 9 months after starting the reactors by measuring its resistance against compression forces, according to the method described by Hulshoff Pol *et al.* (1986), Grotenhuis *et al.* (1991), and Alphenaar (1994). In this method a sample of granular sludge is compressed by a downward movement of a piston which is connected to a load cell. The gradual increase in resistance force drops sharply at the moment the granules disintegrate. The resistance force just before disintegration is characteristic for the granule strength.

### Sludge Volume Index (SVI)

The SVI was determined with about 20 g of wet sludge in a 100-ml measuring cylinder. After 30 minutes of settling, the volume of the sludge was measured. Then, the content of volatile suspended solids (VSS) of the sludge was determined. The SVI is expressed in ml sludge per g VSS. Assays were performed after 9 months of operation.

### Granule size distribution

The granule size distribution was estimated 9 months after starting the reactors using an image analyzer as described by others (Grotenhuis *et al.*, 1991; Alphenaar, 1994). Data were processed using the TEA Image Manager (TIM) software-package (Difa Measuring Systems BV, Breda, The Netherlands) and the analyzed granules were classified in classes of 0.1 mm. A similar method for determining particle size distributions was recently described by Dudley

*et al.* (1992). The mean granule diameter was estimated following a dual approach: 1) by setting the total number of granules to 100% (median diameter); and 2) by setting the total volume to 100%.

### Activity Measurements

The specific methanogenic activity at various acetate concentrations (0-15 g COD.l<sup>-1</sup>) was assessed using the "head space method" activity test (§ 2.4). Control bottles showed very little methane production within the 3-hours test period. Kinetic data were calculated by fitting the activity results to a modified Haldane's equation for substrate inhibition as proposed by Morvai *et al.* (1992), equation (6.3):

$$A = \frac{A_{\max}}{1 + k_m/S + (S/k_i)^n} \quad (6.3)$$

in which A = the measured methanogenic activity, A<sub>max</sub> = maximum methanogenic activity, S = acetate concentration, K<sub>m</sub> = substrate half saturation constant, K<sub>i</sub> = substrate inhibition constant, and n = inhibition-response-coefficient. All computations were based on the concentration undissociated acetic acid which is thought to determine the actual degree of inhibition (Fukuzaki *et al.*, 1990; Morvai *et al.*, 1992). HAC concentrations were calculated by using a pK<sub>a</sub> of 4.80 at 55°C (Sillen and Martell, 1964). Parameters were estimated using the 'Nelder' nonlinear regression routine for parameter estimation (Nelder and Mead, 1965). For one sludge the methanogenic activities were fitted using the non-modified Haldane equation (by omission of n), because no reasonable fit could be obtained with equation (3).

The specific substrate utilization rates were measured 9 months after the start-up of the reactors, using the substrate-depletion activity tests (§ 2.4). Tests were performed in duplicate, except for the sludges from the first and last compartment which were done in triplicate. In these tests acetate, propionate or butyrate were used as the sole substrate in a concentration of 3.0 g COD.l<sup>-1</sup>. Bottles were inoculated with approximately 1.5 g VSS.l<sup>-1</sup>, and samples were taken periodically during 1-4 days. The initial pH was 7.1 ± 0.1.

### Assessment of sludge digestibility

The digestibility of the sludge from the various compartments of reactor R-IV was assessed in triplicate in 320-ml serum bottles. Bottles were filled with 100 ml of substrate-free standard medium (§ 2.3) and brought to a temperature of 55°C. After a 10-ml sludge sample was introduced into each bottle, the bottles were sampled to analyze pH, soluble COD and VFA concentration. The volume of the headspace was determined accurately. Thereafter, the headspace was flushed with N<sub>2</sub>-CO<sub>2</sub> (80:20/vol/vol) and the N<sub>2</sub> and CH<sub>4</sub> concentration in the headspace was followed over a 2-month period. Samples from the headspace were taken after releasing the overpressure in the bottle. The exact amount of methane produced from the unfed sludge was calculated using the measured CH<sub>4</sub> concentration, the exact headspace

volume and the ratio  $N_{2, (t=0)}/N_{2, (t=t)}$ . From the cumulative methane production the endogenous methane production rate was calculated using the slope of the curve at a specific time interval. For this purpose we divided the 50-day test period into 3 experimental parts: i) day 0 to 8, which was not considered because of the conversion of residual soluble COD substrates which were introduced in the serum bottles together with the sludge samples; ii) day 8 to 22, characterized by a relatively high methane production rate after the depletion of the soluble substrate; iii) day 22 to 50, characterized by a relatively low methane production rate under unfed conditions. The VSS content at time  $t$  was calculated from the final VSS concentration (determined after the test) and the amount of digested biomass, assuming that 1 g VSS equals 1.4 g  $\text{CH}_4$ -COD (De Zeeuw, 1985). An 'apparent decay rate' was estimated by calculating the ratio of the methane production rate over the amount of digested biomass in the same period.

### Analyses

Methane,  $\text{Li}^+$ , and all other analyses are described in § 2.5.

Sludge samples were examined periodically with a phase contrast microscope (Olympus BHT, Tokyo, Japan).

**Table 6.3** Performance data reactor R-III and R-IV during months 2-8.

Reactor	period (months)	OLR (g COD.l <sup>-1</sup> .day <sup>-1</sup> )	HRT (h)	COD <sub>in</sub> (g.l <sup>-1</sup> )	COD <sub>out</sub> (g.l <sup>-1</sup> )	VFA <sub>out</sub> (g COD.l <sup>-1</sup> )	Prop <sub>out</sub> <sup>a</sup> (%)
III	3-8	70-80	2-2.5	6-8	0.9-1.5	0.7-1.3	60-80
IV	2-3	70-90	1.7-2.9	6.5-8.5	0.2-0.5	0.03-0.4	50-85
IV	4-6	80-100	2-2.5	7-10	1.2-2.0	1.0-1.7	85-95
IV	6-8	50-70	2.5-3	6-8	0.9-1.4	0.8-1.3	95-100

<sup>a</sup> Propionate concentration in the effluent as percentage of total effluent VFA.

## 6.2.3 Results

### Reactor performance

Table 6.3 summarizes the average performance data of reactors R-III and R-IV in the first 8 months of operation. Start-up procedures and reactor stabilities are described in more detail in Chapter 6.1. Compartment by compartment analysis showed a staged degradation of the various compounds over the height of the reactor (Fig. 6.4). Generally, sucrose (if present) was converted first, followed by butyrate and acetate. Propionate degradation only occurred in the top compartment of both reactors. Table 6.4 shows the local pH and volumetric conversion rates of the above compounds in reactors R-III and R-IV after 5 and 4 months of continuous operation, respectively. At that time both reactors were operated at an OLR of about  $75 \text{ g COD.l}^{-1}.\text{day}^{-1}$ .

**Table 6.4** Local pH and volumetric conversion rates in  $\text{g COD.l}^{-1} \text{ reactor-compartment.day}^{-1}$  of the various compounds along the height of reactor R-III and R-IV. Reactors were operated at an OLR of  $\approx 75 \text{ g COD.l}^{-1}.\text{day}^{-1}$  at time of sampling.

Compartment Reactor	pH <sup>a</sup>		Sucrose <sup>b</sup>		Acetate <sup>c</sup>		Propionate		Butyrate	
	R-III	R-IV	R-IV	R-III	R-IV	R-III	R-IV	R-III	R-IV	
1 (bottom)	7.4	6.5	155	27	2	0	-34 <sup>d</sup>	149	-29 <sup>d</sup>	
2	7.5	6.6	62	69	40	1	0	2	17	
3	7.8	7.2	5	179	129	9	12	1	65	
4	7.8	7.7	0	84	79	6	23	0	8	
5 (top)	7.9	7.7	0	15	44	31	33	0	0	

<sup>a</sup> Influent pH reactor R-III: 6.8, reactor R-IV: 7.5

<sup>b</sup> Sucrose was only present in the influent of reactor R-IV.

<sup>c</sup> The stoichiometric amount of acetate produced in the conversion of sugar, propionate, and butyrate was taken into account. The produced amount of acetate was corrected by means of the COD balance if besides acetate also propionate and butyrate were produced from sucrose.

<sup>d</sup> Negative values: net propionate and butyrate production from sucrose.

### Hydraulic mixing characteristics

The hydraulic behaviour of the USSB reactor system was assessed by means of duplicate tracer experiments on day 54 in reactor R-IV, when the reactor was filled with dispersed sludge and small granular aggregates. Results show that under the conditions prevailing during the start-up, the system can be described by a series of 10.1 completely mixed

compartments (experiment 1, Table 6.5). This high value indicates an almost ideal plug-flow pattern. Obviously, the separate compartments of reactor R-IV were not completely mixed in the start-up period, because the reactor consisted only of 5 compartments and a settler (Fig. 2.2). During the course of the start-up, the flow pattern may be influenced by changes in operational conditions. Also, changes in size of sludge particles, and an increased biogas production rate may influence the hydraulic behaviour (Grobicki and Stuckey, 1992). The hydrodynamic characteristics were assessed again after 2 years of continuous operation. During this period the reactor became completely filled up with thermophilic granular sludge. The tracer experiments were performed both under high loading conditions (experiment 2) as well as under unfed conditions (experiment 3). In both cases the assessed theoretical number of completely mixed compartments ( $N_{th}$ ) was about equal to the actual number of 5 (Table 6.5). Surprisingly, the high biogas production rate even enhanced the plug-flow pattern of the liquid flow (experiments 2 and 3, Table 6.5).

**Table 6.5** Results of the residence time distribution experiments at the various reactor conditions.

Experiment number	$Q_{tot}$ <sup>a</sup>	OLR <sup>b</sup>	Biogas <sup>c</sup>	HRT (h)	Mean, $\phi$ (-)	Variance, $\sigma^2$ (-)	Dead space, $\tau$ (%)	Li <sup>+</sup> -recovery (%)	$N_{th}$ <sup>d</sup>
1	24	32	11	4.34	1.099	0.10	0.3	91 - 95 <sup>e</sup>	10.1
2	40	80	34	2.53	0.866	0.16	22.1	90 - 97 <sup>e</sup>	6.3
3	37	0	0	2.70	0.986	0.19	43.0	58 - 75 <sup>e</sup>	5.2

<sup>a</sup> Total flow ( $Q_{tot}$ ) in  $l \cdot day^{-1}$

<sup>b</sup> Organic loading rate (OLR) in  $g \text{ COD} \cdot l^{-1} \cdot day^{-1}$

<sup>c</sup> Biogas production in  $l \cdot l^{-1} \cdot day^{-1}$

<sup>d</sup> Number of theoretical compartments

<sup>e</sup> Percentage Li<sup>+</sup> recovery, both after 2 and 4 HRT.

### Segregated development of thermophilic anaerobic sludge

A staged degradation over the height of the reactor will only manifest itself when a specific thermophilic biomass develops in each compartment. This will be the case when the exchange of biomass between the compartments is limited. We investigated several physical and biological characteristics of the sludges grown in the various compartments of reactor R-III and R-IV. It should be mentioned here that during the whole experimental period the excess sludge produced was withdrawn only from the top of the reactor once the sludge bed had reached the effluent overflow.

*Physical-chemical sludge characteristics*

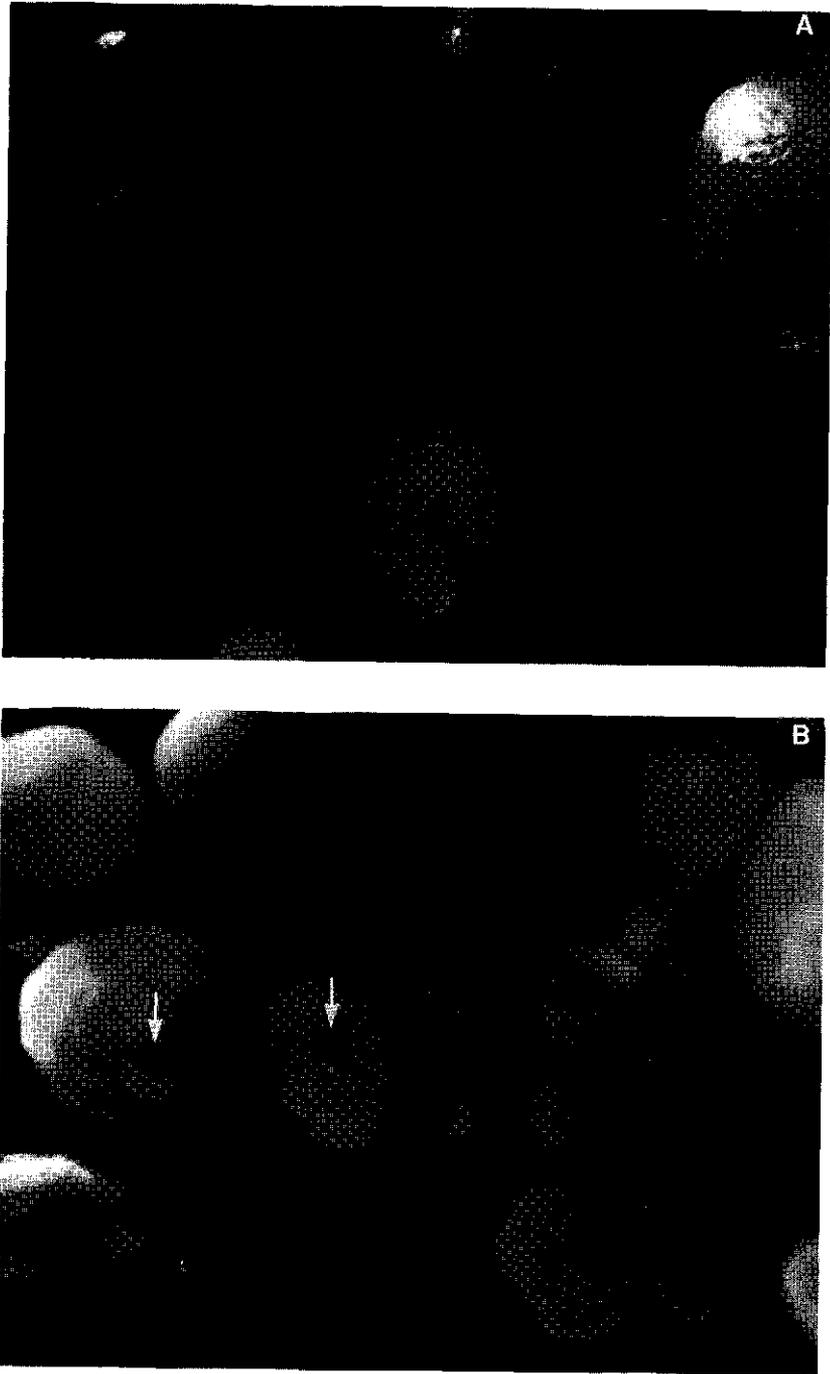
The physical characteristics of sludge from reactor R-III, fed solely with the VFA mixture, and sludge from reactor R-IV, fed with the sucrose-VFA mixture, are summarized in Table 6.6. The results show that in reactor R-III only in the lower compartments small aggregates were found. An increase in the influent butyrate concentration at day 78 onwards did not stimulate granulation of the sludge. According to Grotenhuis (1992) formation of granular sludge might be enhanced by syntrophic associations of acetogenic and methanogenic bacteria. In contrast to reactor R-III, the sludge in all the compartments of reactor R-IV had a granular shape and a median diameter of approximately 1.5 mm. Apparently, granule formation under thermophilic conditions is stimulated by the presence of sugars in the influent. As a consequence of the staged digestion process sucrose conversion is located at the lower compartments of reactor R-IV (Table 6.4). This resulted in the growth of sludge granules with typical properties of acidifying sludge (Alphenaar, 1994; Hulshoff Pol *et al.*, 1986), i.e. a more fluffy and whitish appearance (Fig. 6.7a). Obviously, the composition of this sludge differed significantly from sludge grown in the upper compartments (Fig. 6.7b). Nevertheless, the sludge in the lower compartments can still be characterized as a granular sludge. An important characteristic of the sludge granules from the higher compartments of reactor R-IV were the empty cavities or gas-release-holes (see arrow Fig 6.7b). This feature was already observed by others (Wiegant and De Man, 1986). In reactor R-IV large aggregates of 2-6 mm were also present, particularly in the upper compartments of the reactor. Although these aggregates, which consisted of conglomerated granules, were present in low numbers they contributed significantly to the total biomass (data not shown).

**Table 6.6** Physical characteristics of sludge from the various compartments of reactor R-III and R-IV.

Sludge from compartment: Reactor	granule strength <sup>a</sup> (x 10 <sup>4</sup> N.m <sup>-2</sup> )		ash content (% of dry weight)		median diameter <sup>b</sup> (mm)		SVI (ml.g <sup>-1</sup> VSS)	
	R-IV	R-III	R-IV	R-III	R-IV	R-III	R-IV	
1 (bottom)	0.16 ± 0.03	92.1 ± 0.2	22.6 ± 2.9	0.45	1.70	20.3	12.4	
2	0.21 ± 0.06	67.5 ± 1.6	25.2 ± 1.7	0.45	1.54	16.7	16.7	
3	0.19 ± 0.04	42.2 ± 1.4	34.9 ± 2.3	0.65	1.46	17.4	19.1	
4	0.27 ± 0.07	43.5 ± 1.4	34.6 ± 3.0	0.20	1.44	29.3	21.2	
5 (top)	0.31 ± 0.06	51.0 ± 2.2	34.3 ± 2.1	0.12	1.35	55.7	20.7	

<sup>a</sup> Granule strength only assessed of sludge from reactor R-IV.

<sup>b</sup> The sludge of reactor R-III consisted of weak aggregates.



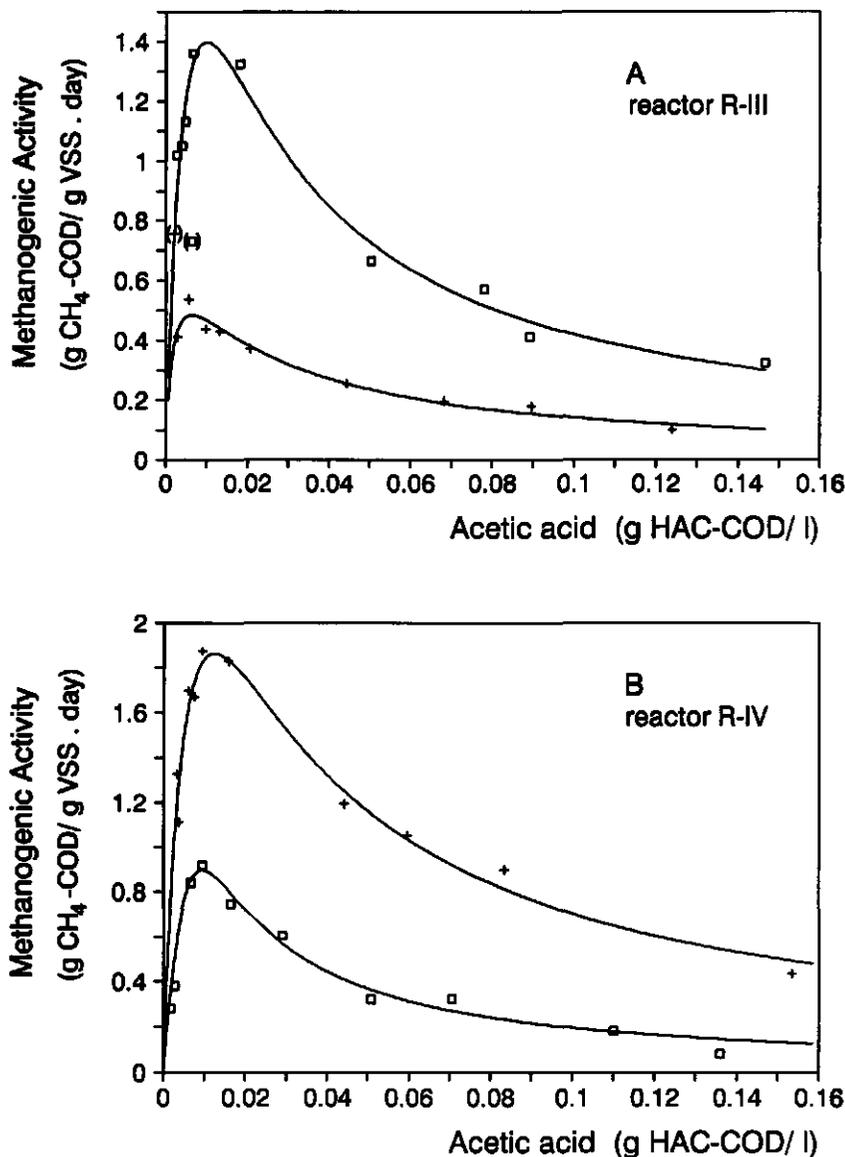
**Fig. 6.7** Sludge granules cultivated in A) the first and B) last compartment of reactor R-IV fed with a sucrose-VFA mixture. Arrows indicate gas-release-holes.

The mechanical strength of the granules only could be assessed for the sludge grown in reactor R-IV. The aggregates formed in reactor R-III were too small and too weak to assess their strength. Results show that the granule strength increased with increasing compartment number (Table 6.6). A weak reciprocal correlation between the granule size and the granule strength (regression coefficient =  $-4.04 \times 10^{-5}$  (N.m<sup>-2</sup>)/mm,  $R^2 = 0.3865$ ) was found. According to Alphenaar (1994) and Hulshoff Pol *et al.* (1986) both these parameters are clearly influenced by the microbiological composition of the granular aggregates. Generally, granules with a large fraction of acidifying biomass are bigger, have a less dense structure, and deteriorate more easily than typical methanogenic granules (Alphenaar, 1994).

Large differences in ash-content and SVI were found between the sludges cultivated in the various compartments of reactor R-III, fed with a VFA mixture. In the compartments 1 and 2 of this reactor, the sludge which developed had an ash-content up to 92% (Table 6.6). The high SVI of the sludge in compartment 5 can be explained by the rather dispersed character of the sludge. The ash content and SVI of the sludges cultivated in the various compartments of reactor R-IV did not vary significantly (Table 6.6).

#### ***Biological sludge characteristics***

Due to the plug-flow conditions in the USSB reactors and the very low extent of sludge mixing, the kinetic properties of the developing methanogenic biomass may differ along the height of the reactor. Between months 2 and 9, the average acetate concentrations in the influent and the effluent of reactor R-III were 2000-3000 and 25-200 mg COD.l<sup>-1</sup>, respectively, and for reactor R-IV 1000-2000 and 10-150 mg COD.l<sup>-1</sup>, respectively. As a result of sucrose conversion, the acetate concentration generally increased by 50% in the first compartment of reactor R-IV (data not shown). Fig. 6.8 shows the assessed specific methanogenic activities at various acetate concentrations for the sludge from compartments 1 and 5 of reactor R-III (Fig. 6.8a) and reactor R-IV (Fig. 6.8b) after 3 months of operation. The results show a clear difference between both reactors with respect to the maximum specific methanogenic activity. A relatively high methanogenic activity was found for the sludge grown in the first compartment of reactor R-III, fed with the VFA mixture. The activity of the sludge grown in compartment 5 was significantly lower. For reactor R-IV, fed with the sucrose-VFA mixture, the opposite was found. Apparently, the acidifying stage of digestion is localized in the first compartment(s) of reactor R-IV, resulting in a low specific methanogenic activity of the sludge.



**Fig. 6.8** Methanogenic activity at various acetate concentrations of sludge cultivated in compartment (□) 1, and (+) 5 of **A**) reactor R-III and **B**) reactor R-IV. Solid lines were computed using equation (3), values between brackets (A) were not considered for the calculations. Substrate concentrations were expressed as undissociated acetic acid because of pH variance between 6.8-7.3.

The computed kinetic constants are shown in Table 6.7. The results of the thermophilic sludge samples reveal a similar sensitivity for high acetate concentrations, i.e. the  $K_i$  was in the same range. However, a somewhat lower  $K_i$  was found for the sludge cultivated in compartment 1 of reactor R-IV. The apparent  $K_m$  was slightly higher for the sludge cultivated in compartment 1 of each reactor. Although this may indicate a selection of different types of acetate-utilizing methanogens along the reactor height, microscopic examinations revealed the dominance of rod-shape methanogenic bacteria in sludge from all compartments (results not shown). Because of the relatively high apparent  $K_m$ - and low  $K_i$ -values, the maximum conversion rates,  $A_{max}$ , calculated by using equation 6.3 (Table 6.7), were higher than the values assessed with the head-space activity test (Fig. 6.8). Compared to the kinetic characteristics of the inoculum MGS, a higher  $K_m$  and a much lower  $K_i$  for acetate conversion was found for the thermophilic granular sludge. Apparently, thermophilic sludge is significantly more sensitive for high acetate concentrations than methanogenic sludge cultivated under mesophilic conditions. In principle, inhibitory effects of sodium acetate might also be attributable to salt toxicity. However, recent experiments with thermophilic methanogenic sludge conducted at various  $Na^+$  concentrations revealed that little if any inhibition effects are found up to 3.4 g  $Na^+$ . $l^{-1}$ , i.e. 11-15 g NaAc-COD. $l^{-1}$  (unpublished results).

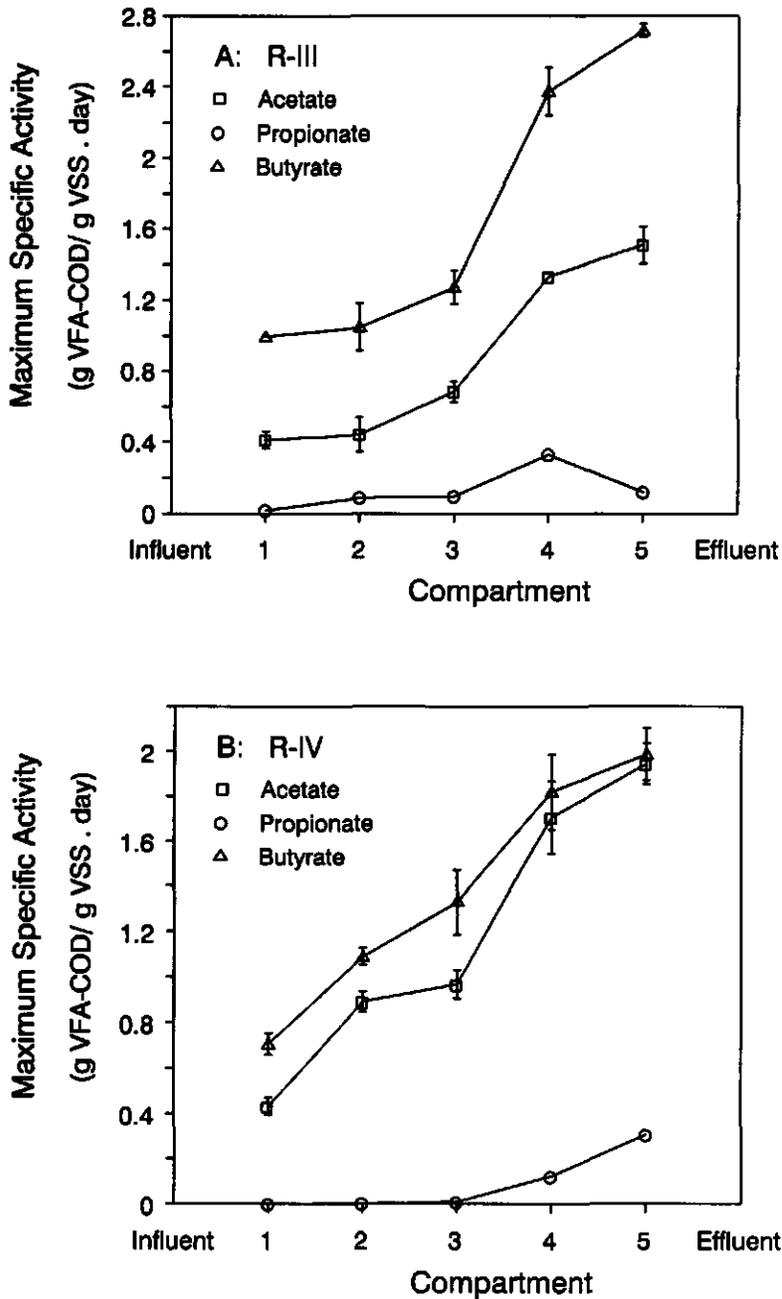
**Table 6.7** Kinetic constants for methanogenesis with acetate as feed<sup>a</sup>. The COD equivalents are used for both  $CH_4$  and acetate.

Sludge-type	$A_{max}$ (g $CH_4$ .g <sup>-1</sup> VSS.day <sup>-1</sup> )	$K_m$ (g Acetate. $l^{-1}$ )	$K_i$ (g Acetate. $l^{-1}$ )	$n$
R-III comp. 1	2.57	0.75	3.70	1.15
R-III comp. 5 <sup>b</sup>	0.74	0.27	3.75	
R-IV comp. 1	2.78	1.76	1.60	1.15
R-IV comp. 5	4.02	1.13	3.38	0.99
MGS (seed)	0.47	0.16	43.0	1.79
SR-MGS <sup>c</sup>	0.18	0.05	21.8	4.18

<sup>a</sup> Constants were computed using equation (3) and the activities presented in Fig. 6.8. At the end of the tests the pH of the serum bottles were in the range 6.8-7.3, except for the experiments with seed-MGS which were in the range 6.3-6.7. Constants were calculated using the fraction undissociated acetic acid. In the table  $K_m$  and  $K_i$  values are expressed as g dissociated acetate. $l^{-1}$  at pH 7.

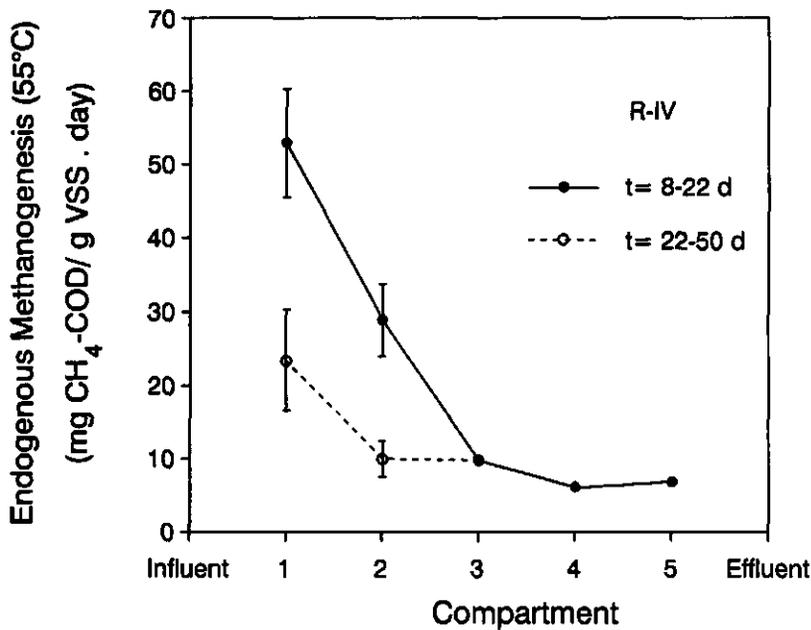
<sup>b</sup> Calculated using the non-modified Haldane's equation for substrate inhibition.

<sup>c</sup> Sulphate Reducing MGS, cultivated by Visser *et al.*, Dept. of Env. Techn. (unpubl. results)



**Fig. 6.9** Specific substrate conversion rates of thermophilic sludge cultivated in the various compartments of (A) reactor R-III and (B) reactor R-IV.

The maximum specific conversion rates for acetate, propionate, and butyrate of the sludges from the various compartments of reactor R-III and R-IV were assessed after 9 months of continuous operation. The results, shown in Fig. 6.9 indicate that the highest specific methanogenic and acetogenic activities are found for the sludges grown in compartments 4 and 5 of each reactor. This is particularly noteworthy for the maximum propionate-utilizing activities (Fig. 6.9). With respect to the maximum acetate conversion rate of the more dispersed sludge from reactor R-III we observed a drop in activity in each serum bottle 2-3 days after starting the test (data not shown).



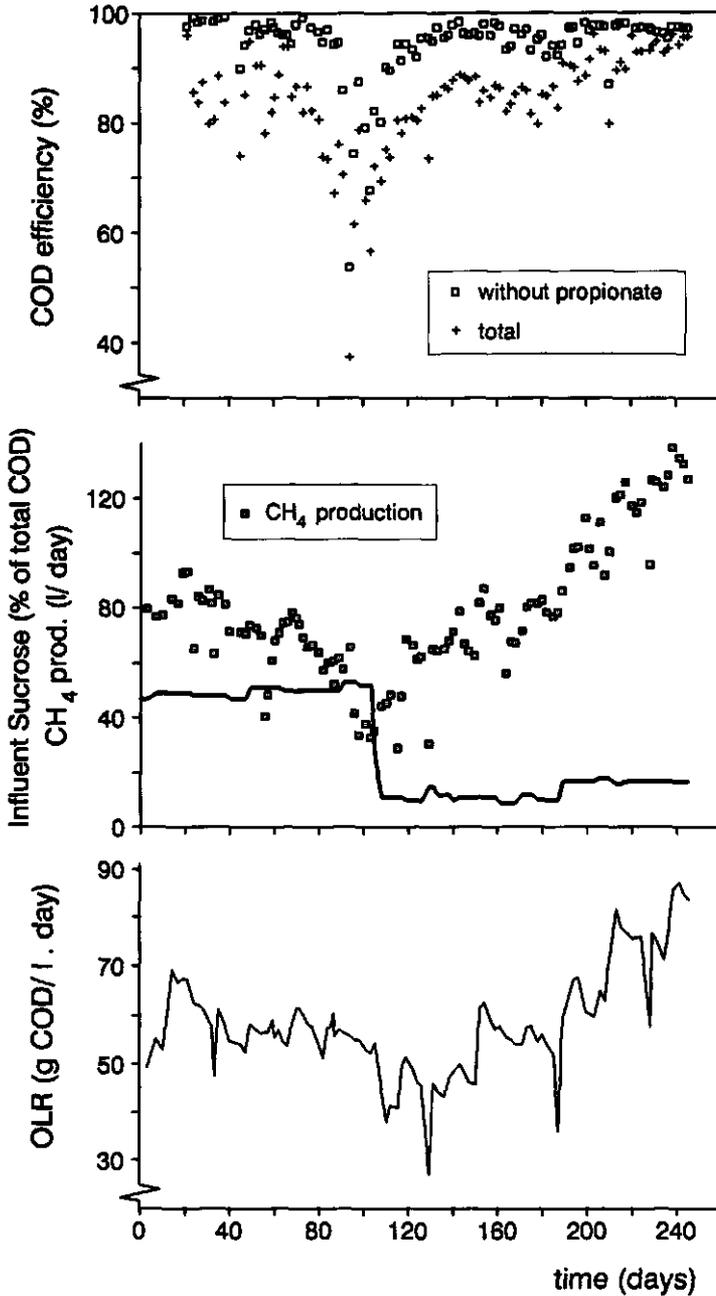
**Fig. 6.10** Digestibility or endogenous methanogenesis of thermophilic sludge grown in the various compartments of reactor R-IV, fed with a sucrose-VFA mixture. Endogenous methanogenesis of sludge from compartments 3, 4, and 5 was the same in both periods.

The sludge digestibility was investigated by means of measuring the methane production rate of the sludge under unfed conditions. Fig. 6.10 shows the digestibility or endogenous methanogenesis of sludge samples grown in each of the 5 compartments of reactor R-IV. During the first weeks of the test period the endogenous methanogenesis of sludge from the compartments 1 and 2 is distinctly higher than that of sludge from the compartments 3, 4 and

5. This presumably can be attributed to the high fraction of acidifying biomass and/or the presence of extracellular polysaccharides in the compartments 1 and 2. The differences in the endogenous methane production rate become insignificant at the end of the test period. The final value probably depends on sludge age and the content of inert organic matter. From the total amount of digested biomass and the endogenous methane production rate, an 'apparent decay rate' of  $0.083 \pm 0.029 \text{ day}^{-1}$  was calculated for the acidifying sludge from compartment 1 and 2. For the stabilized methanogenic sludge from the compartments 3, 4, and 5 a decay rate of  $0.026 \pm 0.003 \text{ day}^{-1}$  was estimated.

### **Influence of acidifying biomass on sludge development**

The sludge development in reactor R-IV was followed for a period of 15 months. Because the excess sludge produced was withdrawn from the top of the reactor the establishment of a steady-state with respect to sludge quality can not be expected in any of the compartments. The dynamic sludge development became apparent from the gradual decrease in the propionate-degrading capacity of reactor R-IV from month 2-3 onwards (Table 6.3). Furthermore, results presented in Table 6.4 show propionate conversion in compartment 3 of reactor R-IV, while this feature was lost after a continuous operation of the system for another 4-5 months (Fig. 6.9b). The 'overall' treatment efficiency of reactor R-IV started to deteriorate after 8 months of continuous operation when propionate contributed to approximately 95% to the effluent VFAs. The thermophilic treatment process was followed for another 8 months in which the effects of changing the sucrose contribution to the influent COD was studied. Fig. 6.11 shows the process performance of the reactor R-IV in this period, starting 7 months after the start-up of the reactor. A severe deterioration of the digestion process occurred at day 80, accompanied by a dramatic change in the sludge characteristics. The whole sludge bed up to the final compartment became whitish, fluffy, and slimy, while the granular structure started to deteriorate. The properties of the voluminous sludge were similar to the sludge found in the lower part of the reactor during the first 8 months of operation (Fig. 6.7a). Apparently, the acidifying sludge gradually replaced the methanogenic granules in the system. Consequently, a recovery of the methanogenic system only can be expected by reducing the sucrose loading rate with or without a concomitant decrease of the total COD loading rate. Restoration of the acetogenic and methanogenic sub-populations will be enhanced when the fraction of unacidified substrate in the feed is reduced. In order to check this hypothesis, the substrate composition of reactor R-IV was changed accordingly. At day 100, the sucrose:VFA COD ratio was changed from 1:1 to 1:9, while the loading rate was decreased from  $55 \text{ g COD.l}^{-1}.\text{day}^{-1}$  for a period of 1 week. Hereafter, the loading rate was increased stepwise up to  $80\text{-}90 \text{ g COD.l}^{-1}.\text{day}^{-1}$  and the sucrose:VFA COD ratio was changed to 1:5. The digestion process indeed recovered completely under these conditions (Fig. 6.11).



**Fig. 6.11** Operation conditions, methane production and COD removal efficiency of reactor R-IV before and after the decrease in influent sucrose concentration. Removal efficiencies are calculated using  $\{(COD_{tot., infl} - COD_{centr., eff.}) / COD_{tot., infl}\}$ . Similar calculations were made excluding the propionate contribution to the influent and effluent.

In comparison to the period preceding the deterioration, the amount of sucrose fed daily to the reactor was halved while the ultimate OLRs were much higher. Also, the granular shape of the sludge was restored and the properties which are thought to be typical for acidifying sludge, were observed again only in the first compartment(s). Similar results were obtained in reactor R-III after changing the feed from the VFA mixture to a sucrose-VFA mixture in the ratio 1:1, on COD basis, for a 6 month period (results not shown). After several months of operation with the sucrose containing feed, the dominance of fluffy and whitish sludge was observed in the entire reactor. This was accompanied by a complete deterioration of the process. Methanogenesis recovered after the sucrose:VFA COD ratio in the feed was lowered to 1:5 (results not shown).

#### 6.2.4 Discussion

Start-up and operation of a compartmentalized upflow reactor for the thermophilic anaerobic treatment of partially acidified and acidified wastewaters leads to a segregated development of methanogenic sludge in the various compartments of the reactor. As a result of this sludge segregation, the digestion process remains very efficient under extreme organic loading conditions, even with respect to the removal of the most problematic VFA, propionate.

The liquid flow in the USSB reactor approximated a plug-flow pattern as was demonstrated in the tracer experiments (Table 6.5). The plug-flow behaviour was more pronounced during the start-up period when the reactor was filled with dispersed sludge. Apparently the flow pattern is influenced by the biomass density, as was already suggested by Grobicki and Stuckey (1992) for the ABR system. Due to granulation, the biomass density becomes much higher. Channelling of the liquid flow through the granular sludge bed, which is indicated by  $\phi < 1$ , may lead to a higher variance in the  $\text{Li}^+$  distribution curve and thus to a lower  $N_{th}$ . In addition to the influence of biomass, it should be noted that, compared to experiment 1 (Table 6.5), experiments 2 and 3 were conducted at flow rates twice as high, which also might contribute to the lower value of  $N_{th}$  (Grobicki and Stuckey, 1992).

The dead-space area increased considerably after prolonged operation (Table 6.5). Because dead-space was negligible during the start-up, the results indicate that the increase in biomass density, by formation of granular sludge, can be seen as the mayor cause for the observed dead-space in experiments 2 and 3 (Table 6.5). According to Grobicki and Stuckey (1992), a biological dead space is minimized by a high biogas production rate. We indeed found a dead-space which was twice as high under unfed conditions, i.e. no biogas production. On the other hand, due to the shape of the reactor and the absence of biogas production, the biological dead-space may be enhanced by a hydraulic stagnant area under the gas-liquid interface in each compartment. Probably as a result of such enhanced biological dead-space area, the fraction of recovered  $\text{Li}^+$  was very low after 2 HRT in experiment 3 (Table 6.5).

Moreover, hydraulic dead-space seems to increase with decreasing HRT (Grobicki and Stuckey, 1992). The latter was also found in our experiments (Table 6.5). Despite the occurrence of biological and/or hydraulic stagnant areas after prolonged operation, it is clear that the USSB reactor may be characterized as a series of at least 5 completely mixed compartments.

The plug-flow pattern leads to a staged degradation of the soluble compounds over the height of the reactor (Table 6.4), particularly under high loading conditions (Chapter 6.1, Fig. 6.4). Consequently, different types of biomass were developing in the various reactor compartments. Bacteria with a high growth rate and a high net growth yield, e.g. acidifiers (Alphenaar, 1994; Pavlostathis and Giraldo-Gomez, 1991), dominate in the first compartments of the reactor. On the other hand, bacteria performing energetically more difficult reactions, e.g. propionate oxidizers (Stams *et al.*, 1992), accumulate in subsequent stages. With respect to reactor R-IV, we calculated in Chapter 6.1 (Fig. 6.6) that for thermodynamic reasons no growth of syntrophic propionate-degrading consortia can be expected in the first compartments of this reactor. The present results indeed show that propionate is only converted by the sludge present in compartments 4 and 5. The dynamic sludge development along the height of the reactor is due to the large differences in kinetic properties between the various bacterial trophic groups involved in the digestion process. Acidifying bacteria, both mesophilic as well thermophilic, are characterized by a higher growth rate and a higher biomass yield than the methanogens and acetogens (Alphenaar, 1994; Henze and Harremoes, 1983; Pavlostathis and Giraldo-Gomez, 1991). For this reason, the maximum specific conversion rates for acetate, butyrate and propionate of the sludge will decrease on the long term in case the influent consists of a large fraction of sucrose due to dilution of acetogenic and methanogenic biomass with acidifying biomass. The decrease in sludge activity in the lower compartments might partly also be attributed to an increase in the fraction of inactive or non-viable organic matter which, under the influence of gravity, accumulates at the bottom of the reactor. The inert matter may originate from the seed material and/or from decaying thermophilic sludge. An accumulation of non-viable biomass probably occurred in reactor R-III treating the completely acidified substrate. In comparison to the situation after 3 months, the methanogenic activity of the sludge from compartment 1 of this reactor dropped to one third after 9 months of operation (Fig. 6.8a, 6.9a). This was accompanied by an increase in the ash content from 35-40% to 90% (Table 6.6). The strong increase in ash content might be enhanced by the relatively high pH increase from 6.5-6.8 to 7.4-7.6 in the first compartment of reactor R-III.

With respect to the sludge from the first compartment of reactor R-IV, fed with the sucrose-VFA medium, a decrease in the maximum acetate conversion rate by more than 50% was observed in a period of about 6 months (Fig. 6.8b, 6.9b). Continuation of the operation for another 4 months resulted in a severe process deterioration due to the decreased conversion capacity of the entire sludge bed (Fig. 6.11a). In predicting the effects of

acidifying biomass on the sludge activity it also should be taken into account that the death rate and decay rate of acidifying bacteria is high (Alphenaar, 1994; Pavlostathis and Giraldo-Gomez, 1991). Therefore, a dominance of acidifiers can only be expected for influents consisting of a relatively large fraction of non-acidified substrate applied at high loading rates (Alphenaar, 1994). Under such conditions, the system is characterized by a low solids retention time (SRT). Based on our observations, a sucrose:VFA COD ratio of 1:1 was obviously too high to maintain a stable thermophilic methanogenic consortium under the extreme OLR applied (Fig. 6.11). A rough estimation of the SRT at an OLR of about 80 g COD. $l^{-1}$ .day $^{-1}$  revealed an 'overall' value of about 8-11 days (data not shown). However, even under such conditions, the excellent performance of the staged reactor system can possibly be conserved by withdrawing biomass from each separate compartment. By using this approach, a steady-state in the segregated sludge development can be achieved similar to conditions with a low sucrose:VFA ratio. As a consequence, the SRT in the last compartments will be much longer than the SRT in the first compartments. Sludge withdrawal from one extraction point, preferentially at the bottom of the upflow reactor, will very likely suffice to ensure stable reactor performance.

Although the dynamic sludge development was much less pronounced for reactor R-III, a clear segregation of different types of biomass was found in the various compartments of both reactors (Table 6.6, Fig. 6.9). In agreement with reactor R-IV, propionate conversion only occurs in the upper compartments of reactor R-III. However, sludge from compartment 5 of the latter reactor exerted a significantly lower propionate-degrading activity than sludge from compartment 4 (Fig. 6.9a). This may be attributed to the very fine structure of the sludge grown in compartment 5 (Table 6.6). The degradation of propionate requires a tight and balanced association of acetogenic and methanogenic bacteria (Mucha *et al.*, 1988; Stams *et al.*, 1992). A decrease in propionate degradation can occur as a result of granule structure disruption (Grotenhuis *et al.*, 1991; Schmidt and Ahring, 1993). The relatively high acetate and butyrate utilization rates found for the sludges from compartment 4 and 5 of reactor R-III (Fig. 6.9) might also be due to the dispersed character of the sludge (Table 6.6). Small aggregates allow far better diffusion of the substrates to the acetogenic and methanogenic bacteria. This hypothesis is in accordance with the results presented in Chapter 4.2, which reveal that physical disruption of intact thermophilic granules leads to a considerable increase of the acetate and butyrate utilization rates, while the propionate conversion rate drops. The thermophilic granules used in the latter experiments displayed a very high activity on acetate and butyrate, while on propionate it was much lower (Chapter 4.2).

The results described here show that staging of the anaerobic treatment process is very beneficial for the application of high-rate thermophilic wastewater treatment. Due to the relatively high susceptibility of thermophilic acetogenic and methanogenic bacteria for intermediate compounds such as acetate and hydrogen (Table 6.7, Chapter 5, Chapter 6.1),

a complete substrate conversion under high loading conditions is impossible in a single stage reactor. The occurrence of a more or less staged degradation of soluble substrate was already observed in an ABR treating a sucrose containing synthetic wastewater under mesophilic conditions (Grobicki and Stuckey, 1991). However, in the mesophilic ABR the separation of the various degradation steps was much less pronounced. This can probably be attributed to the much lower OLRs applied and lower conversion rates achieved. Moreover, the cultivation of specific biomass in the various reactor compartments will also consume considerably more time under mesophilic conditions due to lower bacterial growth rates.

### Appendix

$$\text{Mean} - \phi = \frac{\sum \theta \cdot E_{\theta} \cdot \Delta \theta}{\sum E_{\theta} \cdot \Delta \theta} \quad (6.4)$$

$$\text{Variance} - \sigma^2 = \frac{\sum (\theta - \phi)^2 \cdot E_{\theta} \cdot \Delta \theta}{\sum E_{\theta} \cdot \Delta \theta} \quad (6.5)$$

$$\text{Dead space} - \tau = (1 - (\phi \cdot \epsilon)) \cdot 100\% \quad (6.6)$$

with  $\epsilon$  = the fraction of recovered tracer within 2 residence times.

$$\text{Number of compartments} - N_{st} = \frac{1}{\sigma^2} \quad (6.7)$$

### Acknowledgement

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# Nomenclature

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$a_1$	= energy constant for biosynthesis ( $K^{-1}$ )
$a_2$	= energy constant for microbial decay ( $K^{-1}$ )
$A$	= specific activity per unit biomass ( $\text{g COD.g}^{-1} \text{VSS.day}^{-1}$ )
$A_{\text{max}}$	= maximum specific activity ( $\text{g COD.g}^{-1} \text{VSS.day}^{-1}$ )
$b$	= maintenance and decay rate ( $\text{day}^{-1}$ )
$C_0$	= total amount of tracer per volume of reactor liquid ( $\text{mg.l}^{-1}$ )
$C_t$	= effluent tracer concentration at time $t$ ( $\text{mg.l}^{-1}$ )
$D$	= diffusion coefficient ( $\text{m}^2.\text{s}^{-1}$ ),
$E_\theta$	= normalized effluent tracer concentration (-)
$\Delta G$	= Gibbs free-energy change ( $\text{kJ.mole}^{-1}$ )
$\Delta G^\circ$	= $\Delta G$ under standard conditions: 1 atmosphere, 1 mole. $l^{-1}$ , and $25^\circ\text{C}$
$\Delta G^{\circ\prime}$	= $\Delta G^\circ$ at pH 7 (Thauer <i>et al.</i> , 1977)
$\Delta G'$	= $\Delta G$ at pH 7
$\Delta H$	= change in enthalpy ( $\text{kJ.mole}^{-1}$ )
$k_1$	= temperature related activity constant ( $\text{g COD.g}^{-1} \text{VSS.day}^{-1}$ )
$k_2$	= temperature related decay constant ( $\text{g COD.g}^{-1} \text{VSS.day}^{-1}$ )
$K_b$	= die-off constant ( $\text{h}^{-1}$ )
$K_i$	= substrate inhibition constant ( $\text{g COD.l}^{-1}$ )
$K_{i,a}$	= non-competitive inhibition constant ( $\text{g COD.l}^{-1}$ )
$K_m$	= substrate half saturation constant ( $\text{g COD.l}^{-1}$ )
$n$	= exponent of inhibition or inhibition-response coefficient (-)
$N$	= number of (pathogenic) bacteria
$N_{\text{th}}$	= theoretical number of completely mixed compartments (-)
$\text{pK}_a$	= logarithm of the dissociation constant
$Q_{\text{tot}}$	= total liquid flow ( $\text{l.day}^{-1}$ )
$R$	= gas constant ( $8.314 \text{ J.mole}^{-1}.\text{K}^{-1}$ )
$S$	= substrate concentration ( $\text{g COD.l}^{-1}$ )
$S_0$	= initial substrate concentration ( $\text{g COD.l}^{-1}$ )
$t$	= experimental time (days)
$T$	= absolute temperature (K)
$Y$	= bacterial growth yield ( $\text{g.mole}^{-1}$ )
$X$	= biomass concentration ( $\text{g VSS.l}^{-1}$ )
$x_T$	= temperature correction factor (K)

**Greek symbols**

$\epsilon$	= fraction of recovered $\text{Li}^+$ -tracer within 2 residence times
$\phi$	= mean of the $\text{Li}^+$ -tracer C-curve (-)
$\mu$	= specific growth rate ( $\text{h}^{-1}$ )
$\mu_{\text{max}}$	= maximum specific growth rate ( $\text{h}^{-1}$ )
$\eta$	= liquid viscosity of the solution ( $\text{N.s.m}^{-2}$ ).
$\Theta$	= normalized time (-)
$\Delta\Theta$	= normalized time interval (between 2 collected fractions) (-)
$\Theta_c$	= cell residence time (days)
$\sigma^2$	= variance of the $\text{Li}^+$ -tracer C-curve (-)
$\tau$	= dead space (-)

**Abbreviations**

ABR	= anaerobic baffled reactor
AAFEB	= anaerobic attached film expanded bed
AGLR	= anaerobic gas lift reactor
COD	= chemical oxygen demand ( $\text{g O}_2.\text{t}^{-1}$ )
CZV	= chemisch zuurstof verbruik ( $\text{g O}_2.\text{t}^{-1}$ )
CSTR	= continuous stirred tank reactor
DSFF	= downflow stationary fixed film
FB	= fluidized bed
GFT	= groente-, fruit- en tuinafval
HRT	= hydraulic retention time (h) = volume reactor/total flow
MGS	= mesophilic granular sludge
MTSO-1	= methanogen antigenically strongly related to <i>Methanothrix soehngeni</i> strain Opfikon
MTSO-2	= methanogen antigenically weakly related to <i>M. soehngeni</i> strain Opfikon
OFMSW	= organic fraction of municipal solid waste
OLR	= organic loading rate ( $\text{g COD.t}^{-1}.\text{day}^{-1}$ or $\text{kg COD.m}^{-3}.\text{day}^{-1}$ )
OS	= organische stof
SRT	= solids retention time (days)
SVI	= sludge volume index ( $\text{ml.g}^{-1}$ VSS)
TGS	= thermophilic granular sludge
TSS	= total suspended solids
(U)AF	= (upflow) anaerobic filter
UASB	= upflow anaerobic sludge bed
UFF(R)	= upflow fixed film reactor
USSB	= upflow staged sludge bed
VFA	= volatile fatty acids
VSS	= volatile suspended solids

## Summary and Discussion

Since the eighties, interest in thermophilic anaerobic wastewater treatment has increased considerably. Application of a high process temperature may accelerate anaerobic decomposition of organic matter, which could be attributed to increased reaction rates as well as higher bacterial growth rates. This acceleration of the degradation process, however, is limited within the boundaries of the temperature range of the involved bacteria population. The methanogens of the mesophilic temperature range (20-40°C) rapidly disappear at temperatures exceeding 45°C.

This thesis describes the results of research on a further characterization of the thermophilic anaerobic digestion process. Special attention was given to the start-up and thermostability of the thermophilic treatment process and the microbiological factors involved (Chapters 3 and 4). Research on process optimization (Chapters 5 and 6) concentrated on realizing a stable treatment process with the highest possible efficiency, i.e. the lowest possible concentration of volatile fatty acids (VFA) in the effluent. The results presented in this thesis reveal that low effluent VFA concentrations can be achieved at extreme organic loading rates ( $\approx 100 \text{ kg COD} \cdot \text{m}^{-3} \cdot \text{day}^{-1}$ ). The main research question in this thesis is how an anaerobic thermophilic wastewater treatment reactor can and should be operated under conditions of variations in temperature and organic load.

### 1 Thermophilic Reactor Start-up

The start-up of an anaerobic thermophilic wastewater treatment reactor can be done with close to any mesophilic methanogenic seed sludge. Research done by Wiegant (1986) showed that (digested) cow manure and (digested) sewage sludge is suitable for a thermophilic start-up. Within the research described here, mesophilic anaerobic granular sludge and the digested organic fraction of municipal solid waste (OFMSW) have been examined for their suitability as seed material.

If mesophilic granular sludge is used as inoculum for the start-up of a thermophilic reactor, ( $> 45^\circ\text{C}$ ) the mesophilic population rapidly dies off. The die-off of the mesophiles is followed by the growth of thermophilic organisms, which mainly occurs on the outer surface and in the interstices and/or cavities of the mesophilic granules. Due to the strong adherence of the thermophiles to the sludge granules, the thermophilic reactor can be started at a relatively low hydraulic retention time of about 8 hours (Chapter 3.1). Similar results with mesophilic granular sludge are recently described by Ohtsuki *et al.* (1992). The disadvantage of using mesophilic sludge is the formation of a large volume of inactive, dead biomass.

In contrast to mesophilic granular sludge, the mesophilic activity of the digested OFMSW inoculum was not influenced by a thermophilic period of one month (Van Lier *et al.*, 1993).

The results of the latter experiments are not further evaluated in the context of this thesis.

The rate of granulation of thermophilic biomass, as well as the properties of the formed granules, strongly depend on the composition of the influent. The start-up of a thermophilic UASB reactor inoculated with mesophilic granular sludge and fed with completely acidified substrate (VFA mixture) leads to a disintegration of the mesophilic granules. This is followed by a wash-out of inactive biomass, including the newly grown thermophilic bacteria (Chapter 3). Apparently, the formation of thermophilic granular sludge hardly proceeds with a VFA mixture as the substrate. The inability to produce thermophilic granular sludge with solely VFA as feed has been confirmed by others (Wiegant and Lettinga, 1985; Uemura and Harada, 1993). However, when the influent is not completely acidified formation of thermophilic granules does take place (Chapter 6). Apparently, thermophilic acidifiers and/or their excretion products are of great importance for the formation of thermophilic granular sludge. Also, with digested OFMSW as inoculum, granule formation was found to be strongly influenced by the influent composition. The results reveal that for both types of inoculum a small fraction of the substrate (about 10%) should consist of non-acidified organic matter (e.g. sucrose) in order to promote the formation of active and well settleable granules.

Research performed by Wiegant (1986) proves that the start-up procedure of a thermophilic reactor with unadapted seed sludge is similar to that of a mesophilic reactor. After an adaptation period of several weeks thermophilic methanogenesis starts, followed by a rapid increase in the methane production rate (Chapters 3.1 and 6.1). Because of the high maximum growth rate of thermophilic bacteria the rate of increase in methane production is much faster than during a mesophilic start-up. At high temperatures, the bacterial decay rate increases which can lead to start-up problems when complex (and non-acidified) wastewaters are treated. The latter is of particular importance during a batch-wise start-up of a reactor. For an efficient treatment of complex wastewaters, a balanced consortium of various bacterial subpopulations is necessary. The development of a methanogenic flora will proceed very slowly, due to a lack of substrate, when hydrolysis and/or acidification are the rate-limiting steps in the overall conversion process. Therefore, if complex wastewater is treated, the addition of a simple methanogenic substrate may distinctly enhance the start-up. In the research presented here, simple substrates as mixtures of VFA and sucrose were used and no significant start-up problems were encountered.

## 2 Temperature Optima and Thermo-sensitivity

The temperature characteristics of thermophilic anaerobic sludge are, to a great extent, determined by the applied process conditions. Results presented here reveal that sludge cultivated in high-rate reactors (e.g. UASB) have a temperature optimum which is independent of the cultivation temperature in the range between 45 and 65°C. With respect

to acetate, being the most important methanogenic substrate, only one single temperature optimum was found at 60/65°C for sludge cultivated in UASB reactors at 46, 55 and 64°C (Chapter 4.1). The optimum temperature for propionate degradation is 55/60°C. The methanogenic consortia in the thermophilic UASB reactors were apparently dominated by a single methanogenic species, despite the different cultivation temperatures. Therefore, considering practical implementations, one may expect the cultivation of thermophilic sludge with a high thermostability if reactors with a high sludge retention are used. The optimal process temperature of such a reactor is about 55/58°C with permissible fluctuations of 5°C. However, at temperatures exceeding 60°C, the degradation of propionate becomes the rate-limiting step in the whole process. Results reveal that, if necessary, lower temperatures can also be applied without any problem, e.g. during reduced influent supply. In that case, a temperature rise will result in a direct acceleration of the methanogenic process.

Thermophilic sludge cultivated in completely mixed or batch loaded reactors shows a narrow temperature span, while the temperature optimum is determined by the applied cultivation temperature. Cultivation of thermophilic sludge in batch reactors at 46, 55 and 64°C resulted in sludge with three different temperature optima (Chapter 4.1). Therefore, a high thermo-sensitivity can be expected if this type of process is used for thermophilic treatment.

Whether thermophilic methanogenic sludge with a narrow or wide temperature span is cultivated depends on the prevailing selection criteria for the thermophilic organisms. At high substrate concentrations, the fastest growing species will dominate. High substrate concentrations are not only common in batch loaded and, to a lesser extent, completely mixed anaerobic systems, but also during the start-up of high-rate reactors (Chapter 4.1). Various methanogens with different temperature optima between 45-65°C are described in the literature (Table 4.2). However, for the formation of thermophilic granular sludge, other selection criteria than the actual process temperature are more important, such as the substrate affinity and adherence capacity of the methanogens. Methanogens that fulfil these requirements belong mainly to the genus of *Methanotherix*. Most of the known thermophilic *Methanotherix* species have their optimum growth rate in the temperature range between 60-65°C.

Because of the possible existence of one or more temperature optima, the sensitivity of thermophilic methanogenic sludge for temperature fluctuations also depends on the applied process conditions. In general, temperatures exceeding the optimum growth temperature will lead to much higher decay rates of the microorganisms and thus to a loss of the methanogenic activity. Because sludge retention systems are characterized by a single temperature optimum, these systems are preferable to completely mixed systems. A drop in temperature also leads to a decrease in the maximum activity of methanogenic sludge. However, in the latter case, the activity decrease is completely reversible.

Another factor determining the thermo-sensitivity of thermophilic sludge is the formation of aggregates and/or granules. Results reveal that the maximum methanogenic activity of thermophilic granular sludge is limited by the mass transfer rate of the substrate into the granule (Chapter 4.2). Crushed granular sludge possessed a 2-3 times higher maximum methanogenic activity than the intact granules at high substrate concentrations (Fig. 4.6). This means that even at high substrate concentrations only a part of the granule is active. By lowering the temperature, a larger part of the granule will become active. This can be attributed to the lower activity at lower temperatures which results in a deeper penetration of the substrate into the granule. In effect, the reduction of the maximum methanogenic activity due to a decrease in temperature is compensated by an increase in active biomass that participate in the digestion process. Consequently, the methanogenic activity of thermophilic granular sludge will remain at the same level despite a drop in temperature. Results described in Chapter 4.2 reveal a maximum acetate degrading activity of thermophilic granular sludge of about  $2.5 \text{ g COD.g}^{-1} \text{ VSS.day}^{-1}$  for the whole temperature range between 50 and 65°C. Furthermore, experiments with UASB reactors revealed that the overall activity of the sludge bed hardly decreased when the temperature was lowered from 55 to 35°C. In contrast, the maximum methanogenic activity of dispersed sludge was strongly dependent on the applied process temperature. Due to mass transfer limitation in the sludge granules, the substrate affinity will also increase as a result of a drop in temperature. Consequently, the thermo-sensitivity will be even less at low substrate concentrations. It should be noted that low substrate concentrations can be easily realized by applying high-rate reactors with a high sludge retention.

### 3 Application of Thermophilic Sludge under Mesophilic Conditions

The above results show that thermophilic sludge can be used at lower temperatures. The maximum methanogenic activity of the thermophilic granular sludge remains relatively high in the temperature range between 35 and 40°C, i.e. about  $1 \text{ g COD.g}^{-1} \text{ VSS.day}^{-1}$  (Chapter 4.2). This gives the practical advantage of using the thermophilic sludge under mesophilic conditions and, periodically, at high temperatures, without the loss of active biomass. A decrease in temperature can be applied during periods of low loading conditions. Because the decay rate of thermophilic sludge decreases at lower temperatures, a larger fraction of the thermophilic methanogens will be preserved at conditions of low substrate. Recent research reveals that lowering the temperature from 55°C to 32.5°C for a period of 3 months has no effect on the temperature optimum of the sludge (Van Lier and Lettinga, 1993). A subsequent increase in temperature from the mesophilic to the thermophilic range resulted in a complete recovery of thermophilic methanogenic activity within a few days. The duration of the acclimatization period to the newly installed high temperature is dependent on the duration and extent of the decrease in process temperature. A possible disadvantage of such an application is the higher sensitivity of thermophilic sludge under mesophilic conditions, which

can be attributed to a lack of a 'biomass-buffer'. Due to the much lower specific activity of the thermophilic sludge at low temperatures, substrate conversion is not limited by mass transfer resistance under mesophilic conditions. Therefore, changes in environmental conditions, e.g. temperature, pH, toxic compounds, will directly affect the anaerobic digestion process. If the process temperature is used as a control parameter for the thermophilic treatment, then the start-up period should be completed at high temperatures (55°C) prior to an eventual temperature drop! Otherwise, the appropriate biomass may not yet be cultivated. Because of the possible, more flexible, management of the process, the gained insight distinctly increases the applicability of thermophilic anaerobic treatment. This, however, only applies for high-rate systems and not or hardly for complete mixed systems.

#### 4 Thermophilic Process Technology

So far, thermophilic anaerobic treatment processes are often characterized by high concentrations of VFA, particularly propionate, in the effluent (Chapter 5). Both literature and experimental results reveal that low effluent VFA concentrations are only possible under relatively low loading conditions. The less efficient conversion process under thermophilic conditions can be partly explained by the lower substrate affinity of the thermophilic microorganisms. In addition, the performed research shows that thermophilic acetogenic and methanogenic bacteria are inhibited by high concentrations of intermediates, such as hydrogen and acetate (Chapters 5 and 6). As a consequence, in a thermophilic mixed sludge bed reactor, e.g. UASB, a complete VFA removal is limited by substrate, product, and/or non-competitive inhibition. A single-stage UASB reactor under high loading conditions will also be limited by an excessive carry-over of viable sludge resulting from the extremely high gas load ( $> 20 \text{ m}^3 \cdot \text{m}^{-3} \cdot \text{day}^{-1}$ ).

The results presented in this thesis have led to the development and application of a staged digestion process in a reactor denominated as the Upflow Staged Sludge Bed (USSB) reactor (Chapter 6). In applying such a multiple-step system, with various gas separators along the reactor height, the above mentioned inhibition effects of intermediate products will be minimized. The obtained results reveal that staging the thermophilic high-rate process has a very positive effect on the loading potentials of a thermophilic reactor. This can be attributed to:

- very low concentrations of intermediate compounds, such as acetate and hydrogen, in the final compartments. These compounds are formed during acidification and acetate formation in the lower compartments and are removed from the system due to the compartmentalized set-up. A low concentration of acetate and hydrogen results in a high removal rate of acetate and propionate;
- interim removal of the produced biogas along the reactor height enhances the sludge retention;

- due to staging, well-adapted sludge is developed with different kinetic, physical and morphological properties in the various compartments.

Staging of the thermophilic digestion process leads to the development of sludge which is well adapted to the local conditions of a specific compartment. The sludge development in each compartment is influenced by the sludge development in the previous stage, due to the free connection between the various compartments. Consequently, the properties of the sludge from a specific compartment will change during continuous reactor operation. However, in order to obtain a stable digestion process, establishment of a 'steady state' in the staged sludge development should be pursued. The latter is only possible if the excess sludge produced is withdrawn from each compartment. In using an upflow reactor, like the USSB, sludge withdrawal from one extraction point at the bottom of the reactor will very likely suffice, since bacteria with the highest growth yield will accumulate there.

Both completely and partially acidified wastewater can be treated efficiently at high loading rates using the USSB reactor concept. Treatment of partially acidified wastewater will lead to thermophilic granular sludge with a high specific methanogenic activity and a good settleability. Preservation of the 'high-grade' thermophilic granules in the staged upflow reactor can be achieved by withdrawing the excess sludge produced from the first (acidifying) stage. Treatment of completely acidified wastewater leads to the formation of a more or less dispersed sludge, also with a high specific methanogenic activity. Despite the dispersed nature of the sludge, a high biomass retention is achieved due to the instalment of the various gas-solids separators along the reactor height. In contrast to the results with mixed single-stage sludge bed reactors with dispersed sludge (Chapter 3.1), the thermophilic treatment of VFA in the USSB was not limited by an excessive wash-out of active biomass.

It is recommendable to test the USSB reactor concept on pilot scale with the use of non-synthetic industrial wastewater. A possible up-scaling can be done according to the Biogas-Turm reactor as described recently by Märkl and Reinhold (1994). This new type of anaerobic reactor has, in agreement with the USSB reactor, several gas separators at different heights along the reactor. The Biogas-Turm reactor was particularly designed, for a more efficient gas removal. Very good results were achieved with the treatment of yeast wastewater in a 20 m<sup>3</sup> pilot reactor (Reinhold *et al.*, 1994). The evaluation of the results, however, did not examine the biological characteristics of the cultivated sludge at the different heights of the reactor. In addition to an efficient gas separation, it is also of great importance to create optimal conditions in the various compartments for an efficient biological treatment. As mentioned before, the latter is particularly true for thermophilic conditions. Several researchers described stable process conditions using a thermophilic two-step system (e.g. Kida *et al.*, 1992; Ohtsuki *et al.*, 1994; Lanting *et al.*, 1989, Wiegant *et al.*, 1986), whereby in some cases, the second step was operated under mesophilic conditions

(Kaiser *et al.*, 1994). In all these studies, the thermophilic process was divided over two separate reactors. Results presented in this thesis show that a spatial division of the thermophilic treatment process occurs spontaneously if a staged reactor is applied. The construction of a series of several reactors, which may involve high costs, is not necessary. Based on our results, one reactor subdivided into various compartments is advisable in order to enhance and sustain a good sludge development. In comparison to the choice of using a multiple-step thermophilic digestion process, the matter of using an upflow or side-flow system is of minor importance. In the scaling-up and construction of 'horizontal-flow' systems, the various modules will also be upflow reactors equipped with gas-solids separators.

## Samenvatting en Discussie

Sedert de jaren '80 is de interesse in thermofiele anaërobe zuivering sterk toegenomen. De toepassing van een hogere procestemperatuur kan leiden tot een versnelling van het anaërobe afbraakproces. Immers, naarmate de temperatuur stijgt nemen de reactiesnelheden en bacteriële groeisnelheden in belangrijke mate toe. Deze versnelling van het afbraakproces wordt echter gelimiteerd door het temperatuurbereik van de betrokken microorganismen. Methanogene bacteriën uit het mesofiele gebied (20-40°C) sterven snel af bij temperaturen > 45°C (Hst 3). Thermofiele organismen bezitten hun optimale groeisnelheid juist boven de 45°C.

Dit proefschrift beschrijft de resultaten van onderzoek naar een nadere karakterisering van het thermofiele anaërobe zuiveringsproces. Er is met name aandacht besteed aan de opstart en thermo-stabiliteit van het thermofiele proces en de factoren die hierop van invloed zijn (Hst. 3, 4). Bij het onderzoek naar een verdere optimalisatie van het thermofiele proces (Hst. 5, 6) is de aandacht vooral gericht op het realiseren van een zo stabiel mogelijke procesvoering met een zo hoog mogelijk zuiveringsrendement. Er is met name aandacht besteed aan het behalen van een lage concentratie aan vluchtige vetzuren in het effluent. Uit het onderzoek blijkt dat bij de toepassing van het juiste reactorconcept lage vetzuurconcentraties kunnen worden gehandhaafd bij extreem hoge belastingen ( $\pm 100 \text{ kg CZV} \cdot \text{m}^{-3} \cdot \text{d}^{-1}$ ). De achterliggende vraag bij dit promotie-onderzoek was op welke wijze een thermofiele anaërobe afvalwaterzuiveringsreactor moet/kan worden bedreven bij sterk wisselende belastingen en/of temperaturen. In het onderstaande worden de belangrijkste resultaten nader toegelicht.

### 1 Thermofiele Opstart

De opstart van een thermofiele anaërobe zuiveringsreactor kan worden uitgevoerd met vrijwel elk mesofiel entmateriaal met "enige" methanogene activiteit. Uit het onderzoek van Wiegant (1986) bleek reeds de toepasbaarheid van (vergist) koemest en vergist rioolslib voor een thermofiele opstart. Binnen het kader van het onderhavige onderzoek zijn met name mesofiel anaëroob korrelslib en vergist groente, fruit en tuinafval (GFT) nader onderzocht op hun geschiktheid als entmateriaal.

Bij gebruik van mesofiel korrelslib voor de opstart van een thermofiele reactor (> 45°C) vindt afsterving van de methanogene populatie in de korrels plaats, maar aanwezige thermofiele zullen beter gaan gedijen. De afsterving van mesofielen wordt gevolgd door een toename van thermofiele organismen, echter voornamelijk aan de buitenkant en in de ontstane holten van de "mesofiele" korrels (Hst. 3.2). Omdat hechting van thermofiele organismen aan en in de korrels plaatsvindt kan de reactor bij een relatief lage hydraulische verblijftijd (8 uur) worden opgestart (Hst 3.1). Vergelijkbare resultaten met mesofiel korrelslib zijn recent beschreven door Ohtsuki *et al.* (1992). Het nadeel van het gebruik van mesofiel

korrelslib is echter de vorming van een groot volume aan inactieve (afgestorven) biomassa.

In tegenstelling tot mesofiel korrelslib werd de mesofiele activiteit van het GFT inoculum niet beïnvloedt door een thermofiele periode van één maand (Van Lier *et al.*, 1993). De resultaten van deze experimenten zijn in het bestek van dit proefschrift verder niet besproken.

De snelheid van het thermofiele korrelvormingsproces alsmede de aard van het gevormde korrelslib is sterk afhankelijk van de samenstelling van het influent. De opstart van een thermofiele UASB geënt met mesofiel korrelslib en gevoed met volledig verzuurd substraat (vetzuurmengsel) leidt tot desintegratie van de mesofiele korrels en daarmee tot uitspoeling van afgestorven mesofiel slib met ingegroeid actief thermofiel bacteriemateriaal (Hst. 3). De vorming van thermofiel korrelslib vindt onder deze condities niet of nauwelijks plaats. Dit verschijnsel is door meerdere onderzoekers waargenomen (Wiegant and Lettinga, 1985; Uemura and Harada, 1993). Indien echter het influent níet geheel verzuurd is, vindt wél vorming van thermofiele korrels plaats (Hst. 6). Blijkbaar zijn de thermofiele verzuurders en/of hun afbraakproducten van groot belang voor het thermofiele korrelvormingsproces. Ook bij gebruik van vergist GFT als inoculum wordt het korrelvormingsproces in sterke mate door de influentsamenstelling bepaald. Bij gebruik van beide entmaterialen is het voor de vorming van actief, goed bezinkbaar korrelslib essentieel dat een (geringe) fractie (10%) onverzuurd materiaal (sucrose) aanwezig is.

Zoals reeds bleek uit het onderzoek van Wiegant (1986) is de te volgen opstartprocedure van een thermofiele reactor met niet geadapteerd slib vrijwel gelijk aan die van een mesofiele reactor. Na een adaptatie periode van enkele weken komt het thermofiele afbraakproces op gang, wat wordt gevolgd door een snelle toename van de methaanproductie (Hst. 3.1, 6.1). Door de hogere groeisnelheden van thermofiele bacteriën is de snelheid van deze toename groter dan bij een mesofiele opstart. Echter, aangezien bij hoge temperaturen de afstervingsnelheden ook groter zijn, kunnen 'opstart-problemen' ontstaan bij de behandeling van complexe (en niet-verzuurde) afvalwaters, met name bij een ladingsgewijs uitgevoerde opstart. Voor een efficiënte behandeling van complex afvalwater is een gebalanceerd consortium van diverse bacteriepopulaties noodzakelijk. Indien de hydrolyse of vetzuurvorming de snelheidsbepalende stap is, zal de methanogene populatie zich slechts moeizaam kunnen ontwikkelen door gebrek aan substraat. Bij een dergelijk afvalwater zal toevoeging van een eenvoudig methanogeen substraat de opstart aanzienlijk kunnen versnellen. Door het gebruik van relatief eenvoudige substraten in het onderhavige onderzoek (mengsels van vetzuren en sucrose) zijn geen opstartproblemen van enige betekenis waargenomen.

## 2 Temperatuuroptima en Temperatuurgevoeligheid

De temperatuurkarakteristieken van thermofiel anaëroob slib worden in grote mate bepaald door de opgelegde procescondities. Uit het onderzoek blijkt, dat in het temperatuur gebied

45-65°C thermofiel slib gekweekt in reactoren met een hoge slibretentie (b.v. UASB reactor) een temperatuuroptimum heeft dat onafhankelijk is van de kweektemperatuur. Ten aanzien van de afbraak van azijnzuur, het belangrijkste methanogene substraat, werd slechts één optimum gevonden bij 60-65°C voor slib gekweekt in UASB reactoren bij 46, 55 of 64°C (Hst. 4.1). Voor de afbraak van propionzuur bleek het optimum bij 55-60°C te liggen. De thermofiele slibsoorten uit de UASB reactoren werden, ondanks het grote verschil in de kweektemperatuur, gedomineerd door dezelfde methanogenen. Met het oog op de praktische toepassing betekent dit, dat, thermofiel slib met een hoge thermostabiliteit zal worden verkregen indien thermofiele reactoren met een hoge slibretentie worden aangewend. De optimale procestemperatuur van een dergelijke reactor bedraagt 55-58°C, waarbij temperatuurfluctuaties van 5°C toelaatbaar zijn. Bij temperaturen > 60°C zal de afbraak van propionzuur echter de limiterende factor van het proces worden. Indien nodig kan het thermofiele proces eveneens bij een lagere temperatuur worden bedreven, bijvoorbeeld tijdens een verminderde substraataanvoer. Toepassing van een hogere procestemperatuur leidt dan vervolgens direct tot een versnelling van het methanogene proces.

Thermofiel slib gekweekt in volledig gemengde- of ladingsgewijs (= batch-) bedreven reactoren heeft een nauw temperatuurtraject, waarbij het temperatuuroptimum in grote mate wordt bepaald door de toegepaste kweektemperatuur. Ladingsgewijze procesvoering bij een kweektemperatuur tussen 45-65°C, leidt tot meerdere temperatuuroptima (Hst. 4.1). Hierdoor is een grote temperatuurgevoeligheid te verwachten indien deze procesvoering wordt aangewend voor het thermofiele vergistingsproces.

Het grote verschil in temperatuurgevoeligheid kan worden verklaard uit de geldende selectiecriteria ten aanzien van de thermofiele microorganismen. Bij hoge substraatconcentraties gaan de snelst groeiende organismen in het slib domineren. In de literatuur zijn diverse methanogenen beschreven met een optimale groeitemperatuur tussen 45-65°C (Tabel 4.2). Afhankelijk van de hoogte van de procestemperatuur zullen bepaalde bacteriën gaan domineren. Hoge substraatconcentraties zijn niet alleen kenmerkend voor anaërobe volledig gemengde- en ladingsgewijs bedreven reactoren, maar ook voor de opstartperiode van reactoren met een hoge slibretentie (Hst 4.1). Voor de vorming van thermofiel anaëroob (korrel)slib in reactoren met een hoge slibretentie zijn andere selectiecriteria zoals, hechtingscapaciteit en substraataffiniteit van groter belang dan de actuele procestemperatuur. De methanogenen die aan deze criteria voldoen zijn voornamelijk *Methanothrix spec.* en/of aanverwante soorten. Vrijwel alle bekende thermofiele *Methanothrix spec.* vertonen maximale groei bij 60-65°C.

Overeenkomstig het voorkomen van één of meerdere temperatuuroptima is ook de gevoeligheid van thermofiel slib voor temperatuurfluctuaties afhankelijk van de gekozen procescondities. In het algemeen geldt dat een toename van de temperatuur tot voorbij de maximale temperatuur leidt tot een zeer hoge afstervingsnelheid van de microorganismen en dus tot verlies van methanogene activiteit. Omdat slibretentiesystemen slechts één temperatuur-

optimum in het thermofiele gebied (45-65°C) vertonen, verdienen deze systemen verre de voorkeur boven het gebruik van volledig gemengde reactoren. Een verlaging van de procestemperatuur leidt eveneens tot een lagere maximale activiteit van thermofiel methanogeen slib, maar deze activiteitsafname is geheel reversibel (Hst 4.2); zie ook punt 3.

De gevoeligheid van thermofiel slib voor temperatuurflictuaties is verder afhankelijk van de vorming van aggregaten en/of korrels. Uit het onderzoek is gebleken dat de maximale methanogene activiteit van thermofiel korrelslib wordt gelimiteerd door de diffusiesnelheid van het substraat in de korrels (Hst. 4.2). In vergelijking met intact korrelslib werd met het vermalen korrelslib bij hoge substraatconcentraties een 2-3 maal hogere maximale methanogene activiteit gemeten (Fig. 4.6). Dit betekent dat zelfs bij hoge substraatconcentraties slechts een gedeelte van de biomassa in de korrel actief is. Een verlaging van de temperatuur leidt tot een lagere maximale activiteit waardoor het substraat verder in de korrel kan doordringen en een groter gedeelte van de korrel actief wordt. Met andere woorden de afname van de maximale methanogene activiteit ten gevolge van een temperatuuurdaling wordt gecompenseerd door de grotere hoeveelheid biomassa die kan bijdragen aan het vergistingsproces. In het temperatuurgebied tussen 50-65°C ontplooide thermofiel korrelslib, gekweekt in een 55°C USSB reactor, globaal een zelfde azijnzuur verwijderende methanogene activiteit van  $\pm 2.5$  g CZV/g OS.d (Fig. 4.6). Bij experimenten uitgevoerd in een UASB reactor bleek de 'overall' activiteit van het slibbed nauwelijks af te nemen bij een temperatuurverlaging van 55°C naar 35°C (Fig. 4.9). In vergelijking met thermofiel korrelslib is de maximale activiteit van gesuspendeerd thermofiel slib wel sterk temperatuurafhankelijk en veel gevoeliger voor variaties in de temperatuur.

Ten gevolge van het gelimiteerde massatransport neemt eveneens de substraataffiniteit toe naarmate de temperatuur daalt. Hierdoor zal bij lage substraatconcentraties de temperatuurgevoeligheid verder afnemen. Het verkrijgen van lage substraatconcentraties kan eenvoudig worden gerealiseerd bij de toepassing van systemen met een hoge slibretentie.

### 3 Aanwenden van Thermofiel Slib onder Mesofiele Condities

Uit het voorgaande blijkt dat thermofiel slib eveneens bij lagere temperaturen kan worden aangewend. Met name in het temperatuurgebied 35-40°C is de methanogene activiteit van thermofiel (korrel)slib nog aanzienlijk ( $\pm 1$  g CZV/ g OS.d) (Hst 4.2). Het grote praktische voordeel van het gebruik van thermofiel slib onder mesofiele condities is dat de mogelijkheid bestaat om periodiek bij hogere temperaturen te zuiveren zonder dat het slib afsterft. De mogelijkheid van verlaging van de procestemperatuur kan ook worden gebruikt om een periode van lage belasting te overbruggen. Aangezien ook de afstervingsnelheid van thermofiel slib bij lagere temperaturen afneemt, zal een grotere fractie van de thermofiele me'ia' behouden blijven, indien bij een verminderde substraataanvoer de procestemperatuur wordt verlaagd. Uit recent onderzoek blijkt dat een verlaging van de procestemperatuur van 55 naar 32.5°C gedurende een periode van 3 maanden geen effect heeft op

het temperatuuroptimum van het slib (Van Lier and Lettinga, 1993). Na terugbrengen van de procestemperatuur in het thermofiele gebied werd binnen enkele dagen de methanogene activiteit van vóór de temperatuurverlaging weer bereikt. Evenals bij mesofiel slib is de duur van een acclimatisatieperiode afhankelijk van de duur en mate van de temperatuurverlaging. Een nadeel bij een dergelijke toepassing betreft de grotere gevoeligheid van het thermofiele slib onder mesofiele condities. Dit kan worden geweten aan het feit dat bij lage temperaturen de gehele thermofiele biomassa actief is. Door de veel lagere specifieke activiteit van het thermofiele slib bij lage temperaturen worden de omzettingen niet meer door massa transport gelimiteerd. Een veranderende milieu-omstandigheid (b.v. temperatuur) heeft hierdoor een direct effect op het vergistingsproces. Indien de procestemperatuur wordt gebruikt als controleparameter van het thermofiele afbraakproces, is het noodzakelijk dat de opstartperiode bij hogere temperaturen (55°C) op de juiste wijze tot een goede afronding is gebracht!

De verkregen inzichten vergroten de toepassingsmogelijkheden aanzienlijk want ze bieden de mogelijkheid tot een veel flexibelere bedrijfsvoering van het thermofiele anaërobe zuiveringsproces. Met nadruk willen we er hier echter op wijzen dat door de hoge temperatuurgevoeligheid van volledig gemengde reactoren deze flexibiliteit met name, zo niet uitsluitend, geldt voor reactoren met een hoge slibretentie.

#### 4 Thermofiele Procestechnologie

Bestaande anaërobe thermofiele zuiveringsprocessen worden vaak gekenmerkt door een hoge concentratie aan vetzuren, met name propionzuur, in het effluent (Hst. 5). Lage effluent vetzuur concentraties werden alleen verkregen bij relatief laag belaste systemen. Deze minder efficiënte afbraak kan slechts gedeeltelijk worden verklaard uit de lagere substraataffiniteit van de thermofiele microorganismen. Uit onderzoek is gebleken dat de thermofiele azijnzuurvorming en methanogenese worden geremd door een hoge concentratie aan intermediaire afbraakproducten zoals waterstof en azijnzuur (Hst 5, Hst. 6). Dit betekent dat in een thermofiel gemengd slibbed, b.v. in een UASB reactor, een vergaande verwijdering van vetzuren wordt gelimiteerd door het optreden van zowel produkt-, substraat-, en/of niet-competitieve inhibitie. Een hoogbelaste thermofiele ééntraps UASB reactor wordt bovendien gelimiteerd door een hoge slibuitspoeling ten gevolge van de in het systeem heersende extreem hoge gasbelasting ( $> 20 \text{ m}^3 \cdot \text{m}^{-3} \cdot \text{d}^{-1}$ ).

De verkregen inzichten hebben geleid tot aanpassing van het bestaande UASB-reactorconcept en het aanwenden van een gebalanceerd meerfasesysteem, de zogenaamde 'upflow staged sludge bed' of USSB reactor (Hst. 6). Bij de toepassing van een dergelijk meerfasesysteem met tussentijdse gasafscheiding hebben de afbraakproducten die tijdens de verzuring en azijnzuurvorming worden geproduceerd géén nadelig effect meer op een vergaande verwijdering van deze producten. Uit het onderzoek blijkt dat compartimentering, danwel

fasering van het thermofiele anaërobie 'high rate' proces een zeer positieve invloed heeft op het toepasbare belastingpotentiëel en de te behalen effluent vetzuurconcentraties. Een en ander is toe te schrijven aan:

- zeer lage concentraties intermediaire afbraakproducten (zoals azijnzuur en waterstof) in de laatste compartimenten. Hoge concentraties waterstof, welke ontstaan bij de verzuring van het substraat in de eerste compartimenten, worden in het meerfasesysteem vroegtijdig weggevangen. Een lage concentratie azijnzuur en waterstof leidt tot een vergaande verwijdering van propionzuur en azijnzuur.
- tussentijdse afvangst en afvoer van het geproduceerde biogas over de hoogte van de reactor wat leidt tot een betere slibretentie.
- ontwikkeling van aangepaste slibsoorten met verschillende kinetische, fysische en morfologische eigenschappen in de diverse compartimenten.

Compartimentering leidt tot de ontwikkeling van aan de lokaal heersende omstandigheden goed aangepaste slibsoorten met specifieke eigenschappen. Aangezien de compartimenten niet volledig gescheiden zijn, wordt de slibontwikkeling in elk compartiment beïnvloed door de slibontwikkeling in de voorgaande compartimenten waardoor de eigenschappen van het gekweekte slib gedurende de opstart en continue procesvoering zullen veranderen. Het verkrijgen van een min of meer 'steady state' in deze ontwikkeling is alleen mogelijk indien uit alle compartimenten afzonderlijk slib wordt gespuid. Bij gebruik van een upflow reactor zoals de USSB reactor, kan worden volstaan met het spuien van het slib uit het onderste compartiment, aangezien de bacteriën met de hoogste groeisnelheid en de hoogste groeiopbrengst zich onder in de reactor ophopen. Voor optimalisatie van het proces is het verkrijgen van een 'steady state' in de slibontwikkeling van groot belang.

Bij gebruik van de USSB reactor kunnen zowel gedeeltelijk als volledig verzuurd afvalwater effectief bij hoge belastingen worden behandeld. Behandeling van gedeeltelijk verzuurd afvalwater leidt hierbij tot de ontwikkeling van thermofiel korrelslib met een hoge specifieke methanogene activiteit en een goede bezinkbaarheid. Bij gefaseerde reactoren dient het spuislib voornamelijk uit de eerste (verzurende) fase te worden onttrokken om een hoge specifieke methanogene en acetogene activiteit van het thermofiele slib in de volgende compartimenten te kunnen garanderen. Behandeling van volledig verzuurd afvalwater leidt tot de vorming van een meer dispers slib met een hoge specifieke methanogene activiteit. Door het toegepaste reactorconcept is ook in dit geval de slibuitspoeling beperkt en niet limiterend voor het thermofiele afbraakproces. Dit in tegenstelling tot bedrijfsvoering in reactoren met een volledig gemengd slibbed.

Het verdient aanbeveling om het USSB reactor concept op 'pilot'schaal te testen met gebruik van praktijk afvalwater. Opschaling van het systeem zou kunnen geschieden overeenkomstig de Biogas-Turmreactor die onlangs is beschreven door Märkl and Reinhold (1994). Dit nieuw type anaërobie reactor heeft overeenkomstig de USSB reactor diverse gasafscheiders over de

hoogte van de reactor. De Biogas-Turmreactor is ontworpen ten behoeve van een meer efficiënte gasafvoer. Er werden zeer goede resultaten behaald bij de behandeling van gistaafvalwater in een 20 m<sup>3</sup> pilot reactor (Reinhold *et al.*, 1994). Bij de beschrijving van de resultaten zijn de onderzoekers echter niet nader ingegaan op de biologische karakteristieken van het gekweekte slib over de hoogte van de reactor. Met name onder thermofiele condities is, zoals boven opgemerkt, naast het aanbrengen van een efficiënte gasafvoer, het creëren van optimale lokale condities van groot belang voor het verkrijgen van een vergaande biologische zuivering. Meerdere onderzoekers beschreven een zeer stabiele procesvoering bij gebruik van een thermofiel twee-trap systeem (Kida *et al.*, 1992; Lanting *et al.*, 1989; Ohtsuki *et al.*, 1994; Wiegant *et al.*, 1986), waarbij in sommige gevallen de tweede trap onder mesofiele condities werd bedreven (Kaiser *et al.*, 1994). In al deze studies werd het thermofiele anaërobe proces verdeeld over twee afzonderlijke reactoren. Uit de resultaten van het onderhavige onderzoek blijkt echter dat een ruimtelijke scheiding van het thermofiele afbraakproces vanzelf plaatsvindt indien een gefaseerde reactor wordt aangewend. De constructie van meerdere afzonderlijke reactoren die in serie worden bedreven, hetgeen aanzienlijk hogere kosten met zich mee brengt, is niet nodig. Ten behoeve van een goede slibontwikkeling en slibretentie is fasering in een geïntegreerd één-reactor systeem wenselijk. Bij de toepassing van thermofiele anaërobe afvalwaterzuivering is de keuze tussen een opwaarts- danwel zijwaartsdoorstroomde reactor van veel geringer belang dan de vraag of een meertrap zuiveringsproces moet worden aangewend. Bij de opschaling van een zijwaartsdoorstroomde reactor zullen de afzonderlijke compartimenten worden bedreven als opwaartsdoorstroomde reactoren met afzonderlijke gasafscheiders.

## References

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- Achenbach-Richter L, Gupta R, Stetter KO and Woese CR (1987). Were the original eubacteria thermophiles?. *System Appl Microbiol*, 9: 34-39.
- Ahring BK (1991). Methanogenesis during thermophilic anaerobic digestion with focus on acetate. In: Int. Symp. on Environmental Biotechnology, part 1, Verachtert H and Verstraete W (eds), Oostende (Belgium), Koninklijke Vlaamse Ingenieurs-vereniging VZW, Antwerp, Belgium, pp 275-283.
- Ahring BK (1992). Turn-over of acetate in hot springs at 70°C. In: Thermophiles: Science and Technology, Intern. Conf., Reykjavík, Iceland, 23rd-26th August 1992, IceTec Publ., p 130.
- Ahring BK (1994). Status on science and application of thermophilic anaerobic digestion. *Wat Sci Technol*, 30-12: 241-249.
- Ahring BK (1995). Methanogenesis in thermophilic bioreactors. *Antonie van Leeuwenhoek*, 67: 91-102.
- Ahring BK, Rintala J, Nozhevnikova AN and Mathrani IM (1995). Metabolism of acetate in thermophilic (55°C) and extreme thermophilic (70°C) UASB granules. In: Proc. of International Meeting on: Anaerobic Processes for Bioenergy and Environment, Copenhagen, 25-27 January, 1995.
- Ahring BK, Schmidt JE, Winther-Nielsen M, Macario AJL and Conway de Macario E (1993). Effect of medium composition and sludge removal on the production, composition and architecture of thermophilic (55°C) acetate-utilizing granules from an upflow anaerobic sludge blanket reactor. *Appl Environ Microbiol*, 59: 2538-2545.
- Ahring BK and Westermann P (1984). Isolation and characterization of a thermophilic, acetate-utilizing methanogenic bacterium. *FEMS Microbiol Lett*, 25: 47-52.
- Ahring BK and Westermann P (1985). Methanogenesis from acetate: physiology of a thermophilic acetate-utilizing methanogenic bacterium. *FEMS Microbiol Lett*, 28: 15-19.
- Ahring BK and Westermann P (1987). Thermophilic anaerobic degradation of butyrate by a butyrate-utilizing bacterium in coculture and triculture with methanogenic bacteria. *Appl Environ Microbiol*, 53: 429-433.
- Ahring BK and Westermann P (1988). Product inhibition of butyrate metabolism by acetate and hydrogen in a thermophilic coculture. *Appl Environ Microbiol*, 54: 2393-2397.
- Aitken MD and Mullennix RW (1992). Another look at thermophilic anaerobic digestion of wastewater sludge. *Water Environ Res*, 64: 915-919.
- Alphenaar PA (1994). Anaerobic granular sludge: characterization and factors affecting its functioning. PhD thesis, Department of Environmental Technology, Agricultural University, Wageningen, The Netherlands.
- Alphenaar PA, Pérez MC and Lettinga G (1993). The influence of substrate transport limitation

- on porosity and methanogenic activity of anaerobic sludge granules. *Appl Microbiol Biotechnol*, **39**: 276-280.
- Angelidaki I and Ahring BK (1994). Anaerobic thermophilic digestion of manure at different ammonia loads: effect of temperature. *Wat Res*, **28**: 727-731.
- Aoki N and Kawase M (1991). Development of high-performance thermophilic two-phase digestion process. *Wat Sci Technol*, **23**(7-9): 1147-1156.
- APHA, AWWA, WPCF (1985). Standard methods for the examination of water and wastewater. American Public Health Administration, Washington, D.C., USA
- Bachmann A, Beard VL and McCarty PL (1985). Performance and characteristics of the anaerobic baffled reactor. *Wat Res*, **19**: 99-106.
- Balch WE, Fox GE, Magrum LJ, Woese CR and Wolfe RS (1979). Methanogens: reevaluation of a unique biological group. *Microbiol Rev*, **43**: 260-296.
- Basu AK and Leclerc E (1975). Comparative studies on treatment of beet molasses distillery waste by thermophilic and mesophilic digestion. *Wat Res*, **9**: 103-109.
- Beeftink HH and Staugaard P (1986). Structures and dynamics of anaerobic bacterial aggregates in a gas-lift reactor. *Appl Environ Technol*, **52**: 1139-1146.
- Bendixen HJ (1994). Safeguards against pathogens in Danish biogas plants. *Wat Sci Technol*, **30**-12: 171-180.
- Bleicher K and Winter J (1994). Formate production and utilization by methanogens and by sewage sludge consortia - interference with the concept of interspecies formate transfer. *Appl Microbiol Biotechnol*, **40**: 910-915.
- Blomgren A, Hansen A and Svensson BH (1990). Enrichment of a mesophilic, syntrophic bacterial consortium converting acetate to methane at high ammonium concentrations. In: Belaich JP, Bruschi M and Garcia JL (eds), Microbiology Biochemistry of Strict Anaerobes Involved in Interspecies Hydrogen Transfer, Belaich JP (ed), Plenum Publ. Corp. New York, pp 225-234.
- Bochem HP, Schoberth SM, Sprey B and Wengler P (1982). Thermophilic biomethanation of acetic acid: Morphology and ultrastructure of a granular consortium. *Can J Microbiol*, **28**: 500-510.
- Bolle WL, Van Breugel J, Van Eybergen GC, Kossen NWF and Zoetemeyer RJ (1986). Modelling the liquid flow in up-flow anaerobic sludge blanket reactors. *Biotechnol Bioeng*, **28**: 1615-1620.
- Boone DR (1982). Terminal reactions in anaerobic digestion of animal waste. *Appl Environ Microbiol*, **43**: 57-64.
- Boone DR and Bryant MP (1980). Propionate-degrading bacterium, *Syntrophobacter wolinii* sp. nov. gen. nov., from methanogenic ecosystems. *Appl Environ Microbiol*, **40**: 626-632.
- Boone DR, Johnson RL and Liu Y (1989). Diffusion of the interspecies electron carriers H<sub>2</sub> and formate in methanogenic ecosystems, and applications in the measurement of K<sub>m</sub> for H<sub>2</sub> and formate uptake. *Appl Environ Microbiol*, **55**: 1735-1741.
- Boone DR and Xun L (1987). Effects of pH, temperature, and nutrients on propionate degradation by a methanogenic enrichment culture. *Appl Environ Microbiol*, **53**: 1589-1592.
- Borja R, Martín A, Banks CJ, Alonso V and Chica A (1995). A kinetic study of anaerobic digestion of olive mill wastewater at mesophilic and thermophilic temperatures. *Environm Pollution*, **88**: 13-18.

- Brock TD (ed) (1986). Thermophiles: general, molecular and applied biology. Wiley-Interscience, New York
- Brandis A, Thauer RK and Stetter KO (1981). Relatedness of strain  $\Delta$ H and Marburg of *Methanobacterium thermoautotrophicum*. *Zbl Bakt Hyg I Abt Orig*, C2: 311-317
- Braun R and Huss S (1982). Anaerobic digestion of distillery effluents. *Process Biochem*, 17-4: 25-27.
- Brune G, Schoberth S and Sahn H (1982). Anaerobic treatment of an industrial wastewater containing acetic acid, furfural and sulphite. *Process Biochem*, 17-3: 20-24.
- Bryant MP (1979). Microbial methane production - theoretical aspects. *J Animal Sc*, 48: 193-201.
- Bryniok D and Trösch W (1989). Taxonomy of methanogens by ELISA techniques. *Appl Microbiol Biotechnol*, 32: 243-247.
- Buhr HO and Andrews JF (1977). The thermophilic anaerobic digestion process. *Wat Res*, 11: 129-143.
- Cail R and Barford JP (1985). Thermophilic semi-continuous anaerobic digestion of palm-oil mill effluent. *Agr Wastes*, 13: 295-304.
- Catunda Frassinetti-Cavalcanti P, Van Haandel AC and Lettinga G (1994). Post treatment of anaerobically treated sewage in waste stabilization ponds. In: Paper Pre-prints of the Seventh International Symposium on Anaerobic Digestion, Cape Town, January 23-27, 1994, South Africa, pp 405-415.
- Chartrain M and Zeikus JG (1986). Microbial ecophysiology of whey biomethanation: Characterization of bacterial trophic populations and prevalent species in continuous culture. *Appl Environ Microbiol*, 51: 188-196.
- Cecchi F, Pavan P and Mata-Alvarez J (1992). Fast digester start-up under mesophilic conditions using thermophilic inoculum. *Wat Sci Technol*, 25-4/5: 391-398.
- Cecchi F, Pavan P, Mata-Alvarez J, Bassetti A and Cozzolino C (1991). Anaerobic digestion of municipal solid waste. Thermophilic versus mesophilic performance at high solids. *Waste Manag Res*, 9: 305-315.
- Chang R (1977). Physical chemistry with applications to biological systems. Macmillan Publishing Co., New York.
- Chen YR (1983). Biogas digester design. In: Wise, D.L. (ed.), Fuel Gas Systems. CRC Press, Boca Raton, Fla., USA (1983), pp 23-59.
- Chin KK and Wong KK (1983). Thermophilic anaerobic digestion of palm oil mill effluent. *Wat Res*, 17 993-995.
- Clarens M and Moletta R (1990). Kinetic studies of acetate fermentation by *Methanosarcina* sp. MSTA-1. *Appl Microbiol Biotechnol*, 33: 239-244
- Cooney CL and Wise DL (1975). Thermophilic anaerobic digestion of solid waste for fuel gas production. *Biotechnol Bioeng*, 17: 1119-1135.
- Deboosere S, De Baere L, Smis J, Six W and Verstraete W (1986). Dry anaerobic fermentation of concentrated substrates. In: Anaerobic treatment a grown-up technology, Aquatech '86, september 1986, Amsterdam, The Netherlands. Industrial Presentations (Europe) B.V., Schiedam, The Netherlands, pp 477-488.
- De Zeeuw WJ (1984). Acclimatization of anaerobic sludge for UASB reactor start-up, Ph.D. thesis, Department of Environmental Technology, Agricultural University, Wageningen, The Netherlands.

- De Zeeuw WJ** (1987). Granular sludge in UASB reactors. *In: Lettinga G, Zehnder AJB, Grotenhuis JTC, Hulshoff Pol LW* (eds), Proc. Granular anaerobic sludge; microbiology and technology, GASMAT workshop. Lunteren, October 25-27, 1987, The Netherlands, Pudoc Wageningen, The Netherlands, pp 132-145.
- Disley RS, Walmsley MJ and Forster CF** (1992). Inhibition of gas production by thermophilic anaerobic sludges: The effect of organic compounds. *Environ Technol*, **13**: 1153-1159.
- Dolfing J** (1987). Microbiological aspects of granular methanogenic sludge. Ph.D. Thesis, Department of Microbiology, Agricultural University, Wageningen, The Netherlands.
- Dong Xiuzhu** (1994). The role of formate and hydrogen in the syntrophic degradation of propionate and butyrate. Ph.D. Thesis, Department of Microbiology, Agricultural University, Wageningen, The Netherlands.
- Dubourguier HC, Archer DB, Albagnac G and Prensier G** (1988). Structure and metabolism of methanogenic, microbial conglomerates. *In: Hall ER, Hobson PN* (eds), Anaerobic digestion. Pergamon, Oxford, pp 13-23.
- Dudley BT, Howgrave-Graham AR, Bruton AG and Wallis FM** (1993). Image analysis to quantify and measure UASB digester granules. *Biotechnol Bioeng*, **42**: 279-283.
- Duff SJB and Kennedy KJ** (1982). Effect of hydraulic and organic overloading on thermophilic downflow stationary fixed film (DSFF) reactor. *Biotechnol Lett*, **4**: 815-820.
- Dürre P, Bahl H and Gottschalk G** (1988). Membrane processes and product formation in anaerobes. *In: Erickson LE and Fung DY-C* (eds), Handbook on anaerobic fermentations. Marcel Dekker, Inc. New York, pp 187-206.
- Engeli H, Edelmann W, Fuchs J and Rottermann K** (1993). Survival of plant pathogens and weed seeds during anaerobic digestion. *Wat Sci Technol*, **27-2**: 69-76.
- Fathepure BZ** (1983). Isolation and characterization of an acetoclastic methanogen from a biogas digester. *FEMS Microbiol Lett*, **19**: 151-156.
- Feilden NEH** (1981). A note on the temperature for maximum net gas production in an anaerobic digester system. *Agr Wastes*, **3**: 75-79.
- Fernandez N and Forster CF** (1993). A study of the operation of mesophilic and thermophilic anaerobic filters treating a synthetic coffee waste. *Biores Technol*, **45**: 223-227.
- Field JA, Stams AJM, Kato M and Schraa G** (1995). Enhanced biodegradation of aromatic pollutants in cocultures of anaerobic and aerobic bacterial consortia. *Antonie van Leeuwenhoek*, **67**: 47-77.
- Forsythe WE** (1954). Smithsonian Physical Tables. Smithsonian Miscellaneous Collections, Vol 120. Smithsonian Institution, Washington DC.
- Frankin RJ, Koevoets WAA, Van Gils WMA and Van der Pas A** (1992). Application of the biobed<sup>R</sup> upflow fluidized bed process for anaerobic waste water treatment. *Wat Sci Technol*, **25-7**: 373-382.
- Frankin RJ, Koevoets WAA and Versprille AI** (1994a). Application of the Biobed<sup>R</sup> system for formaldehyde containing dimethyl-terephthalate (=DMT) waste water. *In: Poster Paper Pre-prints of the Seventh International Symposium on Anaerobic Digestion, Cape Town, January 23-27, 1994, South Africa*, pp 244-247.
- Frankin RJ, Van Gils WMA and Wermeling RJF** (1994b). Full-scale anaerobic treatment of Shell waste water containing benzoate with the Biothane<sup>R</sup> UASB process. *In: Poster Paper Pre-prints*

- of the Seventh International Symposium on Anaerobic Digestion, Cape Town, January 23-27, 1994, South Africa, pp 248-251.
- Fukuzaki S, Nishio N and Nagai S (1990). Kinetics of the methanogenic fermentation of acetate. *Appl Environ Microbiol*, **56**: 3158-3163.
- Fukuzaki S, Nishio N, Shobayashi M and Nagai S (1990). Inhibition of the fermentation of propionate to methane by hydrogen, acetate, and propionate. *Appl Environ Microbiol*, **56**: 719-723.
- Garber WF (1977). Certain aspects of anaerobic digestion of wastewater solids in the thermophilic range at the Hyperion treatment plant. *Prog Wat Technol*, **8**: 401-406.
- Garber WF (1982). Operating experience with thermophilic anaerobic digestion. *J Wat Poll Contr Fed*, **54**: 1170-1175.
- Garber WF, Ohara GT, Colbaugh JE and Raksit SK (1975). Thermophilic digestion at the Hyperion treatment plant. *J Wat Poll Contr Fed*, **47**: 950-961.
- Gerhard E, Butsch BM, Marison IW and Von Stockar U (1993). Improved growth and methane production conditions for *Methanobacterium thermoautotrophicum*. *Appl Microbiol Biotechnol*, **40**: 432-437.
- Ghosh S, Klass DL, Christopher RW and Edwards VH (1980). Thermophilic biogasification of biomass. In: Proc. 7<sup>th</sup> Energy Technol. Conf. and Expo., March 24-26, Washington DC.
- Good P, Moundry R and Fluri P (1982). Use of fixed film and CSTR reactor for anaerobic treatment of stillage of wood hydrolysate. *Biotechnol Lett*, **4**: 595-600.
- Gorris LGM, van Deursen JMA, Van der Drift C and Vogels GD (1989). Inhibition of propionate degradation by acetate in methanogenic fluidized bed reactors. *Biotechnol Lett*, **11**: 61-66.
- Grobicki A and Stuckey DC (1991). Performance of the Anaerobic Baffled Reactor under steady-state and shock loading conditions. *Biotechnol Bioeng*, **37**: 344-355.
- Grobicki A and Stuckey DC (1992). Hydrodynamic characteristics of the Anaerobic Baffled Reactor. *Water Res*, **26**: 371-378.
- Grotenhuis JTC (1992). Structure and stability of methanogenic granular sludge. Ph.D. Thesis, Department of Microbiology, Agricultural University, Wageningen, The Netherlands.
- Grotenhuis JTC, Houwen FP, Plugge CM and Zehnder AJB (1986). Microbial interactions in anaerobic sludge. In: Proc. IV Int. Symposium Microbial Ecology, Ljubljana Yugoslavia, pp 163-168.
- Grotenhuis JTC, Smit M, Plugge CM, Xu Y, Van Lammeren AAM, Stams AJM and Zehnder AJB (1991). Bacteriological composition and structure of granular sludge adapted to different substrates. *Appl Environ Microbiol*, **57**: 1942-1949.
- Grotenhuis JTC, Smit M, Van Lammeren AAM, Stams AJM and Zehnder AJB (1990). Effect of interspecies hydrogen transfer on the bacteriological structure of methanogenic granular sludge. In: De Bont JAM, Visser J, Matthiasson B and Tramper J (eds), Int. Symp. Physiology of Immobilized Cells, Wageningen, The Netherlands, Elsevier Science Publ., Amsterdam, pp 95-98.
- Grotenhuis JTC, Smit M, Van Lammeren AAM, Stams AJM and Zehnder AJB (1991). Localization and Quantification of Extracellular Polymers in Methanogenic Granular Sludge. *Appl Microbiol and Biotechnol*, **36**: 115-119.
- Grotenhuis JTC, Stams AJM and Zehnder AJB (1992). Hydrophobicity and electrophoretic mobility of anaerobic isolates from methanogenic granular sludge. *Appl Env Microbiol*, **58**: 1054-

- 1056.
- Grotenhuis JTC, Van Lier JB, Plugge CM, Stams AJM and Zehnder AJB (1991). Effect of ethylene glycol-bis( $\beta$ -aminoethyl ether)-N, N-tertraacetic acid (EGTA) on stability and activity of methanogenic granular sludge. *Appl Microbiol Biotechnol*, **36**: 109-114.
- Gujer W and Zehnder AJB (1983). Conversion processes in anaerobic digestion. *Wat Sci Technol*, **15**: 127-167.
- Harris WL and Dague RR (1993). Comparative performance of anaerobic filters at mesophilic and thermophilic temperatures. *Water Environ Res*, **65**: 764-771.
- Heitzer A, Kohler H-PE, Reichert P and Hamer G (1991). Utility of phenomenological models for describing temperature dependence of bacterial growth. *Appl Environ Microbiol*, **57**: 2656-2665.
- Hensel R and König H (1988). Thermoadaptation of methanogenic bacteria by intracellular ion concentration. *FEMS Microbiol Lett*, **49**: 75-79.
- Henson JM and Smith PH (1985). Isolation of a butyrate-utilizing bacterium in coculture with *Methanobacterium thermoautotrophicum* from a thermophilic digester. *Appl Environ Microbiol*, **49**: 1461-1466.
- Henze M and Harremoes P (1983). Anaerobic treatment of wastewater in fixed film reactors - a literature review. *Wat Sci Techn*, **15**: 1-101.
- Huber H, Thomm M, König H, Thies G and Stetter KO (1982). *Methanococcus thermolithotrophicus*, a novel thermophilic lithotrophic methanogen. *Arch Microbiol*, **136**: 254-561.
- Huber R, Kurr M, Jannasch HW and Stetter KO (1989). A novel group of abyssal methanogenic archaeobacteria (*Methanopyrus*) growing at 110°C. *Nature*, **342**: 833-834.
- Hulshoff Pol LW (1989). The phenomenon of granulation of anaerobic sludge. Ph.D. Thesis, Department of Environmental Technology, Agricultural University, Wageningen, The Netherlands.
- Hulshoff Pol LW, Van de Worp JJM, Lettinga G and Beverloo WA (1986). Physical characterisations of anaerobic sludge. In: Proc. of the NVA/EWPCA conference Anaerobic treatment a grown-up technology, Aquatech '86, september 1986, Amsterdam, The Netherlands. Industrial Presentations (Europe) B.V., Schiedam, The Netherlands (1986), p 89-101.
- Huser BA, Wuhrmann K and Zehnder AJB (1982). *Methanothrix soehngenii* gen. nov. sp. nov., a new acetotrophic non-hydrogen oxidizing methanobacterium. *Arch Microbiol*, **132**: 1-9.
- Isa Z, Grusenmeyer S and Verstraete W (1986). Sulfate reduction relative to methane production in high-rate anaerobic digestion: microbiological aspects. *Appl Environ Microbiol*, **51**: 580-589.
- Jeris JS (1983). Industrial wastewater treatment using anaerobic fluidized bed reactors. *Wat Sci Technol*, **15**-8/9: 169-175.
- Jetten MSM, Stams AJM and Zehnder AJB (1990). Acetate threshold values and acetate activating enzymes in methanogenic bacteria. *FEMS Microbiol Ecol*, **73**: 339-344.
- Jiangrong Z, Yanru Y, Huren A and Yi Q (1994). A study of dyewaste treatment using anaerobic-aerobic process. In: Poster Paper Pre-prints of the Seventh International Symposium on Anaerobic Digestion, Cape Town, January 23-27, 1994, South Africa, pp 360-363.
- Jirka A and Carter MJ (1975). Micro semi-automated analysis of surface and wastewaters for chemical oxygen demand. *Analyt chem*, **47**: 1397-1401.
- Jones WJ, Leigh JA, Mayer F, Woese CR and RS Wolfe (1983). *Methanococcus Janaschii* sp.nov., an extremely thermophilic methanogen from a submarine hydrothermal vent. *Arch Microbiol*, **136**: 254-561.

- Kaiser SK, Harris WL and Dague RR (1993). Initial studies on the temperature phased anaerobic biofilter process. In: Proc. of 66<sup>th</sup> Annual Conference & Exposition of Water Environment Federation, October 3-7 1993, Anaheim, California USA, pp 319-329.
- Kanagata Y and Mikami E (1991). Isolation and characterization of a novel thermophilic *Methanosaeta* strain. *Int J Syst Bacteriol*, **41**: 191-196.
- Kato MT (1994). The anaerobic treatment of low strength soluble wastewaters. Ph.D. Thesis, Department of Environmental Technology, Agricultural University, Wageningen, The Netherlands.
- Kawase M, Nomura T and Majima T (1989). An anaerobic fixed bed reactor with a porous ceramic carrier. *Wat Sci Technol*, **21-4/5**: 77-86.
- Kemp HA, Archer DB and Morgan MRA (1988). Enzyme-linked immunosorbent assay for the specific and sensitive quantification of *Methanosarcina mazei* and *Methanobacterium bryantii*. *Appl Environ Microbiol*, **54**: 1003-1008.
- Kennedy KJ and Van den Berg L (1982). Thermophilic downflow stationary fixed film reactors for methane production from bean bleaching waste. *Biotechnol Lett*, **4**: 171-176.
- Kida K, Ikbal and Sonoda Y (1992). Treatment of coffee waste by slurry-state anaerobic digestion. *J Ferm Bioeng*, **73**: 390-395.
- Kidby DW and Nedwell DB (1991). An investigation into the suitability of biogas hydrogen concentration as a performance monitor for anaerobic sewage sludge digesters. *Wat Res* **25**: 1007-1012.
- Kim B-K and Daniels L (1991). Unexpected errors in gas chromatographic analysis of methane production by thermophilic bacteria. *Appl Env Microbiol*, **57**: 1866-1869.
- Kitaura S, Nishimura N, Mimura A and Takahara Y (1992). Isolation and characterization of a fast-growing thermophilic hydrogenotrophic methanogen. *J Ferment Bioeng*, **74**: 244-247.
- Kobayashi HA, Conway de Macario E, Williams RS and Macario AJL (1988). Direct characterization of methanogens in two high rate anaerobic biological reactors. *Appl Environ Microbiol*, **54**: 693-698.
- Koch M, Dolfig J, Wuhrmann K and Zehnder AJB (1983). Pathways of propionate degradation by enriched methanogenic cultures. *Appl Environ Microbiol*, **45**: 1411-1414.
- König H and Stetter KO (1989). Archaeobacteria. In: Staley JT, Bryant MP, Pfennig N and Holt, JG (eds), *Bergey's Manual<sup>R</sup> of Systematic Bacteriology*, Vol 3, Section 25, Williams & Wilkins Publ., Baltimore, pp 2171-2253.
- Kortekaas S, Doma HS, Potapenko SA, Field JA and Lettinga G (1994). Sequenced anaerobic-aerobic treatment of hemp black liquors. *Wat Sci Technol*, **29-(5/6)**: 409-419.
- Laanbroek HJ, Abee T and Voogd IL (1982). Alcohol conversions by *Desulfobulbus propionicus* Lindhorst in the presence and absence of sulfate and hydrogen. *Arch Microbiol*, **133**: 178-184.
- Lanting J, Jordan JA, Schone MT, Kull A, Carey WW and Kitney BL (1989). Thermophilic anaerobic digestion of coffee wastewater. In: Dalton CS and Wukasch RF (eds), Proc. of 43rd Industrial Waste Conference, May 1988, Lafayette, Indiana, USA. Lewis Publishers, Chelsea Michigan, pp 513-524.
- Lawrence AW and McCarty PL (1969). Kinetics of methane fermentation in anaerobic treatment. *J Wat Poll Contr Fed*, **41-2**: R1-R17.
- Lee MJ and Zinder SH (1988a). Isolation and characterization of a thermophilic bacterium which oxidizes acetate in syntrophic association with a methanogen and which grows acetogenically on

- $H_2$ - $CO_2$ . *Appl Environ Microbiol*, **54**: 124-129.
- Lee MJ and Zinder SH (1988b). Hydrogen partial pressures in a thermophilic acetate-oxidizing a methanogenic coculture. *Appl Environ Microbiol*, **54**: 1457-1461.
- Lema JM, Soto M, Méndez R and Blázquez R (1988). Comparison of mesophilic and thermophilic filters treating very high saline wastewaters. In: Proc. of 5<sup>th</sup> Inter. Symp. on Anaerobic Digestion, Bologna Italy, May 22-26, pp 547-549.
- Leens PM, De Beer D, Cronenberg CH, Houwen FP, Ottengraf SPP and Verstraete W (1993). Heterogenous distribution of microbial activity in methanogenic aggregates: pH and glucose microprofiles. *Appl Environ Microbiol*, **59**: 3803-3815.
- Lescure JP, Delannoy B, Verrier D and Albagnac G (1988). Consequence of a thermal accident on the microbial activity of an industrial anaerobic filter. In: Tilche A, Rozzi A (eds) Poster Papers Fifth Int Symp on Anaerobic Digestion, Bologna, Italy, May 22-26 1988, Monduzzi Editore S.p.A., Bologna, pp 211-214.
- Lettinga G, Field JA, Sierra-Alvarez R, Van Lier JB and Rintala J (1991). Future perspectives for the anaerobic treatment of forest industry wastewaters. *Wat Sci Technol*, **24**: 91-102
- Lettinga G, van Velsen AFM, Hobma SW, de Zeeuw WJ and Klapwijk A (1980). Use of the Upflow Sludge Blanket (USB) reactor. *Biotechnol Bioeng*, **22**: 699-734.
- Levenspiel O (1974). Chemical Reaction Engineering, 2nd edition. Wiley, New York.
- Lide DR (ed) (1992). CRC Handbook of Chemistry & Physics, 73<sup>rd</sup> edition, 1992, CRC Press Inc., Boca Raton, Florida, USA.
- Lin CY, Noike T, Sato K and Matsumoto J (1987). Temperature characteristics of the methanogenesis process in anaerobic digestion. *Wat Sci Technol*, **19**: 299-310.
- Lin C-Y, Sato K, Noike T and Matsumoto J (1986). Methanogenic digestion using mixed substrate of acetic, propionic, and butyric acids. *Wat Res*, **20**: 385-394.
- Lin KC and Yang Z (1991). Technical review on the UASB process. *Int J Environ Studies*, **39**: 203-222.
- Lo KV Liao PH and March AC (1985). Thermophilic anaerobic digestion of screened dairy manure. *Biomass*, **6**: 301-315.
- Lowe SE, Mahendra KJ and Zeikus JG (1993). Biology, ecology, and biotechnological applications of anaerobic bacteria adapted to environmental stresses in temperature, pH, salinity, or substrates. *Microbiol Rev*, **57**: 451-509.
- Lund B, Jensen VF, Have P and Ahring BK (1995). Inactivation of virus during anaerobic digestion of manure in laboratory scale biogas reactors. In: Proc. of International Meeting on: Anaerobic Processes for Bioenergy and Environment, Copenhagen, 25-27 January, 1995.
- Macario AJL and Conway de Macario E (1983). Antigenic fingerprinting of methanogenic bacteria with polyclonal antibody probes. *System Appl Microbiol*, **4**: 451-458.
- Macario AJL and Conway de Macario E (1985). Antibodies for methanogenic biotechnology. *Trends Biotechnol*, **3**: 204-208.
- Macario AJL and Conway de Macario E (1988). Quantitative immunological analysis of the methanogenic flora of digestors reveals a considerable diversity. *Appl Environ Microbiol*, **54**: 79-86.
- Macario AJL, Earle JFK, Chynoweth DP and Conway de Macario E (1989). Distinctive patterns of methanogenic flora determined with antibody probes in anaerobic digestors of different

- characteristics operated under controlled conditions. *Syst Appl Microbiol*, **12**: 216-222.
- Macario AJL, Visser FA, Van Lier JB and Conway de Macario E (1991). Topography of methanogenic subpopulations in a microbial consortium adapting to thermophilic conditions. *J Gen Microbiol*, **137**: 2179-2189.
- Mackie RI and Bryant MP (1981). Metabolic activity of fatty acid-oxidizing bacteria and the contribution of acetate, propionate butyrate and CO<sub>2</sub> to methanogenesis in cattle waste at 40, and 60°C. *Appl Environ Microbiol*, **41**: 1363-1373.
- MacLeod FA, Guiot SR and Costerton JW (1990). Layered structure of bacterial aggregates produced in an upflow anaerobic sludge bed and filter reactor. *Appl Environ Microbiol*, **56**: 1174-1184.
- Mah RA (1982). Methanogenesis and methanogenic partnerships. *Philos. Trans. of the Royal Soc. of London Ser. B.*, **297**: 599-616.
- Mah RA, Smith MR and Baresi L (1978). Studies on an acetate-fermenting strain of *Methanosarcina*. *Appl Environ Microbiol*, **35**: 1174-11184.
- Märkl H and Reinhold G (1994). Der Biogas-Turmreaktors, ein neues Reaktorkonzept für die anaeroben Abwasserreinigung. *Chem-Ing-Tech*, **66-4**: 534-536 (in German).
- Mathrani IM, Johansen K and Ahring BK (1994). Experiences with thermophilic anaerobic digestion of manure, organic industrial and household waste at the large scale biogas plant in Vegger, Denmark. In: Paper Pre-prints of the Seventh International Symposium on Anaerobic Digestion, Cape Town, January 23-27, 1994, South Africa, pp 365-374.
- Mawson AJ, Earle RL and Larsen VF (1991). Degradation of acetic and propionic acids in the methane fermentation. *Wat Res*, **25**: 1549-1554.
- McInerney MJ (1988). Anaerobic hydrolysis and fermentation of fats and proteins. In: Zehnder AJB (ed) *Biology of anaerobic microorganisms*. John Wiley, New York, pp 373-415.
- McInerney MJ and Bryant MP (1981). Basic principles of bioconversion in anaerobic digestion and methanogenesis. In: *Biomass Conversion Processes for Energy and Fuels*. Sofar, S.S. and Zaborsky, O. (eds), Plenum Publ. Corp. New York, pp 277-296.
- McInerney MJ, Bryant MP, Hespell RB and Costerton JW (1981). *Syntrophomonas wolfei*, gen. nov. sp. nov., an anaerobic, syntrophic, fatty acid oxidizing bacterium. *Appl Environ Microbiol*, **41**: 1029-1039.
- McInerney MJ, Bryant MP and Pfennig N (1979). Anaerobic bacterium that degrades fatty acids in syntrophic association with methanogens. *Arch Microbiol*, **132**: 129-135.
- Morvai L, Mihaltz P and Hollo J (1992). Comparison of the kinetics of acetate biometanation by raw and granular sludges. *Appl Microbiol Biotechnol*, **36**: 561-567.
- Mounfort DO and Asher RA (1978). Changes in proportions of acetate and carbon dioxide used as methane precursors during the anaerobic digestion of bovine waste. *Appl Environ Microbiol*, **35**: 648-654.
- Mucha H, Lingens F and Trösch W (1988). Conversion of propionate to acetate and methane by syntrophic consortia. *Appl Microbiol Biotechnol*, **27**: 581-586.
- Mukund S, Xuhong M, Park J-B, Zhou ZH, Blamey JM and Adams MWW (1992). The metabolism of hydrogen by bacteria growing near and above 100°C. In: *Thermophiles: Science and Technology*, Intern. Conf., Reykjavik, Iceland, 23rd-26th August 1992, IceTec Publ., p 129.
- Nagano A, Arikawa E and Kobayashi H (1992). Treatment of liquor wastewater containing high-

- strength suspended solids by membrane bioreactor system. *Wat Sci Technol*, 26-3/4: 887-895.
- Nanba A, Nukada R and Nagai S (1983). Inhibition by acetic and propionic acids of the growth of *Propionibacterium shermanii*. *J Ferment Technol*, 61: 551-556.
- Narayanan B, Suidan MT, Gelderloos AB and Brenner RC (1993a). Treatment of semivolatle compounds in high strength wastes using an anaerobic expanded-bed GAC reactor. *Wat Res*, 27-1: 171-180.
- Narayanan B, Suidan MT, Gelderloos AB and Brenner RC (1993b). Treatment of VOCs in high strength wastes using an anaerobic expanded-bed GAC reactor. *Wat Res*, 27-1: 181-194.
- Nelder JA and Mead R (1965). A simplex method for function minimization. *The Computer Journal*, 8: 308-313.
- Nishimura N, Kitaura S, Mimura A and Takahara Y (1991). Growth of thermophilic methanogen KN-15 on H<sub>2</sub>-CO<sub>2</sub> under batch and continuous culture conditions. *J Fermt Bioeng*, 72: 280-284.
- Novaes RFV (1986). Microbiology of anaerobic digestion. *Water Sci Technol*, 18: 1-14.
- Nozhevnikova AN and Chudina VI (1984). Morphology of the thermophilic acetate bacterium *Methanotherx thermoacetophila* sp. nov.. *Microbiology*, 53: 618-624.
- Nozhevnikova AN and Yagodina TC (1982). A thermophilic acetate methane-producing bacterium. *Microbiology*, 51: 534-541.
- Nyns E-J (1994). A guide to successful industrial implementation of biomethanisation technologies in the European Union, Successes and Guidelines. Report prepared for the European Commission, Directorated general for energy, Thermie programme, DG XVII.
- Ohtsuki T, Tominaga S, Morita T and Yoda M (1994). Thermophilic UASB system start-up and management -change in sludge characteristics in the start-up procedure using mesophilic granular sludge. In: Paper Pre-prints of the Seventh International Symposium on Anaerobic Digestion, Cape Town, January 23-27, 1994, South Africa, pp 348-357.
- Ohtsuki T, Watanabe M and Miyaji Y (1992). Start-up of thermophilic UASB (upflow anaerobic sludge blanket) reactors using microcarrier and mesophilic granular sludge. *Wat Sci Technol*, 26-3/4: 877-886.
- Ollivier B, Lombardo A and Garcia JL (1984). Isolation and characterization of a new thermophilic *Methanosarcina* strain (strain MP). *Ann Microbiol (Inst Pasteur)*, 135B: 187-198.
- Olsen JE, Jorgensen JB and Nansen P (1985). On the reduction of *Mycobacterium paratuberculosis* in bovine slurry subjected to batch mesophilic or thermophilic anaerobic digestion. *Agr Wast*, 13: 273-280.
- Olsen JE and Larsen HE (1987). Bacterial decimation times in anaerobic digestions of animal slurries. *Biol Wast*, 21: 153-168.
- Ono H (1965). Discussion to T. Bhaskaran, Utilization of materials derived from treatment of wastes from molasses distilleries. In: Baars J (ed), *Advances in Water Pollution Research*, Vol II, Pergamon Press, London, pp 101-104.
- Oude Elferink SJWH, Visser A, Hulshoff Pol LW and Stams AJM (1994). Sulfate reduction in methanogenic bioreactors. *FEMS Microbiol Rev*, 15: 119-136.
- Ozturk SS, Palsson BO and Thiele JH (1988). Control of interspecies electron transfer flow during anaerobic digestion: dynamic diffusion reaction models for hydrogen gas transfer in microbial flocs. *Biotechnol Bioeng*, 33: 745-757.
- Patel GB (1984). Characterization and nutritional properties of *Methanotherx concillii* sp.nov., a

- mesophilic aceticlastic methanogen. *Can J Microbiol*, **30**: 1383-1396.
- Patel GB and Sprott GD (1990). *Methanosaeta concilii* gen. nov., sp. nov. ("*Methanothrix concilii*") and *Methanosaeta thermoacetophila* nom. rev., comb. nov.. *Int J Syst Bacteriol*, **40**: 79-82.
- Pavan P, Musacco A, Cecchi F, Bassetti A, and Mata-Alvarez J (1994). Thermophilic semi-dry anaerobic digestion process of the organic fraction of municipal solid waste during transient conditions. *Environ Technol*, **15**: 1173-1182.
- Pavlostathis SG (1994). Anaerobic processes, the 1994 literature review. *Water Environ Res*, **66/4**: 342-356.
- Pavlostathis SG and Giraldo-Gomez E (1991). Kinetics of anaerobic treatment: a critical review. *Critical Rev in Environ Control*, **21** (5,6): 411-490.
- Peillex J-P, Fardeau M-I, Boussand R, Navarro J-M and Belaich J-P (1989). Growth of *Methanococcus thermolithotrophicus* in batch and continuous culture on H<sub>2</sub> and CO<sub>2</sub>: influence of agitation. *Appl Microbiol Biotechnol*, **29**: 560-564.
- Pera A, Vallini G, Frassinetti S and Cecchi F (1992). Co-composting for managing effluent from thermophilic anaerobic digestion of municipal solid waste. *Environ Technol*, **12**: 1137-1145.
- Perry RH and Green DW (eds) (1984). Perry's chemical engineers' handbook, section 14, 6th edition, McGraw-Hill Publishing Co., New York, USA.
- Petersen SP and Ahring BK (1991). Acetate oxidation in a thermophilic anaerobic sewage-sludge digester: the importance of non-aceticlastic methanogenesis from acetate. *FEMS Microbiol Ecol*, **86**: 149-158.
- Petersen SP and Ahring BK (1992). The influence of sulphate on substrate utilization in a thermophilic sewage sludge digester. *Appl Microbiol Biotechnol*, **36**: 805-809.
- Pledger RJ, Crump BC and Baross JA (1994). A barophilic response by two hyperthermophilic, hydrothermal vent *Archaea*: an upward shift in the optimal temperature and acceleration of growth rate at supra-optimal temperatures by elevated pressure. *FEMS Microbiol Ecol*, **14**: 233-242.
- Pohland FG and Bloodgood DE (1963). Laboratory studies on mesophilic and thermophilic anaerobic sludge digestion. *J Wat Poll Contr Fed*, **35**: 11-42.
- Prensler G, Dubourguier HC, Thomas I, Albagnac G and Buisson MN (1988). Specific immunological probes for studying the bacterial association in granules and biofilms. In: Lettinga G, Zehnder AJB, Grotenhuis JTC, Hulshoff Pol LW (eds), Proc. Granular anaerobic sludge; microbiology and technology, GASMAT workshop. Lunteren, October 25-27, 1987, The Netherlands, Pudoc Wageningen, The Netherlands, pp 55-61.
- Reinhold G, Polomski A, Grajetzki R, Sens J and Märkl H (1994). Betriebsverhalten eines Biogas-Turmreaktors im Pilotmaßstab. In: Märkl H und Stegmann R (eds), Anaerobe Behandlung von Festen und Flüssigen Rückständen, Sonderforschungsbereiches 238 der Deutschen Forschungsgemeinschaft, 2-4 November 1994, DECHEMA Chemische Technik und Biotechnologie e.V., Frankfurt am Main, pp 45-62 (in German).
- Rimkus RR, Ryan JM and Cook EJ (1982). Full-scale thermophilic digestion at the West-southwest sewage treatment works, Chicago, Illinois. *J Wat Poll Contr Fed*, **54**: 1447-1457.
- Rintala JA (1992). Thermophilic and mesophilic anaerobic treatment of pulp and paper industry wastewaters. Ph.D. thesis, nr 92, Tampere University of Technology, Tampere, Finland.
- Rintala JA and Ahring BK (1994). Thermophilic anaerobic digestion of source-sorted household solid waste: the effects of enzyme additions. *Appl Microbiol Biotechnol*, **40**: 916-919.

- Rintala JA and Lepistö SS (1992). Anaerobic treatment of thermomechanical pulping whitewater at 35-70°C. *Wat Res*, **26**: 1297-1305.
- Rintala JA, Lepistö SS and Ahring BK (1993). Acetate degradation at 70°C in upflow anaerobic sludge blanket reactors and temperature respons of granules grown at 70°C. *Appl Environ Microbiol*, **59**: 1742-1746.
- Rintala JA and Lettinga G (1992). Effects of temperature elevation from 37 to 55°C on anaerobic treatment of sulphate rich acidified wastewaters. *Environ Technol Lett*, **13**: 801-812.
- Rintala JA, JL Sanz Martin and Lettinga G (1991). Thermophilic anaerobic treatment of sulfate-rich pulp and paper integrate process water. *Wat Sci Technol*, **24-3/4**: 149-160.
- Rinzema A (1988). Anaerobic treatment of wastewater with high concentrations of lipids or sulfate. PhD. thesis, Department of Environmental Technology, Agricultural University, Wageningen, The Netherlands.
- Rinzema A and Lettinga G (1988). Anaerobic treatment of sulfate containing wastewater. In: Wise DL (ed) Biotreatment Systems, Vol. III, CRC press, Inc., Boca Raton, USA, pp 65-109.
- Rinzema A, Van Lier JB and Lettinga G (1988). Sodium inhibition of acetoclastic methanogens in granular sludge from a UASB reactor. *Enzyme and Microb Technol*, **10**: 24-32.
- Robinson RW and Erdős GW (1985). Immunoelectron microscopic identification of *Methanosarcina* spp. in anaerobic digester fluid. *Can J Microbiol*, **31**: 839-844.
- Romero LI, Nebot E, Martínez de la Ossa E and Sales D (1993). Microbiological purification kinetics of wine-distillery wastewaters. *J Chem Tech Biotechnol*, **58**: 141-149.
- Romero LI, Sales D and Martínez de la Ossa E (1990). Comparison of three practical processes for purifying wine distillery wastewaters. *Process Biochem Int*, **25**: 93-96.
- Rudd T, Hicks SJ and Lester JN (1985). Comparison of the treatment of a synthetic meat waste by mesophilic and thermophilic anaerobic fluidized bed reactors. *Environ Technol Lett*, **6**: 209-224.
- Russell NJ and Fukunaga N (1990). A comparison of thermal adaptation of membrane lipids in psychrophilic and thermophilic bacteria. *FEMS Microbiol Rev*, **75**: 171-182.
- Sáez PB and Rittman BE (1992). Model-parameter estimation using least squares. *Wat Res*, **26**: 789-796.
- Schauer NL and Ferry JG (1980). Metabolism of formate in *Methanobacterium formicicum*. *J Bacteriol*, **142**: 800-807.
- Schink B (1984). Fermentation of 2,3-butanediol by *Pelobacter carbinolicus* sp. nov. and *Pelobacter propionicus* sp. nov., and evidence for propionate formation from C<sub>2</sub> compounds. *Arch Microbiol*, **137**: 33-34.
- Schink B (1992). Syntrophism among prokaryotes. In: Balows A, Trüper HG, Dworkin M, Harder W and Schleifer KH (eds), *The Prokaryotes*, Springer Verlag, New York, pp 276-299.
- Schmidt JE and Ahring BK (1993). Effects of hydrogen and formate on the degradation of propionate and butyrate in thermophilic granules from an Upflow Anaerobic Sludge Blanket reactor. *Appl Environ Microbiol*, **59**: 2546-2551.
- Schmidt JE and Ahring BK (1994). Extracellular polymers in granular sludge from different upflow anaerobic sludge blanket (UASB) reactors. *Appl Microbiol Biotechnol*, **42**: 457-462.
- Schmidt JE, Macario AJL, Ahring BK and Conway de Macario E (1992). Effect of magnesium on methanogenic subpopulations in a thermophilic acetate-degrading granular consortium. *Appl Environ Microbiol*, **58**: 862-868.

- Schönheit P, Moll J and Thauer RK (1980). Growth parameters ( $K_s$ ,  $\mu_{max}$ ,  $Y_p$ ) of *Methanobacterium thermoautotrophicum*. *Arch Microbiol*, 127: 59-65.
- Schraa G (1983). Conversion of soluble organic matter with the thermophilic anaerobic attached film expanded bed process. PhD thesis, Cornell Univ., Ithaca, NY, USA.
- Schraa G and Jewell WJ (1984). High rate conversions of soluble organics with the thermophilic anaerobic attached expanded bed. *J Wat Poll Contr Fed*, 56: 226-232.
- Seif HAA, Joshi SG and Gupta SK (1992). Effect of organic load and reactor height on the performance of anaerobic mesophilic and thermophilic fixed film reactors in the treatment of pharmaceutical wastewater. *Environ Technol*, 13: 1161-1168.
- Sen BP and Bhaskaran TR (1962). Anaerobic digestion of liquid molasses distillery wastes. *J Wat Poll Contr Fed*, 34: 1015-1025.
- Sillen LG and Martell AE (1964). Stability constants of metal ion complexes. London: the Chemical Society, Burlington House, W1, London, Great Britain.
- Smith JM (1981). Chemical Engineering Kinetics. McGraw-Hill, Inc. USA.
- Smith MR and Mah RA (1978). Growth and methanogenesis by *Methanosarcina* strain 227 on acetate and methanol. *Appl Environ Microbiol*, 36: 870-879.
- Sonnleitner B (1983). Biotechnology of thermophilic bacteria - growth, products, and applications. In: Advances in Biochemical Engineering and Biotechnology, vol 28, Fiechter A (ed), Springer Verlag, Berlin New York, pp 69-138.
- Soto M, Méndez R and Lema JM (1992). Characterization and comparison of biomass from mesophilic and thermophilic fixed bed anaerobic digesters. *Water Sci Technol*, 25-7: 203-212.
- Souza ME, Fuzaro G and Polegato AR (1992). Thermophilic anaerobic digestion of vinasse in pilot plant UASB reactor. *Wat Sci Technol*, 25-7: 213-222.
- Speece RE and Kem JA (1970). The effect of short-term temperature variations on methane production. *J Wat Poll Contr Fed*, 42: 1990-1997.
- Stams AJM (1994). Metabolic interactions between anaerobic bacteria in methanogenic environments. *Antonie van Leeuwenhoek*, 66: 271-294.
- Stams AJM, Grolle KCF, Frijters CTMJ and Van Lier JB (1992). Enrichment of a thermophilic propionate-oxidizing acetogenic bacterium in coculture with *Methanobacterium thermoautotrophicum* or *Methanobacterium thermoformicum*. *Appl Environ Microbiol*, 58: 346-352.
- Stams AJM, Grotenhuis JTC, Zehnder AJB (1990). Structure-function relationship in granular sludge. In: Hattori T, Ishida Y, Maruyama Y, Morita RY, Uchida A (eds), Recent advances in microbial ecology. Japan Scientific Society Press, Tokyo, pp 440-445.
- Switzenbaum MS and Jewell WJ (1980). Anaerobic attached film expanded bed reactor. *J Wat Poll Contr Fed*, 52: 1953-1965.
- Tafdrup S (1994). Centralized biogas plants combine agricultural and environmental benefits with energy production. *Wat Sci Technol*, 30-12: 133-141.
- Temper U (1983). Methangärung von Klärschlamm und anderen komplexen Substraten bei mesophilen und thermophilen Temperaturen. PhD thesis, Department of Biology, Ludwig-Maximilians-Universität München, Germany.
- Ten Brummeler E, Horbach HCJM and Koster IW (1991). Dry anaerobic batch digestion of the organic fraction of municipal solid waste. *J Chem Technol Biotechnol*, 50: 191-209.

- Ten Brummeler E, Aarnink MMJ and Koster IW (1992). Dry anaerobic digestion of solid organic waste in a BIOCEL reactor at pilot scale. *Wat Sci Tech*, 25-7: 301-310.
- Thauer RK, Jungermann K and Decker K (1977). Energy conservation in chemotrophic anaerobic bacteria. *Bacteriol Rev*, 41: 100-180.
- Thiele JH and Zeikus JG (1988). Control of interspecies electronflow during anaerobic digestion: significance of formate transfer versus hydrogen transfer during syntrophic methanogenesis in flocs. *Appl Environ Microbiol*, 54: 20-29.
- Tholozan JL, Samain E, Grivet JP, Moletta R, Dubourguier HC and Albagnac G (1988). Reductive carboxylation of propionate to butyrate in methanogenic ecosystems. *Appl and Environ Microbiol*, 54: 441-445.
- Torpey WN, Andrews J and Basilico JV (1984). Effects of multiple digestion on sludge. *J Wat Poll Contr Fed*, 56: 62-68.
- Touzel JP, Petroff D and Albagnac G (1985). Isolation and characterization of a new thermophilic *Methanosarcina*, the strain CHTI 55. *Syst Appl Microbiol*, 6: 66-71.
- Tseng S-K and Yang C-J (1994). The reaction characteristics of treatment of wastewater containing nitrophenol by anaerobic biological fluidized bed. *Wat Sci Technol*, 30-12: 233-240.
- Tsien H, Panos Ch, Shockman GD and Higgins ML (1980). Evidence that *Streptococcus mutans* constructs its membranes with excess fluidity for survival at suboptimal temperatures. *J Gen Microbiol*, 121: 105-111.
- Uemura S and Harada H (1993). Microbial characteristics of methanogenic sludge consortia developed in thermophilic UASB reactors. *Appl Microbiol Biotechnol*, 39: 654-660.
- Uemura S and Harada H (1995). Inorganic composition and microbial characteristics of methanogenic granular sludge grown in a thermophilic upflow anaerobic sludge blanket reactor. *Appl Microbiol Biotechnol*, 43: 358-364
- Ugurlu A and Forster CF (1992). The impact of shock loadings on the performance of thermophilic anaerobic filters with porous and non-porous packings. *Biores Technol*, 39: 23-30.
- Vallini G, Cecchi F, Pavon P, Pera A, Mata-Alvarez J and Bassetti A (1993). Recovery and disposal of the organic fraction of municipal solid waste (MSW) by means of combined anaerobic and aerobic biotreatment. *Wat Sci Technol*, 27-2: 121-132.
- Van den Berg L (1977). Effect of temperature on growth and activity of a methanogenic culture utilising acetate. *Can J Microbiol*, 23: 898-902.
- Vanderhaegen B, Ysebaert E, Favere K, Van Wambeke M, Peeters T, Panic V, Vandenlangenbergh V and Verstraete W (1992). Acidogenesis in relation to in-reactor granule yield. *Wat Sci Technol*, 25: 75-81.
- Van Lier JB, Grolle KCF and Lettinga G (1991). Anaerobic digestion at 75°C. In: Poster-papers of the Sixth Int. Symp. on Anaerobic Digestion, Sao Paulo, May 12-16 1991 Brazil, p 163.
- Van Lier JB and Lettinga G (1993). Thermofiele anaërobe afvalwaterzuivering; opstart en optimalisatie. Final report, Department of Environmental Technology, Agricultural University, Wageningen, The Netherlands (in Dutch).
- Van Lier JB, Macario AJL, Conway de Macario E and Lettinga G (1993). Permanent increase of the process temperature of mesophilic Upflow Anaerobic Sludge Bed (UASB) reactors to 46, 55, 64 and 75°C. In: Dalton, CS and Wukasch, RF (eds.), Proc. of 47th Industrial Waste Conference, May 1992, Lafayette Indiana, USA. Lewis Publishers, Chelsea Michigan, USA, p.

- 445-459.
- Van Lier JB, Rintala J, Sanz Martin JL and Lettinga G (1990). Effects of short-term temperature increase on the performance of a mesophilic UASB reactor. *Water Sci Technol*, 22: 183-190
- Van Lier JB, ten Brummeler E and Lettinga G (1993). Thermo-tolerant anaerobic degradation of volatile fatty acids by digested organic fraction of municipal solid waste. *J Ferment and Biotechnol*, 76: 140-144.
- Varel VH (1983). Characteristics of bacteria in thermophilic digesters and effect of antibiotics on methane production. In: Wise, DL (ed.), Fuel Gas Systems. CRC Press, Boca Raton, Fla., USA, pp 19-47.
- Varel VH, Isaacson HR and Bryant MP (1977). Thermophilic methane production from cattle waste. *Appl Environ Microbiol*, 33: 298-307.
- Vellinga SHJ, Hack PJFM and Van der Vlugt (1986). New type 'high rate' anaerobic reactor - first experience on semi-technical scale with a revolutionary and high loaded anaerobic system. In: Anaerobic treatment a grown-up technology, Aquatech '86, september 1986, Amsterdam, The Netherlands. Industrial Presentations (Europe) B.V., Schiedam, The Netherlands, p 547-562.
- Visser A (1995). Anaerobic treatment of sulphate containing wastewater. PhD. thesis, Department of Environmental Technology, Agricultural University, Wageningen, The Netherlands.
- Visser FA, Van Lier JB, Macario AJL and Conway de Macario E (1991). Diversity and population dynamics of methanogenic bacteria in a granular consortium. *Appl Environ Microbiol*, 57: 1728-1734.
- Vlissidis A and Zouboulis AI (1993). Thermophilic anaerobic digestion of alcohol distillery wastewaters. *Biores Technol*, 43: 131-140.
- Vogels GD, Keltjens JT and Van der Drift C (1988). Biochemistry of methane formation. In: Zehnder AJB (ed) Biology of Anaerobic Microorganisms, John Wiley & Sons, New York, pp 707-770.
- Weast RC (1976). Handbook of chemistry and physics. 57<sup>th</sup> edition, CRC press, Boca Raton, Florida, USA.
- Weber H, Kulbe KD, Chmiel H and Trösch W (1984). Microbial acetate conversion to methane: kinetics, yields and pathways in a two-step digestion process. *Appl Microbiol Biotechnol*, 19: 224-228.
- Weimer PJ and Zeikus JG (1978). One carbon metabolism in methanogenic bacteria. Cellular characterization and growth of *Methanosarcina barkeri*. *Arch Microbiol*, 119: 175-182.
- Westermann P (1994). The effect of incubation temperature on steady-state concentrations of hydrogen and volatile fatty acids during anaerobic degradation in slurries from wetland sediments. *FEMS Microbiol Ecol*, 13: 295-302.
- Westermann P, Ahring BK and Mah RA (1989). Temperature compensation in *Methanosarcina barkerii* by modulation of hydrogen and acetate affinity. *Appl Environ Microbiol*, 55: 1262-1266.
- Wheatley A (ed) (1990). Anaerobic digestion: a waste treatment technology. Elsevier Applied Science, London, UK.
- Whitmore TN, Lloyd D, Jones G and Williams TN (1987). Hydrogen-dependent control of the continuous anaerobic digestion process. *Appl Microbiol and Biotechnol*, 26: 383-388.
- Wiegant WM (1986). Thermophilic anaerobic digestion for waste and wastewater treatment. PhD. thesis, Department of Environmental Technology, Agricultural University, Wageningen, The

- Netherlands.
- Wiegant WM (1987). The "spaghetti theory" on anaerobic granular sludge formation, or the inevitability of granulation. *In*: Lettinga G, Zehnder AJB, Grotenhuis JTC, Hulshoff Pol LW (eds) Granular anaerobic sludge; microbiology and technology. Pudoc Wageningen, The Netherlands, pp 146-152.
- Wiegant WM, Claassen JA and Lettinga G (1985). Thermophilic anaerobic digestion of high strength wastewaters. *Biotechnol Bioeng*, 27: 1374-1381.
- Wiegant WM and De Man AWA (1986). Granulation of biomass in thermophilic anaerobic sludge blanket reactors treating acidified wastewaters. *Biotechnol Bioeng*, 28: 718-727.
- Wiegant WM, Hennink M and Lettinga G (1986). Separation of the propionate degradation to improve the efficiency of thermophilic anaerobic treatment of acidified wastewaters. *Wat Res*, 4: 517-524.
- Wiegant WM and Lettinga G (1985). Thermophilic anaerobic digestion of sugars in upflow anaerobic sludge blanket reactors. *Biotechnol Bioeng*, 27: 1603-1607.
- Wiegel J (1990). Temperature spans for growth: hypothesis and discussion. *FEMS Microbiol Rev*, 75: 155-170.
- Wijffels RH, Englund G, Hunik JH, Leenen EJTM, Bakketun Å, Günther A, Obón de Castro JM and Tramper J (1994). Effects of diffusion limitation on immobilized nitrifying microorganisms at low temperatures. *Biotechnol Bioeng*, 45: 1-9.
- Winter J, Lerp, C, Zabel HP, Wildenauer FX, König H and Schindler F (1984). *Methanobacterium wolfei* sp. nov., a new tungsten requiring, thermophilic autotrophic methanogen. *System Appl Microbiol*, 5: 457-466.1
- Winter J and Zellner G (1990). Thermophilic anaerobic degradation of carbohydrates - metabolic properties of microorganisms from the different phases. *FEMS Microbiol Rev*, 75: 139-154.
- Woese CR (1987). Bacterial evolution. *Microbiol Rev*, 51: 139-154.
- Wolin MJ (1982). Hydrogen transfer in microbial communities. *In*: Bull AT and Slater JH (eds), Microbial Interactions and Communities, Acad. Press, London, pp 323-356.
- Wu W-M, Jain MK, Conway de Macario E, Thiele JH and Zeikus JG (1992). Microbial composition and characterization of prevalent methanogens and acetogens isolated from syntrophic methanogenic granules. *Appl Microbiol Biotechnol*, 38: 282-290.
- Wu W-M, Thiele JH, Mahendra KJ and Zeikus JG (1993). Metabolic properties and kinetics of methanogenic granules. *Appl Microbiol Biotechnol*, 39: 804-811.
- Yang M, Ishihara T, Okada M, Nagai S and Sunahara H (1992). Stability and performance of thermophilic anaerobic fixed-bed reactor packed with a saddle-shaped slag biocarrier. *Environ Technol*, 13: 671-678.
- Yeoh BG (1986). A kinetic-based design for thermophilic anaerobic treatment of a high-strength agroindustrial wastewater. *Environ Technol Lett*, 7: 509-518.
- Yoda M, Shin SW, Watanabe A, Watanabe M, Kitagawa M and Miyaji Y (1987). Anaerobic fluidized bed treatment with steady-state biofilm. *Wat Sci Technol*, 19-(1/2): 287-298.
- Yoda M, Kitagawa M and Miyaji Y (1988). Long term competition between sulfate-reducing and methane-producing bacteria for acetate in anaerobic biofilm. *Wat Res*, 21: 1547-1556.
- Young JC and McCarty PL (1969). The anaerobic filter for waste treatment. *J Wat Poll Contr Fed*, 41: R160-R170.

- Young JC and Yang BS (1989). Design considerations for full-scale anaerobic filters. *J Wat Pol Contr Fed*, 61: 1576-1587.
- Zeeman G, Wiegant WM, Koster-Treffers ME and Lettinga G (1985). The influence of the total ammonia concentration on the thermophilic digestion of cow manure. *Agr Wastes*, 14: 19-35.
- Zeeman G and Van Veen HAA (1990). Thermophilic digestion of sewage sludge, a literature review. Department of Environmental Technology, Agricultural University, Wageningen, The Netherlands (in Dutch).
- Zehnder AJB (1978). Ecology of methane formation. In: Mitchell R (ed) Water pollution microbiology, vol 2. John Wiley, New York, pp 349-376.
- Zehnder AJB, Huser BA, Brock TD and Wuhrmann K (1980). Characterization of an acetate-decarboxylating non-hydrogen oxidizing methane bacterium. *Arch Microbiol*, 124: 1-11.
- Zehnder AJB, Ingvorsen K and Marti T (1982). In: Hughes DE et al. (eds), Anaerobic Digestion 1981. Elsevier Biomedical Press, Amsterdam, pp 45-68.
- Zehnder AJB and Wuhrmann K (1977). Physiology of a *Methanobacterium* strain AZ. *Arch Microbiol*, 111: 199-205.
- Zeikus JG and Wolfe RS (1972). *Methanobacterium thermoautotrophicum* sp.nov., an anaerobic, autotrophic, extreme thermophile. *J Bacteriol*, 109: 707-713.
- Zellner G and Kneifel H (1993). Caldopentamine and caldohexamine in cells of *Thermotoga* species, a possible adaptation to the growth at high temperatures. *Arch Microbiol*, 159: 472-476.
- Zellner G and Winter J (1987). Analysis of a highly efficient methanogenic consortium producing biogas from whey. *Syst Appl Microbiol*, 9: 284-292.
- Zhao H, Wood AG, Widdel F and Bryant MP (1988). An extremely thermophilic Methanococcus from a deep sea hydrothermal vent and its plasmid. *Arch Microbiol*, 150: 178-183.
- Zhao H, Yang D, Woese CR and Bryant MP (1993). Assignment of fatty acid- $\beta$ -oxidizing syntrophic bacteria to *Syntrophomonadaceae* fam. nov. on the basis of 16S rRNA sequence analyses. *Int J Syst Bacteriol*, 43: 278-286.
- Zinder SH (1986). Thermophilic waste treatment systems. In: Brock TD (ed.) Thermophiles: general, molecular and applied biology. Wiley-Interscience, New York, pp 257-277.
- Zinder SH (1990). Conversion of acetic acid to methane by thermophiles. *FEMS Microbiol Rev*, 75: 125-138.
- Zinder SH, Anguish T and Cardwell SC (1984b). Effects of temperature on methanogenesis in a thermophilic (58°C) anaerobic digester. *Appl Environ Microbiol*, 47: 808-813.
- Zinder SH, Anguish T, Lobo AL (1987). Isolation and characterization of a thermophilic acetotrophic strain of *Methanotherix*. *Arch Microbiol*, 146: 315-322.
- Zinder SH, Cardwell SC, Anguish T, Lee M and Koch M (1984a). Methanogenesis in a thermophilic (58°C) anaerobic digester: *Methanotherix* sp. as an important aceticlastic methanogen. *Appl Environ Microbiol*, 47: 796-807.
- Zinder SH and Koch M (1984). Non-aceticlastic methanogenesis from acetate: acetate oxidation by a thermophilic syntrophic co-culture. *Arch Microbiol*, 54: 263-272.
- Zinder SH and Mah RA (1979). Isolation and characterization of a thermophilic strain of *Methanosarcina* unable to use H<sub>2</sub>-CO<sub>2</sub> for methanogenesis. *Appl Environ Microbiol*, 38: 996-1008.
- Zinder SH, Sowers KR, Ferry JG (1985) *Methanosarcina thermophila* sp. nov., a thermophilic, acetotrophic, methane-producing bacterium. *Int J Syst Bact*, 35: 522-523.

## Curriculum Vitae

Julius Bernardus van Lier werd op 29 januari 1963 geboren in Reuver. Na wat geknutsel op de lokale bewaarschool en het verkrijgen van basisonderwijs op de Lambertus jongensschool werd in 1981 het Atheneum-B diploma behaald aan de Rijks Scholen Gemeenschap te Roermond. In dat zelfde jaar werd begonnen aan de studie Biologie aan de Katholieke Universiteit Nijmegen. Als lid van de laatste studentenlichting van voor de twee-fase-structuur werd het kandidaatsdiploma in 1984 behaald. In datzelfde jaar werd begonnen aan een 6-maands afstudeervak Regionale Bodemkunde, gevolgd door 6 maanden anaërobe waterzuivering aan de Landbouw Universiteit Wageningen. Het door de KUN niet willen honoreren van een 6-maands "anaërobe" stage in Colombia heeft de auteur doen besluiten om de studie Biologie in Wageningen af te ronden; dat bleek een wijs besluit. Na een laatste 6-maands doktoraalvak bij de vakgroep Microbiologie studeerde hij in 1988 af.

Sinds eind 1988 is hij werkzaam bij de Vakgroep Milieutechnologie van de Landbouw Universiteit Wageningen alwaar hij onderzoek verrichtte naar thermofiele anaërobe afvalwaterzuiveringsprocessen, hetgeen resulteerde in het onderhavige proefschrift. Het onderzoek werd voor 4.5 jaar gefinancierd door de Novem en Paques B.V., Balk. Sinds 1993 is hij werkzaam op projectbasis, hetgeen in 1994 overging in een "tijdelijk-vast-dienstverband" bij dezelfde vakgroep. Naast onderzoek coördineert hij een EEG project op het gebied van de anaërobe zuivering van rioolwater en probeert hij samen met Paques B.V. thermofiele anaërobe zuivering op praktijkschaal toe te passen.