ANAEROBIC GRANULAR SLUDGE:
CHARACTERIZATION, AND FACTORS AFFECTING ITS FUNCTIONING

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Anaerobic Granular Sludge:
Characterization, and Factors Affecting its Functioning

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Voor Jorn Gormbert

Stellingen,

behorend bij het proefschrift "Anaerobic granular sludge: characterization, and factors affecting its functioning" van P. Arne Alphenaar

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1 De conclusie van Van der Haegen et al. dat de vorming van methanogeen korrelslib ook in CSTR reactoren mogelijk is, kan niet uit zijn experimentele resultaten worden afgeleid.
Vanderhaegen B., Ysebaert K., Favere K., van Wambeke M., Peeters T., Panic V., Vandenlangenbergh V. and Verstraete W. (1992). Acidogenesis in relation to in-reactor granule yield. Proc. sixth int. symp. on anaerobic digestion, San Paulo, Brasilia, pp211-230.

2 De toegepaste relatief hoge fosfaatconcentratie in het influent van de gebruikte UASB experimenten leidt tot een overschatting van de betekenis van fosfaatprecipitaten voor de stabiliteit van korrelslib.
Grotenhuis J.T.C., van Lier J.B., Plugge C.M., Stams A.J.M. and Zehnder A.J.B. (1991). Effect of ethylene glycol-bis(ß-amioethyl ether)-N,N-tetraacetic acid (EGTA) on stability and activity of methanogenic granular sludge. Appl. Microbiol. Biotechnol. **36**:109-114.

3 Kosaric et al. concluderen ten onrechte dat korreldesintegratie veroorzaakt wordt door toegepaste opwaartse vloeistofsnelheden hoger dan 0.5 $m.hr^{-1}$.
Kosaric N., Blaszczyk R. and Orphan L. (1990). Factors influencing formation and maintenance of granules in anaerobic sludge blanket reactors (UASBR). Wat. Sci. Technol. **22**:275-282.

4 De vorming en de kwaliteit van methanogeen korrelslib in UASB reactoren gevoed met sucrose houdend influent is afhankelijk van de toegepaste sucrosebelasting van het slib.
Dit proefschrift

5 De bredere toepassing van anaerobe waterzuivering wordt in belangrijke mate gehinderd door het ontbreken van een op de praktijk gericht handboek van deze technologie.

6 Het succes van een opstartprocedure van UASB reactoren waarbij gebruik gemaakt wordt van korrelslib, wordt in belangrijke mate bepaald door de procescondities in de reactor waarin dit entmateriaal gevormd is.
Dit proefschrift

7 Kinderen van promovendi leren al vroeg creatief met wetenschap omgaan.
(eerdere versies van) Dit proefschrift

8 Meer dan door het zo lang mogelijk uitstellen van overlijden blijkt respect voor het leven uit het aanvaarden van de eindigheid ervan.

9 Onderzoek naar de temperatuurtolerantie van tomaten gaat niet over rozen.
Hoek I.H.S., Hänish ten Cate C.H., Keijzer C.J., Schel J.H. and Dons H.J.M. (1993). Development of the fifth leaf is indicative for whole plant performance at low temperature in tomato. Annals of Botany 72:367-374

10 De stelling dat een promotieonderzoek gelijk staat aan 5000 SBU (studie belastings uren) lijkt ontleend aan de inflatiebestrijding volgens de koning van Fop: "vanaf heden bevat een kilo 2000 gram".
(De Volkskrant, december 1992)

11 Door de subsidie voor opvang te beperken tot de kinderen van vrouwelijke werknemers, gaat het ministerie van Landbouw, Natuurbeheer en Visserij er ten onrechte van uit dat het opvoeden van kinderen nog steeds primair de verantwoordelijkheid van de vrouw is.
(Ministerie van Landbouw en Visserij)

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ABSTRACT

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Many UASB reactors are designed in such a fashion that the presence of granular sludge is necessary for a proper purification process. For achieving an optimum wastewater purification with such reactors, knowledge of the factors that determine the growth, retention and disintegration of anaerobic granular sludge is essential. The present research focused on gaining more insight in the factors determining the growth and quality of anaerobic granular sludge.

For determining the total available pore volume and the pore diameter distribution of granular sludge, a method based on size exclusion chromatography has been developed. For most types of sludge, the available pore volume varies between 40% and 80%, granules with a lower porosity probably contain layers that are impermeable to substrate. Small granules were found to have a considerably higher porosity and a higher maximum methanogenic activity than larger granules from the same sludge sample.

In applying higher sludge loads, the average granule diameter increases. A decrease in sludge load or changes in substrate composition result in a weakening of sludge granules, probably due to a lack of substrate for the bacteria in the centre of the granules. In view of the stability of methanogenic granular sludge, this should be attributed to the dying off of the acidifying population inside the sludge granules.

The process in UASB reactors is strongly influenced by the composition and degree of pre-acidification of the wastewater. It was found that non-acidified gelatine can be treated in an one-phase UASB reactor without difficulties up to a sludge load of 1.2 $gCOD.(gVSS.d)^{-1}$. However, sucrose-containing wastewater can be treated only at a sludge load below 0.5 $gCOD.(gVSS.d)^{-1}$. At higher sludge loading rates non-acidified sucrose in the reactor influent can cause problems with regard to sludge retention. However, too much pre-acidification of wastewater can also cause problems. Acidogenic bacteria suspended in the influent may cause very serious flotation of granular sludge.

In treating sulphate-containing wastewater, sulphate-reducing bacteria (SRB) and methane-producing bacteria (MPB) will compete for substrate. It is often assumed that MPB can maintain in high-rate anaerobic reactors because of the poor ability of SRB to attach themselves compared to MPB. However, the present study shows that sulphate-reducing bacteria are capable to maintain in granular sludge. They were even found to be able to attach themselves: on pumice stone as carrier material, purely sulphidogenic aggregates were formed.

A negative effect of a deficiency in phosphate on the methanogenic activity of granular sludge was found to be fully reversible in the presence of phosphate. The phosphorus content of granular sludge from the laboratory reactors fed with gelatine varied between 6 $mgP.gVSS^{-1}$ for sludge from reactors fed with influent that contained almost no phosphate and 10.5 $mgP.gVSS^{-1}$ for sludge from reactors with a sufficient supply of phosphate. Deficiency in phosphate was found to be easily demonstrable: an increase in methanogenic activity after phosphate dosage and/or a rapid uptake of phosphate are clear indications of a deficiency in phosphate in granular sludge.

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CONTENTS

1 INTRODUCTION

1 INTRODUCTION

Anaerobic wastewater treatment

Since the start of industrialization, the production of wastes and wastewater has increased greatly, and at the same time has been concentrated increasingly in relatively small areas. Consequently, many rivers and lakes have changed into open sewer systems. Because surface waters serve various purposes, wastewater treatment has become more and more important, and since the middle of the last century, wastewater treatment plants have been constructed in ever increasing numbers.

Despite the fact that McCarty published his paper "One Hundred Years of Anaerobic Digestion" in 1982, aerobic systems have so far dominated both municipal and industrial wastewater treatment. For a long time, the use of anaerobic treatment systems has been restricted to sludge and slurry digestion. The development of systems based on biomass retention, such as the anaerobic filter at the end of the sixties (Young and McCarty 1969), extended the applicability of anaerobic systems to wastewater streams. Anaerobic wastewater treatment did not become generally accepted immediately however, probably mainly due to the fact that anaerobic systems are, literally and figuratively, black box processes. Anaerobic treatment had, and partly still has, the image of a promising but unproven technology.

However, high energy prices during the energy crisis in the seventies have focused attention on some of the very attractive features of anaerobic wastewater treatment: low energy requirements and, in many cases, the ability to generate biogas as a usable fuel. The real breakthrough of anaerobic wastewater treatment resulted from the development of the Upflow Anaerobic Sludge Bed (UASB) reactor (Lettinga et al. 1980, 1983). Several successful applications of the UASB (and related upflow reactor types) to many different types of wastewater have been reported since then (Hulshoff Pol 1989; Lettinga and Hulshoff Pol 1991; Lin and Yang 1991).

Legal restrictions and high levies on wastewater discharge are among the driving forces behind the development and application of (industrial) wastewater treatment. Economic considerations frequently dictate what treatment systems is chosen.

As already mentioned above, the increasing interest in anaerobic treatment during the seventies was at least partly motivated by the increase of energy prices. In the nineties, one of the major environmental problems is how to get rid of large amounts of solids, such as wastewater treatment sludge, legal restrictions and high levies restrict the solid waste discharge. Today the small excess sludge production of anaerobic treatment systems compared to conventional aerobic wastewater treatment systems is one of the most significant benefits of anaerobic systems.

From an economic point of view, the granular sludge produced in UASB reactors is certainly not a type of waste, but rather a saleable by-product. Although granular sludge is not essential for wastewater treatment using UASB reactors, today almost all full-scale reactors are started up using granular seeding sludge, in order to reduce the start-up period. Moreover, by the use of granular seeding sludge, anaerobic treatment becomes feasible also in the case of wastewater types in which the formation of granular sludge does not proceed well. Particularly because granular sludge can be stored unfed over a long period without serious deterioration (Hulshoff Pol 1989; Wu et al. 1985), anaerobic granular sludge will be regarded more and more as a valuable raw material necessary for optimal wastewater treatment.

Granular sludge

Since the mid-seventies, the transformation of flocculent anaerobic biomass into stable aggregates has been investigated extensively. Although some of these investigations focused on the formation of acidifying aggregates (Beeftink 1987; Mulder 1990; Zoetemeyer 1982; Zoutberg 1990) or on denitrifying sludge granules (van der Hoek 1988), the term "granular sludge" has so far been used almost exclusively for aggregates developed in anaerobic upflow reactors.

Since the first observation of anaerobic granular sludge in 1973 (de Zeeuw 1988; Lettinga et al. 1980), a lot of research has been carried out in order to gain more insight into the mechanism of granulation, and in view of the practical implications of the use of granular sludge in treatment systems (de Zeeuw 1984; Dolfing 1987; Grotenhuis 1992; Hulshoff Pol 1989; Wiegant 1986). The granulation process first became an important issue in comprehensive studies in the Netherlands, but later on also in many other countries. Many studies have shown that the mechanism of the granulation process is based on the specific process conditions applied in upflow reactors: selective wash-out of suspended bacteria initiates autoimmobilization of bacteria into highly settleable aggregates (Guiot et al. 1988a,b; Hulshoff Pol 1989; Wiegant 1988; Wu 1987; Yoda et al. 1989).

Although the mechanism of the granulation process is now fairly well understood, there are still questions in relation to the occurrence of specific problems in full-scale UASB reactors. An understanding of these problems will extend the applicability of anaerobic wastewater treatment to wastewater which, up until now, has been considered unsuitable for anaerobic treatment.

Definition of granular sludge

Granular sludge can be defined as biomass with some very specific properties which make it very suitable for upflow wastewater treatment systems. Granule characteristics pointed out by Hulshoff Pol (1989) are: a high sedimentation velocity, and a high methanogenic activity. Microbiologically, sludge granules can be regarded as well-balanced micro-ecosystems which include all bacterial species necessary for the degradation of the organic pollutants present in the wastewater to which it is exposed.

Unlike temporary particles such as sludge flocs, sludge granules are mechanically stable, separate entities. Morphologically, sludge granules can be characterized as relatively large ($d > 0.5$ mm) particles of more or less regular shape and with a well-defined surface. Together with their relatively high density, their morphology results in excellent settleability.

In contrast with other types of immobilized biomass, inert carrier material neither plays an essential role in the formation of anaerobic sludge granules nor is an important factor with regard to their stability. Because the formation of sludge granules is based on autoimmobilization, granular sludge can consist for the most part of (active) biomass. Table 1 lists the most important granule characteristics.

Table 1 Granule characteristics important to the functioning of (methanogenic) upflow wastewater treatment systems.

Characteristic	Description
Biological activity	Most important to biological wastewater treatment is the conversion and removal of organic wastewater components. In UASB wastewater treatment systems, the specific methanogenic activity and/or the specific sulphate-reducing capacity of the sludge are the most critical factors.
Sedimentation velocity	In upflow reactors, a high sedimentation velocity of sludge granules is essential for biomass retention.
Mechanical strength	A sufficient biomass hold-up in a reactor is crucial for a stable wastewater treatment process. In most UASB reactors, the biomass hold-up is based on the the high settleability of anaerobic granular sludge. To avoid loss of biomass, the formed granules must be sufficiently stable, and resistant to disintegration, (shear) forces, and forces related to internal gas formation.
Development	The development of granular sludge is based on a continuing process of autoimmobilization of bacteria; the sludge granules consists for only a minor fraction of inert carrier material.

Granular sludge quality

As mentioned before, the presence of granular sludge is not essential for wastewater treatment in UASB reactors. Many reactors are designed however in such a way that a granular type of sludge is necessary for the satisfactory functioning of the system. The quality of granular sludge should be related to the extent to which it allows UASB reactors to operate properly. A clear definition of the quality of granular sludge cannot be given, because each situation may require different sludge characteristics.

Both the sludge characteristics themselves and the sludge characteristics needed for proper operation are closely related to the process conditions applied and the composition of the wastewater treated. The relations involved in the granulation process are presented in figure 1. This scheme aims to demonstrate the extreme complexity of the relations between the many microbiological and physical factors involved in sludge granulation. The scheme neither pretends to be complete nor to suggest that it presents the accurate cause-effect relationships between all the parameters involved.

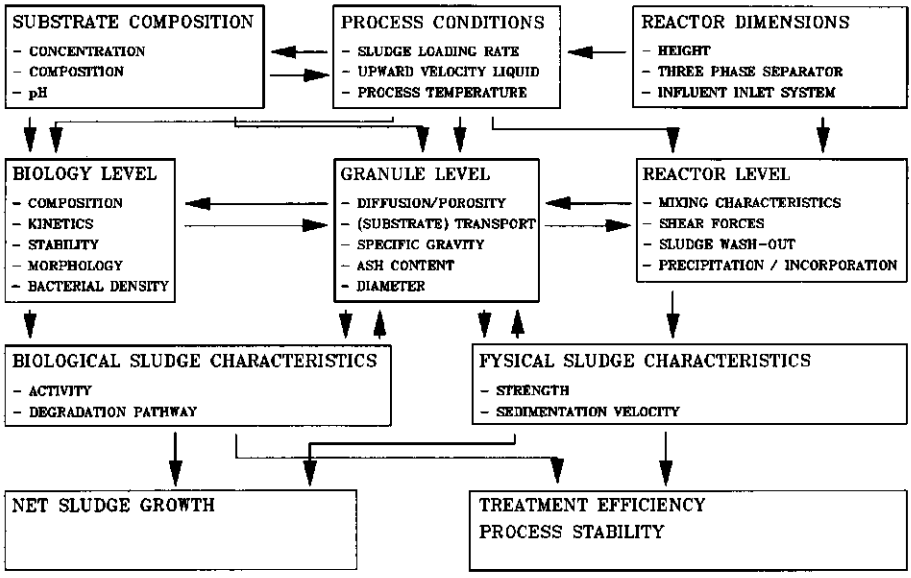

Figure 1 A scheme of the complex relations between wastewater characteristics, process conditions, and granule quality.

Granular sludge characterization

As already mentioned above, the quality of granular sludge depends on a combination of microbiological and physical characteristics that are advantageous for the performance of upflow wastewater treatment systems. The most important physical properties of granular sludge with respect to its behaviour in a UASB reactor are: settleability (mainly related to the size and density of the particles), and mechanical strength. Of course, its biological performance is mainly determined by the composition and distribution of the microbial population, but other factors are also important, for example the granules' porosity, pore size distribution, and permeability for substrate and products (chapters 3 and 4).

Through adequate and accurate sludge characterization, relevant predictions with regard to the functioning of a granular sludge type can be made. However, so far few methods for sludge characterization have been available. The mechanical strength of granular sludge can be assessed using a method based on compression (Hulshoff Pol et al. 1986) or by measuring the abrasion with a Couette vessel or a continuously stirred vessel (Tramper et al. 1984). The granule size distribution also can be determined on the basis of two different principles. Hulshoff Pol et al. (1986) developed a method based on the sedimentation velocity of granular sludge. A method based on the image analyzing technique was developed by Tramper et al. (1984) and Grotenhuis et al. (1992a).

Many researchers regard mass transfer resistance in biofilms and/or granules as one of the major factors controlling the reactor performance (Alibhai and Forster 1986a,b; Dolfing 1985; Grotenhuis et al. 1991a). Mass transfer values play an important role in modelling studies concerning granular sludge and biofilms (Beeftink 1987; Canovas-Diaz and Howell 1988); Rittmann and Manem 1992 and Stevens 1988). Recently, Goodwin et al. (1991); Kitsos et al. (1992) and Nilsson and Karlsson (1989) developed methods for assessing the diffusion coefficient in anaerobic biofilms. However, at present there are little experimental data on substrate diffusion in granular sludge.

Mass transfer limitation is related to the porosity of sludge granules. We have developed a method for assessing both the available pore volume and the pore size distribution of sludge granules (chapter 3). Chapter 4 relates the porosity data, maximum specific methanogenic activity, and substrate affinity data of several types of granular sludge to the results of a simple method for assessing the diffusion constant in granular sludge.

The porosity and density of granular sludge can also be estimated optically using microscopy. Especially scanning electron microscopy gives very illustrative spatial information on the granule organization and morphology of the dominant species.

However, for analysing the internal structure of granular sludge, cross-sectioning is necessary. Unfortunately, in the case of large and weak objects such as anaerobic sludge granules, this method leads to a serious disruption of the structure. Therefore, in order to obtain more usable information about the structure of granules, a cryo-fractioning method has been developed which makes smooth and unaffected sectioning surfaces possible (chapter 5).

The effect of sludge loading rate on granular sludge

One of the objectives of our investigations was to identify the most important process conditions affecting the granular sludge quality and granular sludge growth in UASB reactors. We have paid special attention to those factors which can be regulated by system design or process control. One of the most important process conditions in this respect is the sludge loading rate applied.

Morvai et al. (1990) found a positive relation between the sludge loading rate and the average granule size. Grotenhuis et al. (1991a) found a positive relation between the influent concentration and the average granule size. However, it is very difficult to discriminate between sludge loading rate- and influent concentration effects, since loading rates and influent concentration are often related.

It is likely that granule size is related to substrate penetration depth. Beeftink (1987) developed a model for the autoimmobilization of acidogenic bacteria in gaslift reactors, in which particle size and mechanical strength of the developed particles are regulated by lysis of internal organisms due to substrate limitation. For methanogenic sludge granules, we presume an analogous mechanism.

Substrate limitation occurs (and negatively affects the granule quality) when granule size and substrate penetration are not balanced (Dolfing 1985; Grotenhuis et al. 1986; Kosaric et al. 1990b; chapter 4 of this thesis). For this reason, the relation between the sludge loading rate applied and the granule quality in laboratory-scale UASB reactors was investigated (chapter 6). Especially with regard to the start-up procedure of UASB reactors, it is very likely that there is a significant influence of loading rate changes on granular sludge quality.

Suspended material in the reactor influent

The presence of suspended matter in influent may be a major cause of problems in anaerobic wastewater treatment in UASB reactors (Hulshoff Pol 1989; Lettinga et al. 1985; Lin en Yang 1991; Sayed et al. 1988). Due to the presence of SS (Suspended Solids), insufficient growth of new granular sludge is likely to occur, while also the granule characteristics with respect to strength, settleability and methanogenic activity may become detrimentally affected.

Since the presence of suspended acidogenic bacteria is one of the typical aspects of pre-acidified wastewater, suspended acidogenic bacteria belong to the most common types of suspended material present in the influent of methanogenic UASB reactors. chapter 7 presents the results of investigations in which we assessed the effects of suspended acidogenic bacteria present in the influent of methanogenic UASB reactors on the granular sludge quality and process stability.

Wastewater pre-acidification

Anaerobic wastewater treatment can be controlled by regulating the degree of pre-acidification of wastewater fed into a methanogenic reactor. For many years, the possible benefits of using wastewater pre-acidification for optimization of anaerobic wastewater treatment systems have been a point of discussion. A higher volumetric loading rate in the methanogenic reactor and a lower sensitivity to toxic compounds in the wastewater were designated as the main benefits of the two-step treatment by Cohen et al. 1979, 1985; Dinopoulou and Lester 1989 and Komatsu et al. 1991. On the other hand, the poor granule formation in methanogenic reactors treating acidified wastewater (Hulshoff Pol 1989; Sam-Soon et al. 1988; Vanderhaegen et al. 1992) and the high investment costs of two-step systems (Lettinga and Hulshoff Pol 1991) were considered as serious drawbacks already in the first reports on anaerobic wastewater treatment. Furthermore, little information is available about the influence of non-acidified substrate on the process stability and granule quality. Problems related to the formation of bulking sludge and the occurrence of sludge flotation in UASB reactors treating non-acidified sucrose were reported by Anderson et al. (1991); Hulshoff Pol et al. (1983b) and Mendez-Rapin et al. (1986).

In a study, which is described in chapter 8, we investigated the effects of non-acidified substrates on the granular sludge quality in UASB reactors by conducting a number of experiments using gelatin and sucrose as non-acidified model substrates.

Substrate competition between sulphate-reducing bacteria and methane-producing-bacteria

In anaerobic wastewater treatment, either sulphate reduction or methanogenesis can be the final step in the degradation sequence. Both methane-producing bacteria (MPB) and sulphate-reducing bacteria (SRB) are capable of using acetate and hydrogen as substrates, resulting in a competition between SRB and MPB for these substrates. Based on thermodynamic and kinetic data, SRB should be able to win this competition. Studies of sediments and studies using CSTR reactors confirm this prediction (Lovley and Klug 1986; Middleton and Lawrence 1977; Rinzema and Lettinga 1988; Robinson and Tiedje 1984 and Smith and Klug 1981). However, in high-rate anaerobic reactors it has

frequently been observed that acetate-degrading MPB are very well able to compete with SRB. Even complete conversion of acetate into methane has often been found under sulphate-reducing conditions (Hoeks et al. 1984; Mulder 1984 and Rinzema and Lettinga 1988). According to Isa et al. (1986a,b), a superior attachment capacity of MPB in relation to SRB results in a selective wash-out of SRB from high-rate anaerobic reactors. In this way, MPB can compete successfully with SRB. Immobilization of methane-producing bacteria (MPB) has already been discussed extensively by Dolfing 1987, Grotenhuis 1992, Hulshoff Pol 1989. However, so far little is known about the immobilization of sulphate-reducing bacteria (SRB) in anaerobic reactors.

Methods to control the competition between SRB and MPB in the anaerobic treatment of sulphate-containing wastewaters are very valuable for two reasons. In most situations, the production of toxic (hydrogen) sulphide is a great disadvantage of anaerobic treatment: it can cause major problems (corrosion, bad smell, toxicity), and reduces the amount of COD removed from the wastewater. On the other hand, there is a growing interest in the development of wastewater treatment systems based on sulphate reduction, because such treatment systems can be used for removing heavy metals from wastewater by stimulating the precipitation of metal sulphides or can be used in order to remove S-compounds from wastewater (Buisman 1989).

Many industrial wastewaters, such as those of the fermentation-, edible oil-, and paper and pulp industry, contain high concentrations of sulphate, sulphite or other S-compounds (Rinzema and Lettinga 1988). For such wastewaters, the occurrence of sulphate reduction is an important factor: if the wastewater composition and process conditions are not advantageous enough in a selective sense to MPB, SRB may remove the MPB present.

The investigations described in chapter 9 concern UASB experiments in which different hydraulic retention times (HRT) and superficial liquid velocities were applied in order to assess the influence of selection pressure on granule formation in systems that are both methanogenic and sulphidogenic. One hypothesis for the poor competitive properties of SRB in high-rate treatment systems is based on the possibly inferior attachment capacity of SRB. Chapter 10 presents the results of a study of the granule formation capacity of SRB in the presence and absence of methanogens. Chapter 11 describes experiments for finding out whether the formation of pure sulphidogenic granules can be enhanced by using a suitable carrier material, such as pumice, methanogenic granular sludge, and inactivated methanogenic sludge granules.

Nutrient limitation

Insufficient sludge growth is one of the factors which may upset a stable biological treatment process. Since in general the availability of nutrients is important to microbial

growth, insufficient sludge growth may be induced by nutrient deficiency of the wastewater concerned (Hulshoff Pol et al. 1983a; Speece 1987).

As phosphorus is an essential nutrient, it often needs to be added to nutrient-deficient industrial wastewater in order to make it feasible for anaerobic treatment. In practice, phosphate is added to wastewater in concentrations ranging from 2 to more than 50 mg $P.l^{-1}$, generally without correlating the added amount with the applied loading rate or the wastewater composition.

Obviously, overdosage of phosphorus is unacceptable from both an economic and an environmental point of view. Chapter 12 presents the results of a study performed to investigate the effect of different phosphorus dosage regimes on the growth, methanogenic activity, and physical characteristics of granular sludge.

Scope of this thesis

The Upflow Anaerobic Sludge Bed (UASB) reactor is presently by far the most common anaerobic wastewater treatment system. The benefits of the UASB and related systems over other anaerobic systems can be partially attributed to the formation of granular sludge in the former systems.

Knowledge of the factors affecting the quality and growth of anaerobic granular sludge will become increasingly important, especially because today almost all reactors are started up using granular seeding sludge. Some process conditions and/or types of wastewater may cause a serious decrease in granule quality or an insufficient growth of granular sludge. Also changes in process conditions may cause an instability in the treatment process.

The present research focused on gaining more insight into the factors determining the growth and quality of anaerobic granular sludge. The first part focuses on methodology. Chapter 2 describes the materials and methods used during the experiments, and chapters 3-5 discuss some newly developed methods for the determination of granule porosity, and substrate transport in granules. In the second part of this thesis (chapters 6-12), special attention is paid to those aspects of granular sludge quality which can be regulated by system design or process control: the sludge loading rate applied (chapter 6), the presence of suspended acidogenic bacteria and the degree of wastewater pre-acidification (chapter 7 and 8 respectively), the competition between methanogenic bacteria and sulphate-reducing bacteria (chapters 9-11), and the effect of phosphorus limitation (chapter 12).

Chapter 13 summarizes the results of the investigations carried out, and includes a general discussion, while chapter 14 summarizes and discuss both the introduction and the results in Dutch.

2 MATERIALS AND METHODS

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MATERIALS

UASB Reactors

Several types of UASB (Upflow Anaerobic Sludge Bed) reactors were used during the experiments present in this thesis:

- 3.2 litre liquid volume plexiglass UASB reactors (height approx. 420 mm, internal diameter 95 mm) with an external phase separator (figure 1a) (chapters 7 and 8). This reactor type was operated in batch-fed recirculation mode also (figure 1b) during the experiments described in chapter 7.
- 3.2 litre liquid volume reactors identical to those described above, but equipped with a conventional (internal) 3-phase separation (figure 1c). These reactors were used during the experiments described in chapters 6 and 12.
- 1.1 litre liquid volume PVC UASB reactors (height 400 mm, internal diameter 60 mm) with a conventional (internal) 3-phase separator (figure 1c) were used during the experiments described in chapters 9, 10 and 11.
- 0.2 litre liquid volume glass UASB reactors (height approx. 170 mm height, internal volume 39 mm). A glass marble was serving as a three-phase separator in this reactor type (according to Rinzema et al. (1989)) (figure 1d). These reactors were used in experiments described in chapter 12.

The methane production was measured using wet gas meters (Meterfabriek Dordrecht, Dordrecht, the Netherlands) placed in series with a 3-5% (w/v) NaOH solution and a soda-lime pellets column to remove CO_2 and H_2S from the biogas in all UASB reactor except those of 0.2 litre liquid volume. In the 0.2 litre reactors the methane production was monitored by liquid replacement using Mariotte flasks filled with 3% NaOH (w/v).

CSTR and batch reactors

- 2.5 litre liquid volume, intermittently stirred (30 s pulse, 3 min. pause), plexiglas batch reactors (figure 2c) were used to measure the substrate affinity and the phosphate uptake velocity (described in chapter 4 and 12 respectively). The liquid was sampled periodically with a fraction collector, 2112 Redirac fraction collector, LKB Bromma, Sweden. Methane production was measured by use of Mariotte flasks filled with 3% (w/v) NaOH solution.

- a 20 litre acidogenic CSTR (Continuously Stirred Tank Reactor) operated at a HRT of 24 hour and fed with a sucrose solution was used to produce suspended acidogenic bacteria used in the experiments described in chapter 7. In order to obtain a stable acidification pattern the pH of the CSTR reactor was controlled at pH 5.8 according the results of Zoetemeyer (1982).
- 0.5 litre and 1 litre serum flasks batch reactors were used to assess the maximal methanogenic activity. The methane production rate in the tests was monitored with modified Mariotte flasks containing 3% of NaOH (w/v).

Schemes of the batch reactors used in the activity assays are given in figure 2. The activity assays are described below.

All reactors used were placed in a temperature controlled room (30 ± 1°C). To prevent premature degradation the influent of the continuous flow reactors was stored at 4°C. When separate influent flows were used (organic substrate, nutrients and/or dilution water) only the degradable organic substrate solution was cooled.

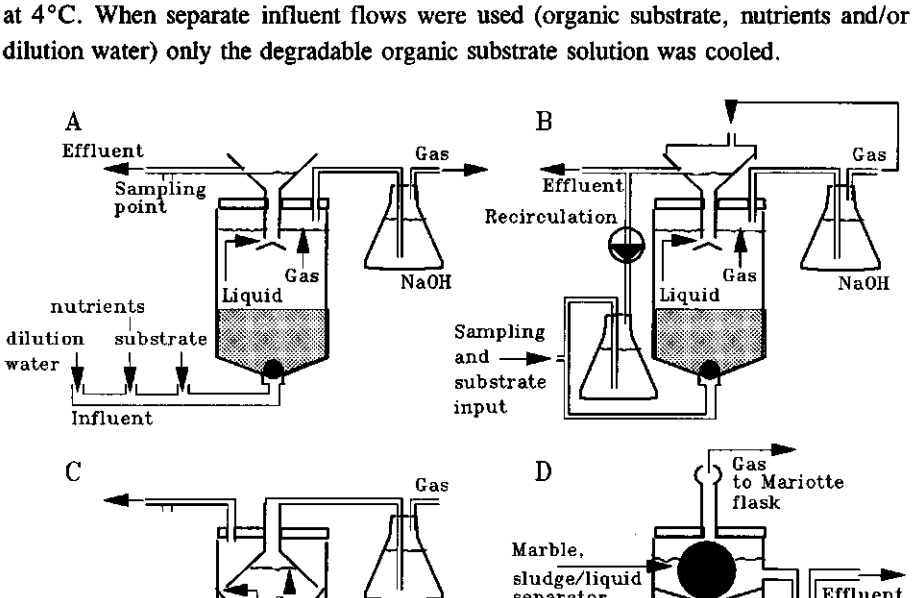


Figure 1 Schematic view of the used laboratory scale UASB reactor types. A: UASB reactor equipped with an external three-phase separator; B: identical reactor as presented in figure 1a, but operated in batch-fed mode; C: UASB reactor equipped with an internal three-phase separator, D: UASB reactor equipped with a marble functioning as solid/liquid phase separator.

Media

Nutrients were supplied in the concentrations mentioned in table 1, except when mentioned otherwise (chapter 12). Trace elements were added in all experiments using a stock solution composition adapted from Huser (1980).

All chemicals used are of PA quality (Merck AG, Darmstadt, Germany) except the yeast extract (Oxoid extract, Unipath Ltd, Basingstroke, England), the $NaHCO_3$ and the NH_4Cl except when mentioned otherwise (chapter 12). Tap water was used for all media except when mentioned otherwise.

Table 1 Nutrient composition of the substrates used[a].

Composition and concentration of the nutrient used in the continuous flow experiments[b].		Composition and concentration of the nutrients used in the batch assays[c].			
		For all assays except those presented in chapters 9-11		For assays used in the studies presented in chapters 9-11	
component	conc. $mg.l^{-1}$	component	conc. $mg.l^{-1}$	component	conc. $mg.l^{-1}$
NH_4Cl	1040	NH_4Cl	280	NH_4Cl	280
KH_2PO_4	170	KH_2PO_4	250	$NaH_2PO_4.2H_2O$	795
$(NH_4)2SO_4$	170	$MgSO_4.7H_2O$	100	K_2HPO_4	600
$MgCl_2.6H_2O$	150	$CaCl_2.2H_2O$	10	$MgSO_4.7H_2O$	110
KCl	270	$NaHCO_3$	400	$CaCl_2.2H_2O$	10
Yeast extract	18	Yeast extract	100	$NaHCO_3$	2000
$NaHCO_3$[d]	0.5			Yeast extract	20

[a] Different nutrient compositions were used in several experiments to link up the obtained results much as possible to corresponding studies at our department

[b] The nutrients were added using a 1000 : 6 concentrated stock solution

[c] The nutrients were added using a 5 : 1 concentrated stock solution

[d] $g.gCOD^{-1}$

Table 2 Composition of the trace element stock solution used[a].

$FeCl_2.4H_2O$	2000	$mg.l^{-1}$	$ZnCl_2$	50	$mg.l^{-1}$
$MnCL_2.4H_2O$	500	$mg.l^{-1}$	$NH_4MoO_{24}.4H_2O$	50	$mg.l^{-1}$
Rezasurine	500	$mg.l^{-1}$	$AlCl_3$	50	$mg.l^{-1}$
Mg-EDTA	500	$mg.l^{-1}$	$CoCl_2.6H_2O$	50	$mg.l^{-1}$
$NaSeO_3$	100	$mg.l^{-1}$	$CuCl_2.2H_2O$	50	$mg.l^{-1}$
H_3BO_3	50	$mg.l^{-1}$	HCl	1	$ml.l^{-1}$

[a] In the continuous flow experiments 1 $ml.l^{-1}$ of this solution is used. In the batch experiments 0.1 $ml.l^{-1}$ substrate is used. The composition is adapted from Huser (1980)

Biomass

Granular sludge was used to seed the methanogenic reactors, with exception of the studies to the formation of granule formation in sulphidogenic and methanogenic systems, which were seeded with crushed granular sludge (chapters 9 - 11). The granular sludges were elutriated before use applying an upward velocity of 15 $m.hr^{-1}$. The sludge was stored at 4°C before use. Different granular sludge types were used:

- Granular sludge from a full-scale UASB reactor treating wastewater of an alcohol industry (Nedalco, Bergen op Zoom, The Netherlands) (all experiments).
- Granular sludge from a full-scale UASB reactor treating wastewater of the potato industry (Aviko, Steenderen, The Netherlands) (chapters 3 - 5, one experiment presented in chapter 12).
- Granular sludge from a full-scale UASB reactor treating wastewater of a starch factory, (Latenstein, Nijmegen, the Netherlands).
- Granular sludge from a pilot scale EGSB (Expanded Granular Sludge Blanket) reactor processing municipal wastewater (Bennekom, the Netherlands).
- Granular sludge from a full-scale UASB reactor treating wastewater of a recycle paper factory (Roermond Papier, Roermond, The Netherlands.

METHODS

Activity

The maximal methanogenic activity is defined as the methane production rate of the sludge under optimal conditions (gCH_4-COD.$(gVSS.d)^{-1}$). Similarly, the maximal sulphate reducing capacity is defined using the S^{2-}-COD production or the SO_4^{2-} decrease.

The maximum sludge activity assays were performed in non-stirred, 500 or 1000 ml serum flasks placed at 30°C. A mixture of acetate, propionate and butyrate (1:1:1, based on COD-values) was used as substrate. At the start of the maximum methanogenic sludge activity assays 3 gCOD.l^{-1} of substrate (VFA) was added. The initial pH was set at pH 7. The sludge concentration during the activity tests was approx. 1.5 gVSS.l^{-1}. Nutrients and trace elements were added as mentioned in table 1 and 2. The activity was assessed by monitoring the methane production rate using a liquid replacement system consisting of a 3% (w/v) NaOH solution (figure 2a).

In the studies present in chapters 9 - 11 some modifications were applied. The methanogenic and the sulphidogenic activity of the sludge were measured using the same set up, but only 2 gCOD.l^{-1} of substrate, and for the assays in the presence of sulphate, 4 g SO_4^{2-}.l^{-1} was added to the medium. The sludge activities were measured by monitoring the substrate, sulphate and sulphide concentrations in the liquid and the methane concentration in the headspace (figure 2b).

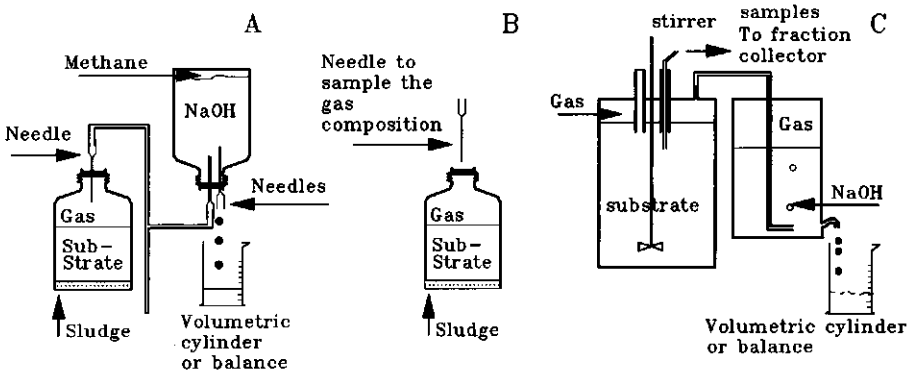


Figure 2 A schematic view of the batch reactors used for the assessment of the maximal methanogenic sludge activity. A: Serum bottle "reactor". Methane production was measured applying liquid displacement from a serum bottle; B: Serum bottle "reactor". Methane production was measured by monitoring the concentration in the headspace; c: Stirred batch reactor system. Methane production was measured using a Mariotte flask.

Microscopy

Scanning electron microscopy (SEM) (Jeol JSM 5200) was used to examine the internal and external structures of the sludge types studied. Granules and flocculant sludges were fixed for 2 hours at 20°C in 2.5% glutaraldehyde. After washing, fixation continued in 1% osmium tetroxide for 1.5 hours. Subsequently, granules were dehydrated in a graded ethanol series (10% - 30% - 50% - 70% - 96% - 100%) and critical point dried, mounted on stubs with colloidal silver (Biorad) and sectioned using razor knife. The samples were sputter coated with gold/palladium before observation in the SEM.

Pictures at low magnifications were made by use of an Olympus ZS40 zoom microscope.

Size distribution of the sludge granules

The size distribution of the sludge was determined by the image analyzing technique adapted from Grotenhuis et al. (1991a). For sampling, the total sludge bed was removed from the reactor. After decanting most of the fluid followed by mixing the sludge to avoid segregation by sedimentation, sludge samples of approx. 1 ml were brought in to 3.5 cm petri dishes. The sludge particles were fixed in kaisergelatin (Merck AG, Darmstadt, Germany). Pictures of the dishes (minimal 4 plates per sample and minimal 500 particles per sample) were digitalized and analyzed by image analyzing software (TEA Image Manager (TIM), Difa Measuring Systems BV, Breda, the Netherlands). Assuming ideal spherical shapes of the particles the radius and volume were calculated from the two-dimensional projection of the particles.

Specific gravity of granular sludge

The granule specific gravity measurements were performed by use of a pycnometer according to Standard Methods (method 213e). Surface attached water was removed by spreading the sludge sample during 5 minutes on a grid placed on a hydroscopic tissue (paper napkin). Subsequently, sludge was placed for 0.5 hour at H_2O saturated, 30°C air.

Strength of the granular sludge

The granular strength of a sludge sample was measured as the resistance against compression forces according Hulshoff Pol et al. (1986). A scheme of the measurement is given in figure 3a. The force which correlates with a downward movement of the piston is measured by a load cell, and is printed with a recorder. Above a critical compression, the granules disintegrate and the force increase per piston movement changes (point Y, figure 3b). A short time before this point is reached, the first biomass

is observed in the space between piston and cylinder (point X, figure 3b). Since this point was observed to be more reproducible, the force recorded at this moment, expressed as $N.m^{-1}$ is assumed to be characteristic for the granular strength.

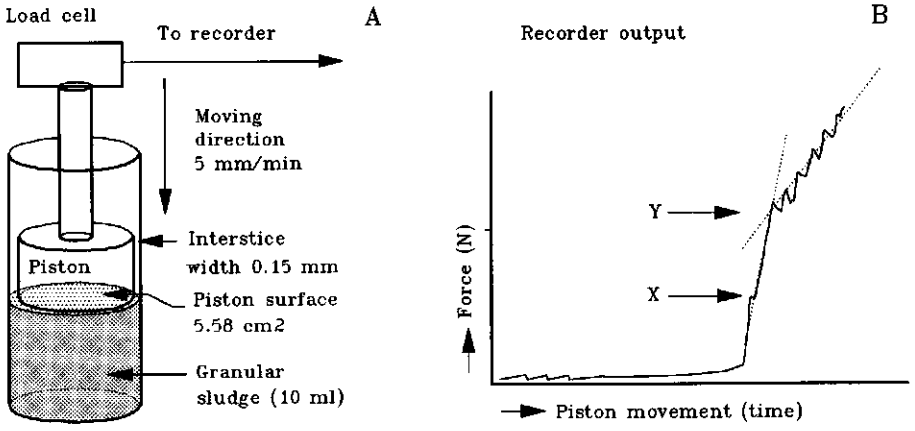


Figure 3 Schematic view of the set-up of the granular strength measurement (A) and the recorder output of the measurement (B).

Sludge bed sampling

The sludge amount in the reactors was evaluated by sampling the total sludge bed, and measuring the volume after 5 minutes sedimentation in a two litre volumetric cylinder. Samples for sludge analysis were taken after decanting the fluid and subsequently continuously mixed during sampling to avoid segregation by sedimentation. The VSS and TSS (volatile suspended solids c.q. total suspended solids) concentration were determined on a well mixed sample of this amount (the volume was measured in a 100 ml volumetric cylinder after replacing the reactor fluid):

Total VSS amount (g) = sludge volume (ml) x VSS concentration ($gVSS.ml^{-1}$)

VSS and TSS were analyzed according the Dutch Standard Methods (NEN 32355.3). The total solids were measured after drying at 105°C (over night), the ash content after 2 hr at 550°C. The VSS content was calculated as the difference between total solids and ash.

Volatile fatty acids (VFA)

Volatile fatty acids (VFA) were analyzed with a Packard 417 and a HP 5890 gas chromatograph equipped with a 2 m x 4 mm (i.d.) glass column packed with Supelcoport (100-120 mesh) coated with 10% Fluorad FC 431. Temperature of the column, injection port and flame ionization detector were 120, 220 and 240°C, respectively. Nitrogen saturated with formic acid was used as carrier gas at a flow rate of 50 ml/min. All samples were centrifuged (4 min., 15 600 g) and fixed by adding 3% formic acid (dilution 1:1).

Chemical oxygen demand (COD)

The COD was determined according to the "micro method" as described by Jirka and Carter (1975). The sample was oxidized with dichromat in H_2SO_4 (18M) under pressure at 160°C in closed 20 ml glass vessels during 2 hr. The sample was analyzed colorimetrical. The sulphide present in the samples from the reactors was removed prior to the COD measurement by flushing with nitrogen gas.

Sulphate and sulphide

Sulphate was measured with a high pressure liquid chromatograph (Spectra Physics), equipped with a 100 mm x 3 mm Ionosphere A column and a conductivity meter (Waters 431). The temperature of the column and the detector were 30°C. Samples were centrifuged first and diluted with demineralized water to the appropriate concentration. 20 μl of sample was injected. The eluent was 0.027 M potassium biphtalate, pH 9, at a flow rate of 0.4 $ml.min^{-1}$.

Sulphide was measured colorimetrically using a method adapted from Trüper and Schlegel (1964).

3 DETERMINATION OF THE PERMEABILITY AND POROSITY OF ANAEROBIC SLUDGE GRANULES BY SIZE EXCLUSION CHROMATOGRAPHY

This chapter consists of a modified edition of:

Determination of the permeability and porosity of anaerobic sludge granules by size exclusion chromatography.
P.Arne Alphenaar, M.Cecilia Pérez, Willem J.H. van Berkel and Gatze Lettinga
Appl. Microbiol. Biotechnol. (1992) 36:795-799

3 DETERMINATION OF THE PERMEABILITY AND POROSITY OF ANAEROBIC SLUDGE GRANULES BY SIZE EXCLUSION CHROMATOGRAPHY

ABSTRACT

A new application of size-exclusion chromatography is described for assessment of the permeability and internal pore distribution of anaerobic sludge granules.
The fractionation range and adsorption characteristics were investigated for a series of standard proteins and dextrans. To determine possible adsorption of solutes and stability of the sludges, the pH and salt concentration of the mobile phase were varied.

Good results were obtained using dextrans as solutes and tap water as mobile phase. To inhibit the sludge activity without affecting the granule characteristics the experimental arrangement was operated at 4°C.

Three granular sludge types were investigated. The permeability of the granular sludges varied from 7% to 96%. Also the exclusion limit expressed as molecular mass showed large differences. For two sludges, molecules greater than 80 000 Da cannot penetrate the pores; for one sludge the exclusion limit is 1 300 Da. Experiments using acetic acid as an indicator of permeability gave similar results.

INTRODUCTION

The upflow anaerobic sludge blanket (UASB) reactor is the most widely applied anaerobic waste-water treatment system (Lettinga et al. 1987). The benefits of the UASB over other anaerobic systems are partially related to autoimmobilization of the bacteria (Beeftink 1987; Grotenhuis et al. 1987; Lettinga et al. 1987; Hulshoff Pol 1989), which results in a granular sludge with particles up to 6 mm in diameter. The volatile solid (VSS) content of these granules varies widely (20-99%), but usually a percentage of 70 - 80% is found.

The major part of VSS is bacterial biomass. In granules, considered as a typical form of biofilm, transport of substrate and biogas will be an important factor (Harremoës 1978).

Porosity and pore size distribution are closely related to substrate and biogas transport. For large aggregates, transport limitation of substrate and products will have a substantial influence on the fraction of bacteria that will be active, and so on the quality of the granules (Beeftink 1987; de Beer 1990).

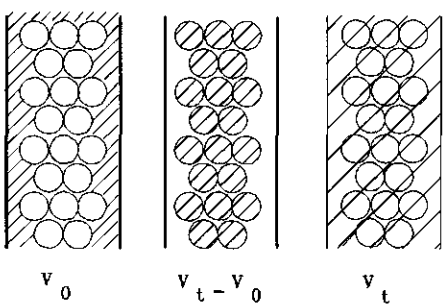
The extent to which substrate can penetrate a granule (and reach the bacteria) probably is one of the main factors influencing the granule characteristics, and thus:

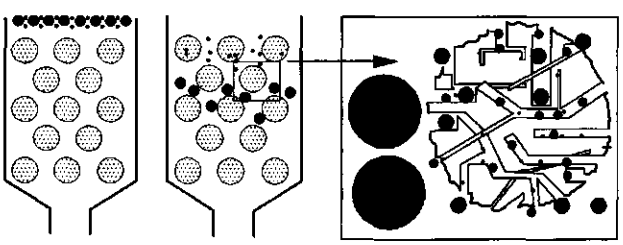
- For more accurate modelling of the granular growth an accurate estimation of the porosity is essential. In combination with the substrate diffusion coefficient it will determine the growth and efficiency of granules. .
- Under reactor conditions, insufficient substrate transport in a granule causes a low specific activity due to substrate limitation of a significant part of the biomass.
- For large particles, substrate-limitation can initiate bacterial lysis in the centre, which can lead to disintegration or wash out of the granular biomass.

Size exclusion chromatography (SEC), introduced by Flodin (1961) and Porath and Flodin (1959), is a separation method based on the relative molecular mass, or more correctly, relative molecular size of molecules.

Figure 1

A: Diagrammatic representation of the main parameters of size-exclusion chromatography (SEC), adapted from Fischer (1971):
(V_o), void volume; (V_t), total volume.





B: Principle of SEC used fo determination of pore size distribution of pore size distribution of anaerobic sludge granules

A stationary phase of uncharged porous beads is used as the chromatographic support. The liquid mobile phase, or eluant, flows through the bed. A column constructed from such beads has the following definite volumes (figure 1a): total volume (V_t), calculated from the length and diameter of the bed; void volume (V_o), consisting of the volume of the liquid between the beads in the bed; internal volume (V_i), the volume of the liquid within the open pores of the beads; volume of beads (V_g), obtained as the difference between (V_t) and ($V_o + V_i$); elution volume (V_e), the volume of eluant that is available (and required) to carry the molecules of substrate through the column; $V_o \leq V_e \leq V_t$.

The sample to be separated must be soluble in the mobile phase used. Large molecules are excluded from the internal volume. Because of the difference in available volume, molecules flow through the bed at different velocities: large molecules emerge first, while smaller molecules are retarded, depending on their abilities to enter pores of the stationary phase. The dimensions important to SEC are the diameters of the pores that gives access to the internal volume and the hydrodynamic diameter of the molecules (figure 1b).

The fractionation range of the size-exclusion matrix is defined as the approximate range of molecular mass in which maximal separation of molecules is achieved.
One of the features of SEC is the good correlation between molecular mass and V_e. For globular molecules (e.g. proteins) and within the fractionation range, V_e is approximately a linear function of the logarithm of the molecular mass (Flodin 1961). The partition coefficient (K_{av}), represents the fraction of the V_i that is available for diffusion of a given solute species:

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

As a consequence, the SEC principle can be used to study the characteristics of the porous beads by using granular sludge as a stationary phase.

For some sludges, closed pores results in a non-neglectable V_g. For a conventional use of SEC the K_{av} must be modified for this volume. In the presented study, however, the K_{av} of the internal standards, flavine mononucleotide (FMN) and acetic acid (CH_3COOH), is used as an indicator of the available porosity. A similar application of the method was described for investigating the porosity of cotton matter (Martin et al. 1971). They used chromatographic columns filled with finely divided fibrous cotton to determine the relative V_e of sugars ranging in discrete molecular mass.

As substrate and gas transport take place during anaerobic digestion, granular sludge must have a porous structure that allows penetration of solutes and gas. As in size-exclusion chromatography, the access to these pores for solutes is related to their molecular sizes and shapes. In this research, a method to determine the porous structure

characteristics and the internal volume of the sludge granules has been developed by using granular sludge as a size-exclusion chromatographic support. With this aim, the sludge was packed in a column, and V_e of standard proteins and dextrans of different molecular mass (size) were measured.

MATERIALS AND METHODS

Columns of 2.6 cm diameter and 40 cm height (Pharmacia LKB Biotechnology, Uppsala, Sweden) were packed with granular sludge. The eluant was pumped by a peristaltic pump (Watson & Marlow 101u, Falmouth, England) at a constant flow-rate of 0.2-1.0 $ml.min^{-1}$. The eluate was collected continuously with a fraction collector (2112 Redirac LKB Bromma, Uppsala, Sweden).

Experiments were carried out at 4°C to minimize the activity of the microorganisms in the sludge.

The first tests were carried out using downward flow and standard proteins ranging from 14 000 to 67 000 Da in molecular mass. Subsequent tests were carried out using dextrans ranging from 1000 to 80 000 Da. To avoid clogging in these tests upward flow was used.

Table 1 Some characteristics of the granular sludge types used.

Parameters	Units	Sludge types		
		I[a]	II[a]	III[a]
TSS[b]	%	9.4	9.3	9.3
VSS[b]	%	6.8	8.5	6.8
Activity	$gCH_{4\text{-}COD}/(gVSS.d)$	0.6	0.8	0.22
Specific gravity	$kg.m^{-3}$	1040.6	1039.0	1026.4
Diameter	mm	1.0-2.0	1.4-2.0	3.2-4.0

[a] The sludges used originate from full-scale upflow anaerobic sludge blanket reactors, processing wastewater from: (I) a starch factory, (Latenstein, Nijmegen, the Netherlands); (II) an alcohol producing factory (Nedalco b.v., Bergen op Zoom, the Netherlands); (III) a pilot scale expanded granular sludge bed reactor processing municipal wastewater (Bennekom, the Netherlands)

[b] TSS, total solids; VSS, volatile solids, i.e. organic matter, both in percentages of leaked fresh sludge

[c] The sludges were separated by sieving. The fraction used is given

[d] COD, chemical oxygen demand

The cofactor FMN (450 Da) was used as yellow-coloured internal standard for determination of total available porosity of the granules as well as acetic acid (60 Da). Blue dextran ($>10^6$ Da) was used as a reference to determine the void volume of the column. FMN and Blue dextran were used in all experiments. For all tests 1.0 ml of a solute solution with a concentration of 10 $mg.ml^{-1}$ was used.

All dextrans were from Fluka Chemie AG (Buchs, Switzerland). Myoglobin, trypsin inhibitor and bovine serum albumin were from Serva AG (Heidelberg, FRG) and cytochrome C, ovalbumin and FMN were from Sigma (St. Louis, USA).
An UV-VIS spectrophotometer (LKB-Biochrom ultrospec II) was used to measure the relative protein concentration at 280 nm. The relative concentration of FMN was measured at 445 nm. The dextran concentration was determined colorimetrically as carbohydrate by the phenol-H_2SO_4 reaction (AOAC 988.12). Acetic acid was determined with a gaschromatograph equipped with a 2 m x 4 mm column, packed with 100-120 mesh Supelcoport and coated with 10% Fluorad FC431.

In this work, the characteristic V_e is defined as the eluted volume at maximal solute concentration. The pore size distribution and the exclusion limit of the sludge granules are expressed in molecular mass.

To discriminate for possible adsorption phenomena and for the sludge stability, experiments were carried out with different mobile phase conditions: tap water; 25 mM, 50 mM or 100 mM NaCl; phosphate buffers pH 5.0, 6.0 or 7.0. Granular sludges from three different UASB reactors were used in this research to test the method (table 1).

Total solids (TSS) and volatile solids (VSS) were analyzed according to Nederlandse Norm (1988; Dutch standard methods), and are expressed as percentage of the fresh sludge. The sludge density was determined using a picnometer. The sludge fractions were separated by test sieves (Retsch, Haan, FRG).

Methanogenic activity measurements were performed in 1 litre glass flasks at 30°C. The flasks were supplied with tap water, nutrients and a methanogenic substrate. As substrate a mixture of volatile fatty acids was used, neutralized to pH 7. The chemical oxygen demand (COD) at start of the tests was 3 $gCOD.l^{-1}$, using 1 $g.l^{-1}$ COD of acetic acid, propionic acid and butyric acid, respectively. The activity was calculated from CH_4 production, monitored with modified Mariotte flasks.

RESULTS

In preliminary experiments the microbial activity was inhibited by treating the sludge with 3% glutardialdehyde. Disintegration of granules was observed, however, probably caused by effects on the granule structure. To avoid these effects, experiments were carried out at 4°C to inhibit the activity of microorganisms in the sludge.

The exclusion limit and the total available volume of the sludges used are given in table 2 (determined using tap water and elution in an upflow mode). As an example, the results of tests with sludge I and dextrans are shown in figure 2.

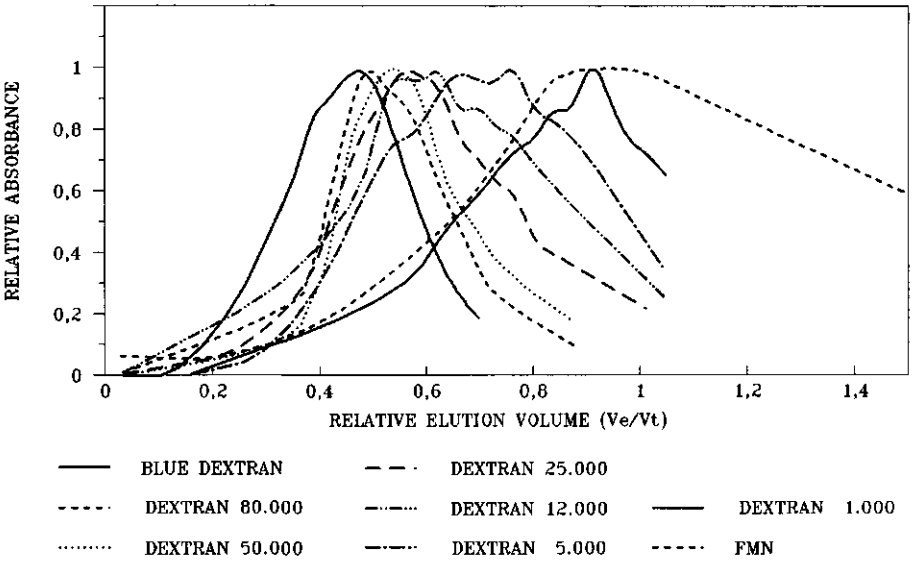


Figure 2 Chromatogram of tests with sludge I and dextran series: tap water as eluant; flow rate: 1.0 ml.min^{-1} (upflow); molecular mass of the dextrans used: Blue dextran (>2x10^6 Da), Dextran 80.000 (80 900 Da), Dextran 50.000 (48 600 Da), Dextran 12.000 (11 600 Da), Dextran 5.000 (5220 Da) and Dextran 1.000 (1 270 Da); molecular weight of flavin mononucleotide (FMN) (450 Da). V_e: elution volume; (V_t): total volume

The relation between K_{av} and log of the molecular mass, as an indication of the pore size distribution, is shown in figure 3. Both dextrans and proteins showed a correlation between molecular mass and their elution volumes in parallel experiments carried out with sludge I. The differences in K_{av} most probably are related to the different molecular shapes (and so diffusion coefficients) of both types of molecules (figure 3). For all sludges FMN and acetic acid tests (pH 7.0, tap water, upflow mode) indicated a comparable available porosity (table 2).

Table 2 Porosity characteristics of granular sludges using tap water as the eluant.[a]

Sample	Solute[a]	Exclusion limit (as molecular mass)	Available porous volume (K_{av} x 100)	
			FMN[b]	CH_3COOH
Sludge I	Proteins	67000 Da	96%	96%
Sludge I	Dextran	80900 Da	96%	96%
Sludge II	Dextran	80900 Da	36%	42%
Sludge III	Dextran	1270 Da	7%	7%

[a] Flow rate 0.4 ml.min^{-1} for protein tests and 1.0 ml.min^{-1} for dextran tests

[b] FMN, flavin mononucleotide

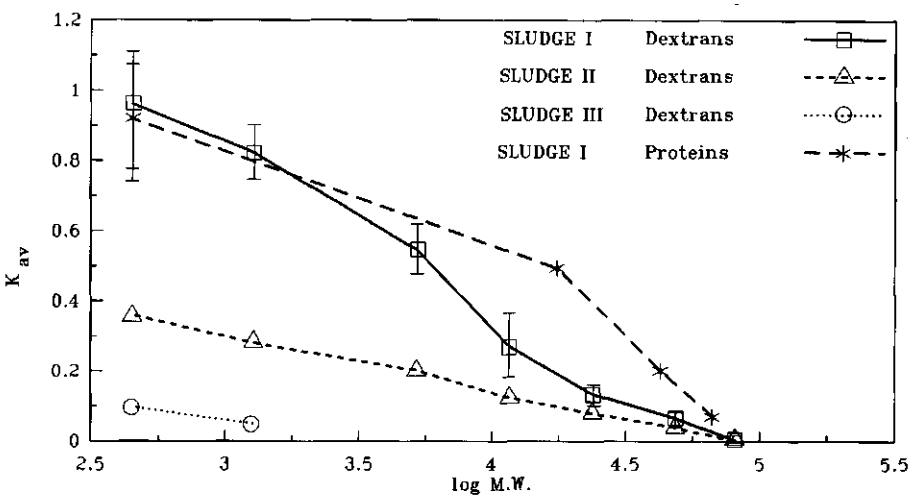


Figure 3 Log of molecular mass (M.W.) vs. partition coefficient (K_{av}) correlation curves for sludge I, II and III. Experiments with proteins (sludge I) and dextran series (sludge I, II and III) and tap water as eluant. The figure indicates the total available pore volume, the exclusion limit and the pore size distribution expressed as log M.W. The vertical bars indicate the K_{av} calculated from V_e / V_t at 95% of the maximal peak height (figure 2). The dextrans used and FMN are described in figure 2. Of the proteins investigated myoglobin (17 600 Da), ovalbumin (43 000 Da) and bovine serum albumin (67 000 Da) are used in the test presented in figure 3. Cytochrome C (13 370 Da) and trypsin inhibitor (22 400 Da) were not useful due to strong nonspecific adsorption.

Experiments performed in phosphate buffers (pH 5.0-7.0) showed problems of turbidity in the eluate and the quality of the curves decreased with time, most probably as a consequence of the conditions used (sludge I, experiments with proteins as well as dextrans solutes, down flow mode). Buffered eluants at pH above 7.5 were not tested because preliminary experiments showed a serious decrease in sludge quality.

Tests with 0.025 M, 0.05 M and 0.1 M NaCl solutions as eluants did not show a time-dependent turbidity increase in the eluate. Compared with tests in tap water also no effect on adsorption phenomena was found. Neither the V_e, nor the elution patterns were affected by the salt concentration (sludge I, experiments with proteins as well as dextrans solutes, upflow mode).

Within the experimental range (0.2-1.0 ml.min^{-1}), the flow rate did not affect the results obtained with sludge I and FMN as solute.

DISCUSSION

The results presented in this chapter show that the intended objective of the method, determination of the pore size distribution in granular sludge, is feasible.
FMN elution patterns showed tailing in all experiments. Since the flow rate does not effect the V_e, the retardation of FMN is not an effect of a slow diffusion. Using an inert and non-porous medium as the stationary phase (glass beads, 2 mm) to examine the behaviour of the system, this tailing, however, did not occur. The commercial FMN used contains 10% of a far more hydrophobic riboflavin (Nielsen et al. 1983), and the irregular elution patterns are probably a consequence of non-specific adsorption. Several proteins investigated also showed this non-specific adsorption.

Both protein as well as dextran reference standards yielded analogous chromatographic results in tests with sludge I. The globular molecular shape of proteins results in higher K_{av} values when compared to dextran molecules (linear molecular shape) of corresponding molecular mass. Dextrans are favoured because their strong similarity in molecular structure and non-adsorptive properties permit a better comparison of their chromatographic behaviour. For the described application of SEC a more complete molecular mass range was available for dextrans as for proteins (figure 3).

The exclusion limit values for the granular sludges I and II were quite similar; the total porosity of sludge II was however distantly lower. For sludge I, the calculated volume correlated with the percentage of dry matter; the volume represented by this matter was not available for FMN or acetic acid. For the second sludge however, the measured maximal available volume (K_{av}) is much lower (36%), whereas the TSS and VSS percentages did not vary considerably from sludge I. A possible explanation for

this high 'dead' volume might be a "blockade" in the surface area of the granules, such that a fraction of the internal pores was not available. Further research to this is being carried out.

Sludge III has a very low porosity and the test revealed a complete absence of larger pores. However, microscopic examination of cross-sections of these granules showed large hollow parts, especially in the centre of the particles. A possible explanation could be the presence of an impermeable layer enclosing the empty inner regions of the particles. Probably the limited substrate transport allowed growth of bacteria merely at the surface. The cross-sections indeed show a dense black layer, located just beneath the granule surface. The large granules (the diameter can be up to 6.4 mm) all seemed to have hollow centres. Moreover, their maximal specific methanogenic activity was low, which is also an indication of inactive organic matter or exclusion of active biomass from supply of substrate.

The porosity is only one of the factors that is related to the activity. In this study no simple correlation between methanogenic activity (table 1) and the available porosity (table 2) was found. Sludges I and II were separated on granule size and the larger granules were tested. For these granules the major amount of viable bacteria are probably orientated in the surface region (due to substrate limitation). Inactive biomass in the centre of the granules used might reduce the maximal methanogenic activity. Further research on this problem is being carried out.

Substrate transport within the granule is related to the effective molecular size of the substrate molecules, the pore size distribution of the granular sludge and the availability of the pores. Besides the determination of the granule pore size distribution, the hydrodynamic diameter of the actual substrates must also be investigated to give an indication of the transport possibilities into the granules.

For the sludges investigated in this study, acetic acid behaviour was similar to that of FMN (table 2). It can be concluded that for the method developed acetic acid might serve as well as FMN as an internal standard to indicate the maximal volume available.

The pore size distribution might influence the area of the granule in which the degradation takes place during waste-water treatment. For granules with an exclusion limit lower than the molecule radius of the substrates, degradation to smaller molecules should take place at the surface area of the granules. The porosity and the pore size distribution will control the distribution and growth of the different groups of bacteria over the granule radius.

4 THE INFLUENCE OF SUBSTRATE TRANSPORT LIMITATION ON POROSITY AND METHANOGENIC ACTIVITY OF ANAEROBIC SLUDGE GRANULES

This chapter consists of a modified edition of:

The influence of substrate transport limitation on porosity and methanogenic activity of anaerobic sludge granules.
P.Arne Alphenaar, M.Cecilia Pérez, and Gatze Lettinga
Appl. Microbiol. Biotechnol. (1993) 39:276-280

4 THE INFLUENCE OF SUBSTRATE TRANSPORT LIMITATION ON POROSITY AND METHANOGENIC ACTIVITY OF ANAEROBIC SLUDGE GRANULES.

ABSTRACT

The relation between porosity, diameter and methanogenic activity of anaerobic granules has been investigated.

Experiments with different granular sludges revealed that substrate transport limitations increase with the diameter of the granules. As a consequence, autolysis can occur in the core of the granule, producing hollow granules. The porosity measurements revealed that the hollow centre is not available for substrate transport. Possibly as an effect of bacterial lysis, the porosity decreases in the more interior layers of the granules. This results in a inactive inner part of the large granules, which is not involved in the treatment process; the specific methanogenic activity decreases with granule size. No marked difference in substrate affinity is observed between granules of different sizes, which probably indicates that for large granules only the exterior is biological active.

INTRODUCTION

During the past two decades the use of anaerobic treatment systems for the purification of large (industrial) wastewater streams has increased strongly. This improvement is mainly related to the development of the upflow anaerobic sludge blanket (UASB) reactor. (Lettinga et al. 1980; De Zeeuw 1988). One of the most serious drawbacks of high rate anaerobic systems is the extended start-up operation. For the UASB reactor this drawback has been overcome by the possibility of using granular sludge as seed material. This can reduce the start-up period from months to days. In some cases, however problems related to the granule stability occur during the start-up period. These problems seems to be related to substrate concentration changes in the granule during the start-up period and to the history of the granules.

Grotenhuis et al. (1991a) observed a positive relation between the granule diameter and the influent concentration. Research conducted in our laboratory also indicated a relationship between substrate concentration in the reactor and granule size. The diameter is probably controlled by substrate diffusion in the biomass. Substrate limitation in the granule centre will reduce bacterial growth there, or even cause lysis, which will weaken the granule and result in breaking up of the structures. The substrate concentration in a granule is related to the substrate utilization rate, the distance from

the granule surface, the external substrate concentration and the substrate transport velocity in the granular biomass.

In literature, the transport potential of substrate and products is mentioned as a fundamental biofilm and granule characteristics. Morvai et al. (1992) reported an increasing K_s (substrate affinity constant) value during granule development. Other authors have also mentioned the relation between granule stability and substrate gradient in the granule. Dolfing (1985, 1987) however reported a minor effect of substrate diffusion limitation on granule characteristics. De Beer (1990) mentioned the pH gradient in methanogenic biofilms as being more important. Presumably granule characteristics such as porosity and biomass density of the granules provide useful indicators of the substrate transport possibilities (chapter 3).

The aim of this study was to investigate some factors that are involved in the substrate transport trough the granule. The relationships between granule porosity, granule size and methanogenic activity can possibly partly reveal the mechanisms of granule disintegration. This knowledge may lead to an optimal start-up of granular sludge reactors.

MATERIALS AND METHODS

Biomass

This study was carried out with granular sludges originating from full-scale UASB reactors treating different wastewaters: Latenstein, starch factory, Nijmegen, The Netherlands (sludge I); Roermond, recycle paper factory, Roermond, The Netherlands (sludge II); Aviko, potato processing industry, Steenderen, The Netherlands (sludge III); Nedalco, alcohol producing industry, Bergen op Zoom, The Netherlands (sludge IV). The sludge samples were size-fractionated by wet sieving in water.

Activity and K_s

The methanogenic activity was measured as the acetate degradation velocity using 2.5 *l*, intermittently stirred batch reactors (chapter 2, figure 2c). The experiments were carried out at 30°C. The pH was controlled automatically between Ph 7.2 and pH 7.5 by NaOH and HCl (0.1 M).

The initial substrate level of the tests was 3 g chemical oxygen demand (COD).l^{-1} of acetic acid. The mineral medium contained (in g.l^{-1}): NH_4Cl, 2.8; KH_2PO_4.3 H_2O, 3.3; $MgSO_4.7H_2O$ 1.0; $CaCl_2$, 0.076; $NaHCO_3$, 4.0; and yeast extract, 0.1. Per litre of substrate, 200 ml of the described medium and 1 ml of a trace element solution according to Zehnder et al.(1980) were added. Two substrate feedings were given.

Subsequently, the granules were crushed anaerobically using a syringe needle (Microlance 21g1½ 0.8x40), and fed again.

The activity was calculated both from the CH_4 production rate, monitored with modified Mariotte flasks, and from the decrease in substrate concentration. To evaluate the K_s of the granular and crushed sludge samples, the activity (based on concentration decrease) was plotted against the substrate concentration.

The sampling frequency was 6 hours. Samples were collected by an automatic fraction collector (FRAC-100 Pharmacia Fine Chemicals, Uppsala, Sweden); 3% formic acid (dilution 1:1) was present in the collector sampling tubes for fixation.

Volatile fatty acids (VFA) were analyzed by use of a gaschromatograph (HP 5890, Hewlett Packard, Switzerland) (chapter 2). All samples were centrifuged (15 600g for 4 min.; IEC Centra-M Centrifuge, Needham Mass, USA) and fixed with 3% formic acid (dilution 1:1). COD was determined according the micromethod of Jirka and Carter (1975) (described in chapter 2).

Release velocity

Substrate transport velocities were calculated from substrate desorption measurements. For this purpose the sludge samples were treated with a buffered acetic acid solution (approx. 3 g COD.l^{-1}, pH 7.0) for 1-2 hours. After removing the acetate solution, the sludge was dispersed into demineralized water (sludge/liquid = 1 g/1 ml). The acetate concentration in the bulk of the liquid was followed under continuous stirring. The tests were carried out at 4°C in order to minimize methanogenic activity.

Assuming spherical granules consisting of concentric layers of equal thickness, a simplified discrete simulation model based on concentration gradients between these layers has been developed (equation 1).

$$F = \frac{\Delta t}{\Delta x} . D'' . (A_n . (S_{n+1} - S_n) - A_{n-1} . (S_n - S_{n-1})) \qquad (1)$$

where:

F	= transport (mol)	Δt	= time step (s);
D''	= release coefficient ($m^2.s^{-1}$)	A_n	= external surface area layer n (m^2)
S_n	= substrate concentration in layer n ($mol.m^{-3}$)	Δx	= layer thickness (m).

The bulk of the external liquid is considered as the most external layer. For subsequent time steps, till $\Sigma\Delta S$ is equal to the experimental sample time, the concentration in the external liquid is calculated. The liquid volume is corrected for the sample volume.

The substrate release velocity for specific granules was obtained by fitting the experimental data to the model and used to approximate the diffusion coefficient. Results of a typical example are presented in figure 1.

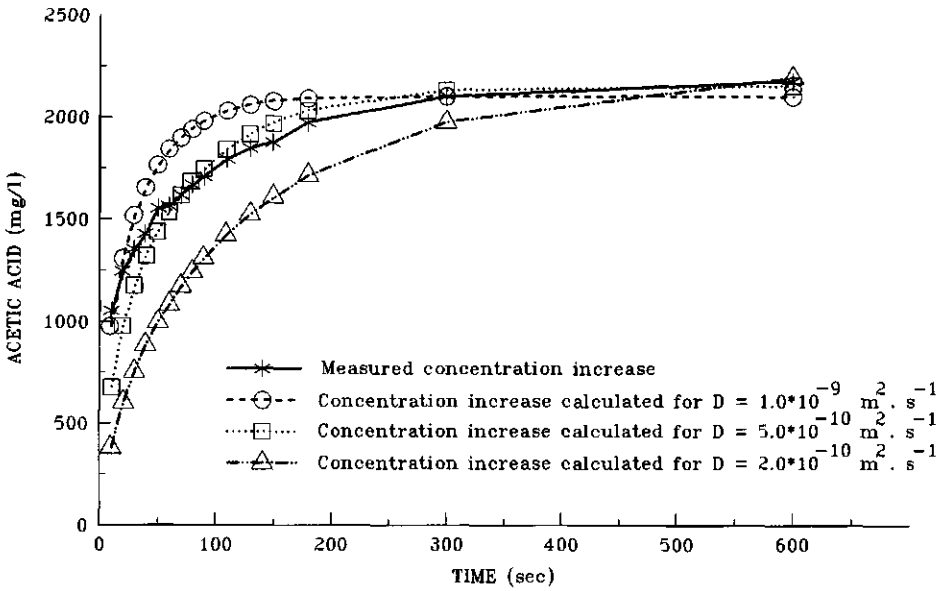


Figure 1 Comparison between the experimental substrate release velocity of acetate for sludge I, fraction 1-2 mm, and the theoretical results according to a simulation model based on concentration gradients between concentric layers of the granule. The release velocity is used as a measure of the diffusion coefficient.

Physical characteristics

Porosity tests were made by size exclusion chromatography according Alphenaar et al. (1992), using dextrans, flavin mononucleotide (FMN) and Blue Dextran as solutes. Total solids (TSS) and volatile solids (VSS) were analyzed according to Nederlandse Norm NEN 6621 (Dutch Standard Norms 1988).

The specific gravity of the granules was determined by use of a pycnometer, according to American Public Health Association (1985) standard methods (method 213e). Before measurement, surface water was removed by spreading the sludge sample on a grid placed on a hydroscopic tissue (paper napkin). Subsequently, sludge was placed for a 30 min. at H_2O-saturated air at 30°C.

RESULTS AND DISCUSSION

From microscopic observations of cross-sectioned granules the non-homogeneous structure of granules from full scale UASB is evident; layered structures are common. In many cases, especially for large granules, the density seems to decrease toward the centre, and even large holes are common. In the literature these structures are explained by different mechanisms (Beeftink, 1987; Kosaric et al. 1990a; MacLeod et al. 1990; Grotenhuis et al. 1991a).

Substrate will be converted at first in the more external granule layers. As a consequence of diffusion limitation of substrate, the internal layers will receive a much lower substrate concentration. Using Monod kinetics, this results in a lower biomass growth. In extreme cases even a decrease of population in the granule core due to autolysis can take place. As a consequence, these granules may disintegrate (Grotenhuis et al. 1991a, this thesis chapter 6) or wash-out by flotation (Kosaric et al. 1990b; and unpublished results). The disintegration of hollow granules is related to the internal and external forces; for low loaded reactors with a very diluted influent, a stable performance is observed while large, hollow granules with a low specific methanogenic activity are formed (results not shown). The composition and performance will be not homogeneous over the granule radius; consequently every layer has its own specific porosity, biomass growth, and, for most substrates, its own population. The measured granule characteristic, as solids concentration, activity, porosity and density, are therefore averages of the actual values in each layer.

The main physical characteristics of the used sludge samples are summarized in table 1. The TSS concentration increases with the granule diameter. For most sludges the percentage organic matter decreases slightly with increasing granule size. Probably due to mineralization in the inner part of the granule, the VSS/TSS ratio decreases with increasing diameters. The measurements also show a decreasing maximal porosity for increasing granule size (table 2).

The pore-size distribution strongly indicates mass transport limitation for large granules (figure 3). Optically however, for large granules a decreasing density of the granule core is observed (figure 2). The empty space in these granules is not available, however. Because the holes are generated by substrate limitation and bacterial deterioration, the low available porosity measured for these large granules may be related to non-permeable lysis products that block substrate transport. The high porosity of the small (young) granules indicates a high permeability of viable bacteria for small molecules.

Table 1. The organic matter percentage and specific gravity for different (by sieving separated) size-fractions of the used granular sludge samples.

Sludge type	Fraction[a] (mm)	Total solids (gTSS)	Volatile solids (gVSS)	VSS/TSS ($g.g^{-1}$)	Specific gravity $kg.m^{-3}$
I	1.0-2.0	10.76	9.67	0.89	1042.5
I	>2.0	11.93	11.04	0.92	1039.7
II	0.5-1.0	11.67	10.25	0.88	1055.0
II	1.0-2.0	14.09	11.89	0.84	1052.1
II	>2.0	13.32	11.20	0.84	1055.5
III	0.5-1.0	7.76	6.55	0.84	1027.6
III	1.0-2.0	9.00	7.61	0.85	1026.1
III	>2.0	9.07	7.29	0.80	1029.1
IV	0.5-1.0	8.22	7.40	0.90	
IV	1.0-2.0	11.20	9.71	0.87	1030.5
IV	>2.0	12.11	10.35	0.85	1030.5

[a] The size fractions were separated by sieving. For sludge sample identification, see Materials and methods

Substrate diffusion in the granule is a physical process. In this study it is measured as the release velocity of the substrate at low temperatures conditions (4°C) and at high substrate concentrations (figure 1); microbiological inactivity and inhibition can be assumed. Probably these conditions also change the transport characteristics in the biofilm.

The mass transport capacity and velocity in the granule can be expected to be related to the porosity and the pore size distribution of the granule (chapter 3, Alphenaar et al. 1992). Contrary to the supposed relationship, the release velocity seems to increase with the granule diameter and, consequently, with decreasing porosity (table 2). An explanation for the high release velocity for large granules could be non-homogeneous granule organization. Supposing that the porosity of a granule decreases as a consequence of clogging by lysis products, the exterior layers of the granule will have the same high porosity conditions as observed for small granules. The availability of the volume in the granule core, as measured by the porosity measurement, will be very limited. The average porosity will be lower than for small granules.

The substrate diffusion velocity will be overestimated because it is calculated over the total granule radius whereas actually only the external layers are involved. The tests for the release velocity can be related to the theoretical diffusion coefficient of acetate in pure water at 4°C ($1.24 * 10^{-9} m^2.s^{-1}$), calculated according the Wilke equation on the basis of the Stokes-Einstein equation (Bird et al. 1960)

Table 2 Granule characteristics related to the substrate transport and the biological activity for different fractions of the studied granular sludge samples.
The granular porosity as measured by size exclusion chromatography indicate the total available volume, the acetate release velocity approximate the substrate diffusion velocity constant. The maximal acetate utilising methanogenic activity indicate the total amount of viable methanogens present. The differences in substrate affinity between crushed- and intact granules indicate the substrate transport limitation in the investigated granular sludges.

Sludge type	Fraction (mm)	Maximal porosity[a]		Release velocity[c] ($m^2.s^{-1}$)	Granules		Crushed granules	
		FMN[b]	C2[b]		Activity[d]	Ks[e]	Activity[d]	Ks[e]
I	1.0-2.0	0.96	0.95	$0.5E^{-9}$	0.59	500	0.59	340
I	>2.0	0.72	0.78	$1.0E^{-9}$	0.44	510	0.45	310
II	0.5-1.0	0.93	1.06	--	0.48	500	0.46	200
II	1.0-2.0	0.86	0.98	$0.8E^{-9}$	0.36	--	0.39	250
II	>2.0	0.75	0.78	$1.0E^{-9}$	0.30	500	0.36	320
III	0.5-1.0	1.01	1.04	$0.8E^{-9}$	0.59	160	0.58	180
III	1.0-2.0	0.86	0.90	$0.6E^{-9}$	0.57	150	0.65	200
III	>2.0	0.58	0.69	$0.6E^{-9}$	0.53	180	0.56	200

[a] K_{av}: partition coefficient (size exclusion chromatography), the fraction of the sludge granule volume which is available for diffusion of the used solute

[b] C_2: acetate, FMN = flavin mononucleotide. Used as solute in gel permeation chromatography to determine the total porosity

[c] Substrate (acetate) release velocity is used to approximate the substrate diffusion coefficient.

[d] Maximal methanogenic activity ($gCH4\text{-}COD.(gVSS.d)^{-1}$)

[e] Substrate (acetate) affinity coefficient ($mgCOD.l^{-1}$)

For the granule samples, effective overall diffusion coefficients were found to be between 40% and 80% of the diffusion coefficient in pure water (figure 1). Nilsson and Karlsson (1989) reported values of 22-33% for a matrix of methane-producing microorganisms using lithium chloride; Kitsos et al. (1992) found the effective diffusivity of acetate in an anaerobic film to be only 7% of that in water. The apparently high results obtained here from the release velocity experiments are probably a consequence of the strong mixing conditions imposed.

The measured K_s values did not show a significant relationship with the granule diameter (table 2). However, the maximal methanogenic activity (gCH_4-COD.(g VSS.day)$^{-1}$) for all sludges decreased with increasing granule size; crushing did not have an important effect on the methanogenic activity. In combination with the K_s data we can conclude that substrate limitation does not affect the activity in the whole granules investigated.

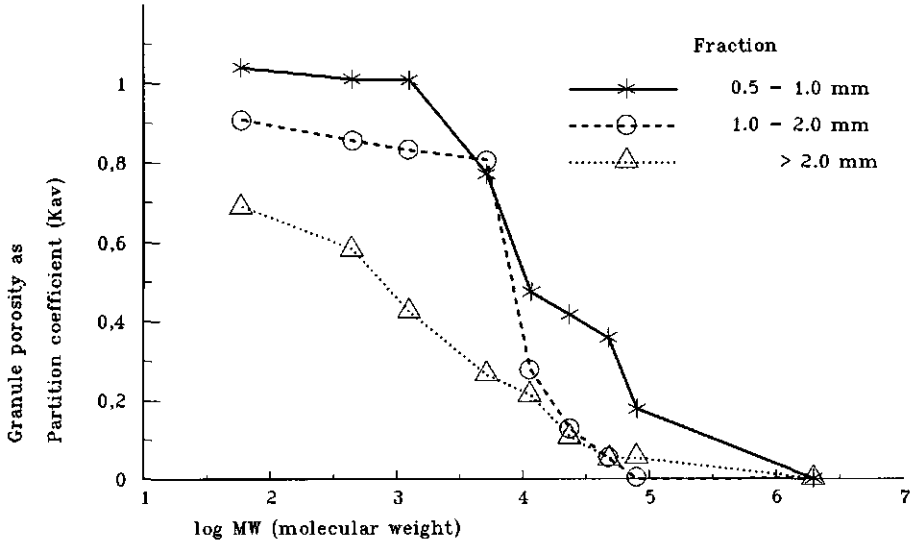


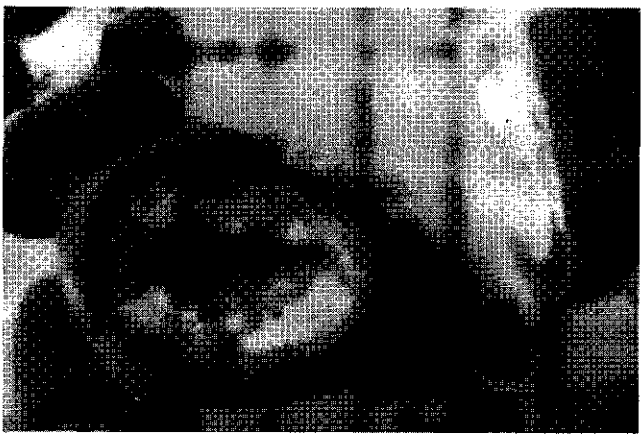
Figure 2 The porosity and pore-size distribution for different fractions of sludge III determinated by gel permeation technique (Alphenaar et al. 1992). The results indicates a decreasing porosity for larger sludge granules, which is an evidence of mass transport limitation. The porosity is presented as the distribution coefficient, where K_{av} = 1 indicate 100% porosity.

The decreasing activity must result from the presence of inert organic material, plausibly produced from cell lysis in the granule core. For the large granules investigated, viable bacteria exist only in the peripheral zone.

It is important to note that the experiments were carried out with sludges originating from full-scale UASB reactors treating industrial waste-waters. For large granules formed under laboratory conditions, the granule interior presumably will contain only substrate-limited, but still viable, bacteria. Studies with such sludges then will reveal completely different results with respect to K_s and activity values related to the granule size. Probably this explains different results found in the literature; further research will be carried out in the near future.

The results indicate that only a limited part of the biofilm of anaerobic granules is actually active. When lysis has a negative effect on porosity and consequently on mass transport capacity, this enhances the development of an inactive granule core for large granules. In many cases the cores of anaerobic sludge granules will be completely inactive due to substrate limitation and autolysis. At common treatment conditions, an important percentage of the biomass present in large granules is not involved in the treatment process.

Figure 3
Photographs (by Olympus ZS40 zoom microscope) of cross-sectioned granules. bars and grid indicating 1 mm



a: Granule cultivated in a laboratory UASB grown on gelatin as substrate. The formation of the central hole (H) was related to substrate limitation due to process condition changes (chapter 6).

b: Very large granules (5 mm diameter in average) were cultivated on sewage wastewater. The empty spaces (H) were not available for substrate transport, as indicated by an extreme low porosity (approx. 7%) measured by gel permeation technique (chapter 3). Possibly impermeable layers (arrow) limited the substrate transport.

5 SCANNING ELECTRON MICROSCOPICAL METHOD FOR INTERNAL STRUCTURE ANALYSIS OF ANAEROBIC GRANULAR SLUDGE

This chapter consists of a modified edition of:

Scanning electron microscopical method for internal structure analysis of anaerobic granular sludge.

P.A. Alphenaar, N. Groeneveld and A.C. van Aelst,
In Press, MICRON

5 SCANNING ELECTRON MICROSCOPICAL METHOD FOR INTERNAL STRUCTURE ANALYSIS OF ANAEROBIC GRANULAR SLUDGE

ABSTRACT

The development and disintegration of anaerobic granular sludge are crucial factors for successful application of anaerobic wastewater treatment using Upflow Anaerobic Sludge Bed (UASB) reactors. Knowledge of the internal structure of anaerobic sludge granules is essential for understanding these processes. The scanning electron microscope is widely used in biofilm research because of the three dimensional view. However, sectioning is necessary for internal structure research but induces severe artifacts.

In this study a method was developed to minimize sectioning artifacts during preparation for scanning electron microcopy research on anaerobic granular sludge.

Cryo-fixation was used to increase the mechanical strength of the samples. To avoid ice crystal damage, and to improves the sectioning result, DMSO and ethanol were used as cryo-protectants. Best results were obtained using DMSO as a cryo-protectant. Smooth section surface areas were obtained using 50% DMSO-treated, fast frozen granules sectioned at -110°C.

INTRODUCTION

During the last three decades several high rate anaerobic waste water treatment systems have been developed. All systems are based on the immobilization of biomass, to obtain separate hydraulic retention time and biomass retention time. The Upflow Anaerobic Sludge Bed (UASB) reactor has become the most widely applied system (Lettinga *et al.* 1987 and Lin and Yang 1991). The success of the UASB reactor is founded to the great extent on autoimmobilization of the active biomass to a granular sludge in these reactors (Hulshoff Pol 1989). The granular sludge can be stored without feeding for a prolonged period. One of the most important disadvantages of anaerobic treatment, the extended start-up period, has been overcome by application of granular sludge as inoculum to accelerate the start of new reactors. However the mechanical stability of granules decreases, resulting in disintegration or flotation of the granules and consequently loss of activity from the system (Kosaric and Blaszcyk 1990; this thesis, chapter 6). Structural analysis of the internal granules is essential for understanding the population dynamics and ecology of the bacteria in the granules. With this knowledge the relations between structure and function of the anaerobic sludge can be interpreted, and the consequences of specific process conditions can be predicted.

Thin sectioning of embedded sludge granules and observation with transmission electron microscopy is a useful method to investigate the inner structure of anaerobic sludge granules (Grotenhuis *et al.* 1991c). Scanning Electron Microscopy (SEM) is widely used to examine the biofilm and granular sludge research (Eighmy *et al.* 1983; Alleman *et al.* 1985; Richard and Turner 1984; Kosaric and Blaszcyk 1990 and Anderson *et al.* 1991) because this method enables the visualization of the spatial organization and the morphology of bacteria.

Cutting or cleaving is required to investigate spatial arrangement inside the granule. However, the lack of mechanical strength of sludge granules induces dramatic changes of the structural arrangement due to cutting or cleaving transversely through the granule. A useful method to increase the mechanical strength is the use of cryo-sectioning (Alleman *et al.* 1985; Haggis 1988; Steinbrecht and Zierold 1987 and van Aelst and Wilms 1988). Due to the size of the sludge granules, up to 4 mm diameter, serious ice crystal damage occurs when cryo-techniques are applied on sludge granules. The use of a cryo-protectant prevents structural damage caused by ice crystals (Robards and Sleytr 1985).

The aim of our research was to develop a sectioning method for anaerobic sludge granules for the ambient temperature scanning electron microscope. In this paper we compare the results of dry sectioning and cryo-sectioning. For cryo-sectioning we compare the applicability of the cryo-protectants dimethylsulfoxide (DMSO) and ethanol.

MATERIALS AND METHODS

Granular sludge was obtained from the UASB reactor of the paper factory at Roermond, The Netherlands. Samples were separated in two classes by sieving; small (1.0 - 1.4 mm) and large (2 - 3.2 mm).

Granules were fixed for 2 hours in 2.5% glutaraldehyde, overnight in 1.5% ruthenium red and postfixed for 1.5 hours in 1% osmium tetroxide. Sodium cacodylate buffer, 0.2 M (pH 7.1) was used in all fixation and rinsing steps.

For the dry-sectioning method, granules were dehydrated immediately after fixation in an ethanol series (10% - 30% - 50% - 70% - 90% - 100%, 20 min. per step) and subsequently critical point dried with CO_2. The dried granules were mounted on stubs with colloidal silver (Biorad) and sectioned with a laboratory made sledge microtome (Keijzer 1993). The knife of this microtome exist of a razor blade fitted in a stainless steel holder. Finally the samples were sputter coated with gold/palladium.

For the cryo-fracture method two different treatments after fixation were utilized. Half the amount of sludge granules was dehydrated in an ethanol series 10% - 30% -

50% to 70% ethanol (20 min. per step). The other half was treated with a series of DMSO 15% - 30% to 50% DMSO in water. Single granules of the different size classes of both treatments were mounted on rivets with cyano-acrylate superglue.

The samples were frozen by plunging in liquid propane (-185°C) with a spring-powered injector. Some samples were frozen in a low temperature freezer (-70°C). Transverse fractures of the granules were achieved by sectioning (1 μm) with glass knifes with a Reichert cryo-ultramicrotome FC4D.

The temperature conditions for specimen and knife during sectioning were varied between -100°C and -130°C.

Subsequently the sectioned material was thawed in 70% ethanol or 50% DMSO, respectively. The granules in 70% ethanol were dehydrated up to 100% ethanol. The granules thawed in 50% DMSO were "rehydrated" stepwise to pure water via 30% - 15% DMSO, and then completely dehydrated in graded ethanol series. All samples were critical point dried with CO_2 and sputter coated with gold/palladium before observation in the SEM (JEOL 5200).

RESULTS

The dry-sectioning method resulted in a seriously disrupted surface area (figure 1). The deformation seems to be related to the sectioning direction and results in a kind of scattering pattern parallel to the knife edge (figure 1, arrows). Also surface deformation caused by the scratches on the (razor) knife edge are present. These deformations are manifest parallel to the section direction (figure 1, asterix).

Slow freezing of cryo-protected granules resulted in many small cracks in the entire fracture surface of granules. These small cracks are randomly distributed and will separate parts of the granule and individual bacteria from each other. Ethanol was less suitable as cryo-protectant, the use of ethanol resulted in an uneven section-surface.

Transversely sectioned, fast frozen granules treated with 30% DMSO showed a reasonably good preservation of larger surface areas but cracks parallel to the knife edge can be observed (figure 2). Identical cracks were observed in ethanol treated granules. Higher magnifications of these cracks show domains which are different in height (figure 3). Granules treated with 70% DMSO were not sectioned successfully. Similar to the "dry sectioning" method, a lack in mechanical strength led to disruptions.

In general all described artifacts are more prominently apparent in the larger granules than in the smaller granules. Especially the large fast frozen granules treated with 50% DMSO show large cracks after fracturing, which can divide whole granules into pieces (figure 4).

The fracture surface areas of fast frozen cryo-sectioned DMSO (50%) granules are

completely flat and smooth (figure 4). In the fracture plane all bacteria are sectioned almost without damage (figure 5,6).

Best sectioning results were obtained with the ultra cryo-microtome with a knife temperature of -120°C and a sample temperature of -110°C.

In the transversely sectioned granules the dominant organism in the granule centre are morphologically similar to *Methanothrix* (figure 5,6,9) but also areas with other bacteria species were observed (not shown). All bacteria are very tightly packed (figure 3,5,6). In the granules a few small holes were observed, most of them were filled with bacteria with a morphologically different as the bacteria dominant in the solid areas (figure 7,8). Many granules shown a zonation pattern visible as shells (figure 4). In some granules fungal spores are visible in one shell (figure 8).

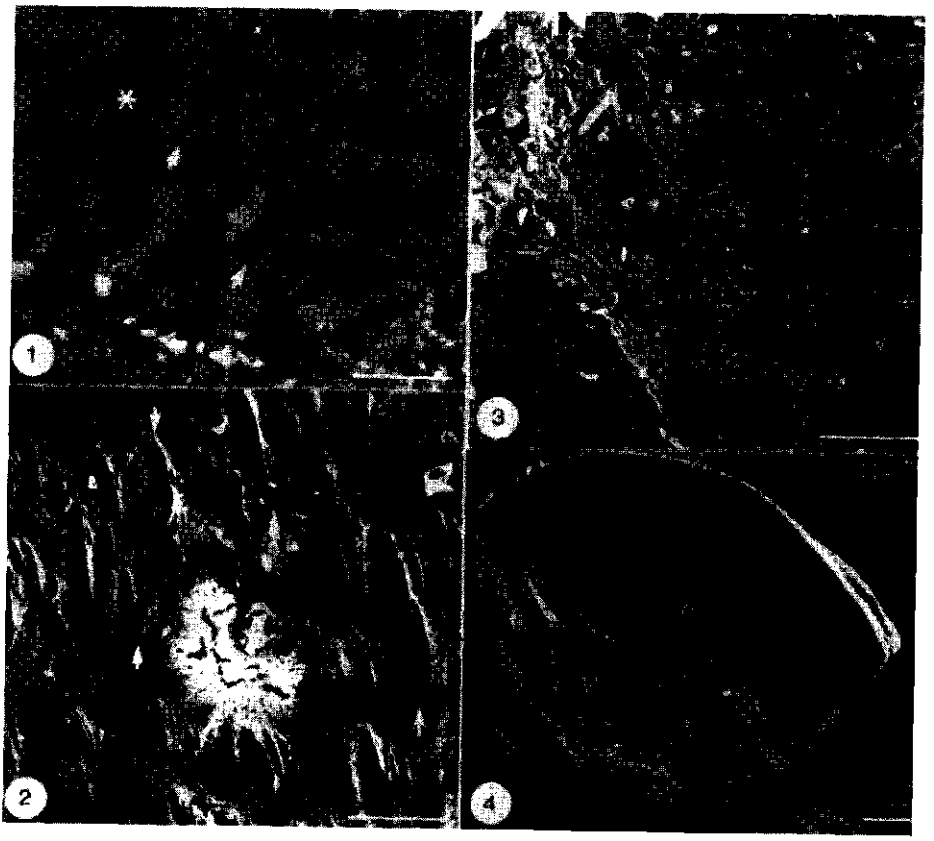


Figure 1 The disrupted surface of a small sludge granule, transversely 'dry' sectioned after critical point drying. A scattering pattern parallel to the knife edge is present (arrows). Bar = 10 μm.

Figure 2 Surface area of a sludge granule transversely sectioned with a cryo-microtome, fast frozen with 30% DMSO. Cracks are present in the surface (arrows). Bar = 20 μm.

Figure 3 Surface area of a sludge granule transversely sectioned with a cryo-microtome, fast frozen with 30% DMSO. Bar = 2 μm.

Figure 4 Surface area of transverse fractured large granule fast frozen with 50% DMSO. Large cracks split the granule. A zonation pattern is visible which indicate a change in structural organization. Bar = 200 μm.

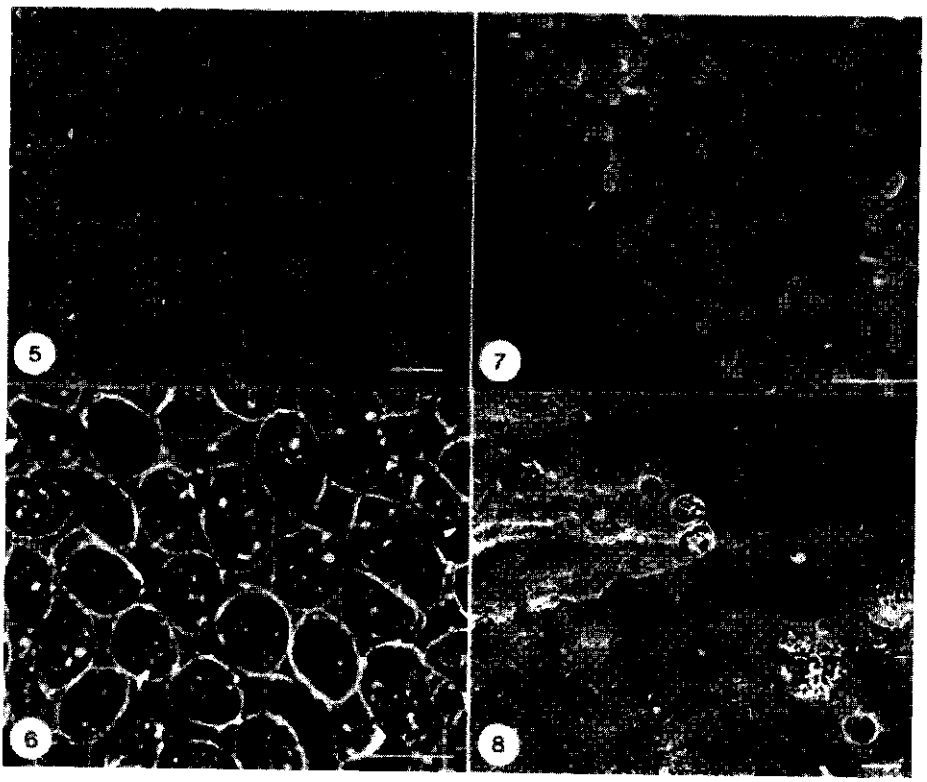


Figure 5 Section plane of the centre a fast frozen small granule treated with 50% DMSO, Bar = 2 μm.

Figure 6 Detail of section plane of the centre of a fast frozen small granule treated with 50% DMSO. Bar = 1 μm.

Figure 7 Detail of a (gas?) cavity in the section of a fast frozen sludge granule (50% DMSO). The bacteria in the cavity might be hydrogen consuming bacteria which avail the elevated substrate supply. Bar = 5 μm.

Figure 8 Section plane of the surface area a fast frozen large granule treated with 50% DMSO. Fungi spores (S), a cavity (C) as well as a less dense packed *Methanothrix* (M) spot are visible, Bar = 10 μm.

DISCUSSION

The evaluation of the developed method generally is distinguished between freezing and cryo-sectioning. After critical point drying the fragile structures of the granules become very sensitive to mechanical deformation. The absence of supporting material around the fragile structures in the sludge granule causes displacement of the bacteria.

To support the internal structures, the use of embedding media to support sensitive structures during sectioning is well known. Solid water as embedding medium is used in cryo-microtomy (Steinbrecht and Zierold 1987) and cryo-scanning electron microscopy (Van Aelst *et al.* 1989 and Jeffree and Read 1991). The disadvantage of the application of solidified water is the occurrence of ice crystals. Due to the slow freezing rate this occurs especially in large objects. To reduce the crystal size below the detectable level of the SEM, cryo-protectants are used (Steinbrecht and Zierold 1987). This study revealed the freezing speed is less critical using DMSO. However after slow freezing small crystals were present. The use of a low temperature freezer (-70°C) was less suitable for freezing cryo-protected sludge granules than fast freezing by plunging in liquid propane(-185°C).

Another advantage of the use of cryo-protectants is the introduction of increased plasticity in the sample. This plasticity permits some reversible deformation in the shear zone in front of the knife edge at low temperatures (Robards and Sleytr 1985). This results in more sectioning than fracturing during cryo-microtomy at low temperatures. On the other hand fracture planes introduced by the knife at low concentration of cryo-protectants follow a rather free way through the material. The difference in hight in the surface areas of the ethanol treated material probably is due to these fracture planes. Also periodic deformation originating from a lack of plasticity during sectioning can affect the surface area as is observed with the ethanol and 30% DMSO treated material.

For 50% DMSO treated samples the plasticity resulted in smooth sectioned surfaces. High DMSO concentrations (70%) however resulted in "smeared" surfaces. This "smearing" effect is related to the lack of rigidity.

In this study, the standard SEM fixation using glutaraldehyde was used. Glutaraldehyde may induce artefacts due to the removal of exopolymeric matter (Richard and Turner 1984; Howgrave-Graham and White 1990). However the use of these fixatives is not essential for the freezing and sectioning method.

The presented study was mainly focused on the development of a good sectioning method, nevertheless some preliminary results related to anaerobic wastewater treatment research can be made. All sectioned granules showed a very dense packing of the bacteria. The dominant species observed in the tangential fracture planes were morphologically similar to *Methanothrix*. Although *Methanothrix* seems to be the dominant organism, other species, some embedded in exopolymeric matter, were

observed in large areas. Only in the few shrinkage cracks a "spaghetti" like organization as reported by other authors was observed (Wiegant 1988 and Hulshoff Pol 1989). The dominant illustration of *Methanothrix* in morphological studies on granular sludge can partly be explained by the possibility to identify especially this species in damaged section areas and cleaving cracks induced by the conventionally used methods. The cleaving path in general follows these less dense parts. This directly points to the main dizadvantage of the developed method. The obtained smooth sectioning surface results to a great extend in the loss of the three-dimensional view of SEM microscopy, as illustrated by figure 9. The organism in the crack customary is morphologically identified as *Methanothrix*. Identification of the same organism in the bottom part of the figure, and especially in figure 5 and 6 will be much more difficult.

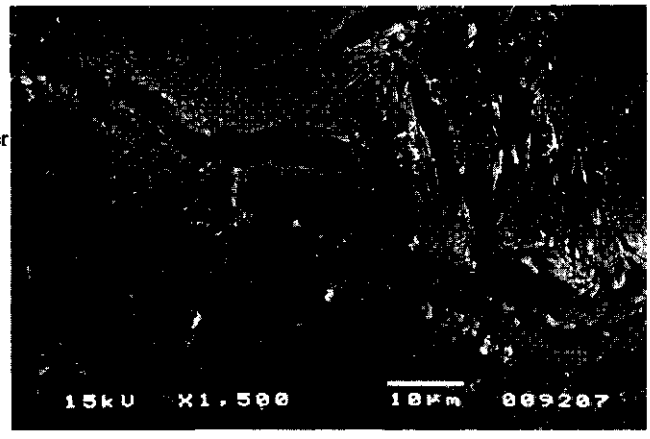


Figure 9.
Section plane of the surface area a fast frozen large granule treated with 50% DMSO. loosely packed (upper part) and dense packed (bottom part) *Methanothrix* bacteria are visible.
Bar = 10 μm.

The studied granules showed a layered structure. In literature those structures are related both to a multi-layered population organization (Guiot *et al.* 1992b) and to condition changes (Hulshoff Pol 1989). The last mechanism is supported by the presence of fungal spores just between two shells. Apparently the UASB reactor for a short period is supplied with a fungal spores containing wastewater.

In most granules larger holes are randomly distributed over the fractioning area. Presumably these holes were developed secondary as gas transport canals. The loosely packed species observed in these holes might be hydrogen consuming bacteria which take advantage of the elevated hydrogen concentration in these regions.

Summarising, the developed cryo-protection and cryo-sectioning method is suitable for biofilms and anaerobic granular sludge. The use of DMSO as cryo-protectant will reveal more detailed information about the spatial organization of the involved microbial populations.

Freezing rate of the treated samples has to be fast as possible, using powered injection into liquid propane. The optimal sectioning was achieved using 50% DMSO as cryo-protectant. Ethanol was less suitable as cryo-protectant. For cryo-microtomy, the knife temperature of -120°C and sample temperature of -110°C were optimal.

6 THE EFFECT OF LOADING RATE VARIATIONS ON STABILITY OF ANAEROBIC GRANULAR SLUDGE

This chapter consists of a modified edition of:
The effect of loading rate variations on stability of anaerobic granular sludge
P. Arne Alphenaar, Meinard E. Eekhof, Frans Visser, M. Cecilia Pérez and Gatze Lettinga.
Submitted for publication

6 THE EFFECT OF LOADING RATE VARIATIONS ON STABILITY OF ANAEROBIC GRANULAR SLUDGE

ABSTRACT

The influence of organic loading rates on granule characteristics was studied in laboratory scale UASB (Upflow Anaerobic Sludge Bed) reactors fed with a gelatin-containing substrate. The applied sludge loading rates were approx. 0.25 and 0.75 gCOD.(gVSS.d)$^{-1}$; (COD = chemical oxygen demand, VSS = volatile suspended solids). A positive correlation between the average granule diameter and the applied sludge loading rate was found.

Loading rate reduction induced a decrease of granule stability, possibly due to substrate limitation in the granule. Problems with the biomass retention during start-up operations using granular seeding sludge may be related to a start-up regime which is over cautious. The results indicate the advantage of applying the design loading rate already during start-up operations of UASB reactors seeded with granular sludge, in order to prevent granule disintegration.

INTRODUCTION

During the past two decades anaerobic treatment systems have been developed for the purification of large wastewater streams, e.g. the Anaerobic Filter (A), the Upflow Anaerobic Sludge Bed (UASB) reactor and the Fluidized Bed reactor (FB), (van den Berg and Kennedy 1983; Hickey et al. 1991; Lettinga and Hulshoff Pol 1991). Due to the low sludge yield of anaerobic bacteria, all anaerobic wastewater treatment systems have extended start-up periods. For the UASB reactor, the most widely applied system so far, this drawback has been overcome by using granular sludge as seeding material (de Zeeuw 1988; Lettinga et al. 1985). Although this procedure generally is very successful, in some cases exposure of the granular seed sludge to a different operating regime results in a serious decrease in granule quality (Lettinga et al. 1985; Hickey et al. 1991).

In general, difficulties during start-up operations of full-scale UASB reactors are thought to be related to changes in the wastewater composition (Lettinga et al. 1987; Morgan et al. 1990). In practice however, sometimes difficulties related to the granule stability occur despite a great similarity in wastewater composition of the seeded reactor and the reactor from which the seed sludge is obtained. In those situations probably the changing process conditions affects the granular sludge characteristics.

One of the major characteristic parameters of granular sludge with regards to the functioning of the sludge in UASB reactors is the size distribution (Grotenhuis et al. 1991a). Several factors affecting the granule size distribution are reported in the literature. A low pre-acidification, and specially a high carbohydrate fraction in the feed stimulates the formation of large-sized granules (Harada et al. 1988; Hickey et al. 1991). Grotenhuis et al. (1991a) reported a positive relation between influent concentration and the average granule size. The liquid upward velocity in the reactor was reported to affect the granule size both positively (Guiot et al. 1992a) and negatively (Kosaric et al. 1990a). Contradictory relations are also reported with regard to the organic loading rate; Morvai et al. (1990) noted a positive relation, while others (Hulshoff Pol 1989; Switzenbaum and Eimstad 1987) pointed to the negative influence of high loading rates on granule size.

Granular growth is related to bacterial growth, while the maximum thickness of the active and stable biomass is limited by the maximum substrate penetration depth (Beeftink and Staugaard 1986). The positive relations between the granule size and both loading rate and influent concentration possibly are related to correspondingly higher substrate concentrations in the reactor at higher loading rates or influent concentrations. However, internal gas production and external (shear) forces related to biogas production may induce deterioration or even disintegration of the granules (Christensen et al. 1989; Liu and Pfeffer 1991). The granule size distribution in a sludge bed will depend upon the retention time of the various sludge fractions and the sludge removal by excess-sludge discharge and wash-out.

With regards to the functioning of UASB reactors in practice, changes of the granule size distribution may give an early indication of major problems related to the granule stability. The aim of the present study was to investigate the effect of loading rate changes and loading rate changes as occurring during start-up operations in the case of using granular sludge, on the characteristics of the sludge granules. Special attention has been paid to changes in the granule size distribution.

MATERIALS AND METHODS

Reactors

The experiments were performed in lab-scale UASB reactors. A scheme of the reactors is given in figure 1, chapter 2.

Reactor A and B - 3.2 litre liquid volume UASB reactors (height approx. 420 mm, internal diameter 95 mm) with an external 3-phase separator. (figure 1c, chapter 2)

Reactor C and D - UASB reactors identical to A and B but equipped with an internal 3-phase separator (figure 1a, chapter 2)

Reactor C_a, C_b and D_a - 1.1 litre liquid volume PVC UASB reactors (height 40 cm, internal diameter 6 cm) with an internal phase separator (figure 1c, chapter 2).

Three separate influent flows were used in all experiments; an organic substrate stock solution (stored at 4°C to prevent pre-acidification), a nutrient/trace element stock solution and dilution water (tap water, 30°C). These flows were mixed just before the reactor inlet. The experiments were conducted in a temperature controlled room (30 ± 1°C). The methane production was monitored by a wet gasmeter (Meterfabriek Dordrecht, Dordrecht, the Netherlands) after removing CO_2 by use of 5% (w/v) of NaOH solution.

The experimental set-up of the different reactor experiments is given in table 1.

Biomass

Reactor A, B, C and D were seeded with granular sludge originating from a full-scale reactor treating wastewater from an alcohol producing industry (Nedalco, Bergen op Zoom, The Netherlands). The sludge was elutriated at an upward velocity of approx. 15 $m.hr^{-1}$ before use. Reactors C_a, C_b and D_a were seeded with granular sludge from reactors C and D respectively (table 1). Sludge samples were stored at 4°C.

Media

Gelatin was used as sole COD source. 0.5 g $NaHCO_3.(gCOD)^{-1}$ was used as buffer. The mineral medium and the trace element solution (according to Huser 1981) used in the continuous reactor experiments are described in chapter 2. All chemicals were of analytical grade (Merck AG, Darmstadt Germany) except the gelatin (food quality, 150 Bloom, Sanofi Brussel, Belgium) and the Oxoid yeast extract (Unipath Ltd, Basingstroke, England). The media were prepared in tap water.

Methods

Flocculent and granular sludge were separated by repeated (approx. 10 times) mixing and subsequent decanting of the sludge sample after sedimentation for 1 minute in a 100 ml volumetric cylinder (height approx. 25 cm). The separation is assumed to be

comparable with separation based on elutriation with a liquid velocity of approx. 15 $m.hr^{-1}$. After separation, the VSS and TSS of the flocs and granules were measured.

All other used methods are described in chapter 2

Experimental set-up

Table 1 Experimental set-up of the experiments to the relation between sludge load and granule characteristics.

Aim of the experiment:	*Assessment of the effect of sludge loading rate and loading rate change at constant hydraulic retention time*					
	day 0 to 90			day 90 to 170		
Reactor	load[a]	conc.[a]	HRT[a]	load	conc.	HRT
A	0.3	370	2.3	0.53	890	2.3
B	0.65	1010	2.4	0.31	460	2.3

Aim of the experiment:	*Assessment of the influence of the sludge loading rate on the granule quality at constant influent concentration*			
	day 0 to 128			
Reactor	load	conc.	HRT	
C	0.65	1600	2.8	Sludge transferred[b] to Reactors C_a and C_b
D	0.25	1450	8.5	Sludge transferred[b] to Reactor D_a

Aim of the experiment:	*Assessment of the effect of changes of sludge loading rate and/or of influent concentration on granular sludge quality with sludge developed in reactor C and D*					
	day 0 to 56			day 56 to 78		
Reactor	load	conc.	HRT	load	conc.	HRT
C_a	0.26	1350	8.8	0.6	1450	4.0
C_b	0.25	450	3.2	0.55	1000	3.2
	day 0 to 29			day 29 to 78		
D_a	0.25	1450	9.2	0.25	500	3.2

[a] load = sludge loading rate ($gCOD.(gVSS.d)^{-1}$); conc. = influent concentration ($mgCOD.l^{-1}$); HRT = hydraulic retention time (hr)

[b] The sludge was stored at 4 °C for 12 days

RESULTS

The results of the UASB experiments conducted in the present study indicate that higher sludge loading rates correspond to larger granule diameters. In the first experiments the difference in loading rate was established by difference in the influent concentration at constant HRT (hydraulic retention time). Sludge loading rate variation of the reactors during the experiment resulted in a corresponding change of the size distribution of the granular sludge (figure 1).

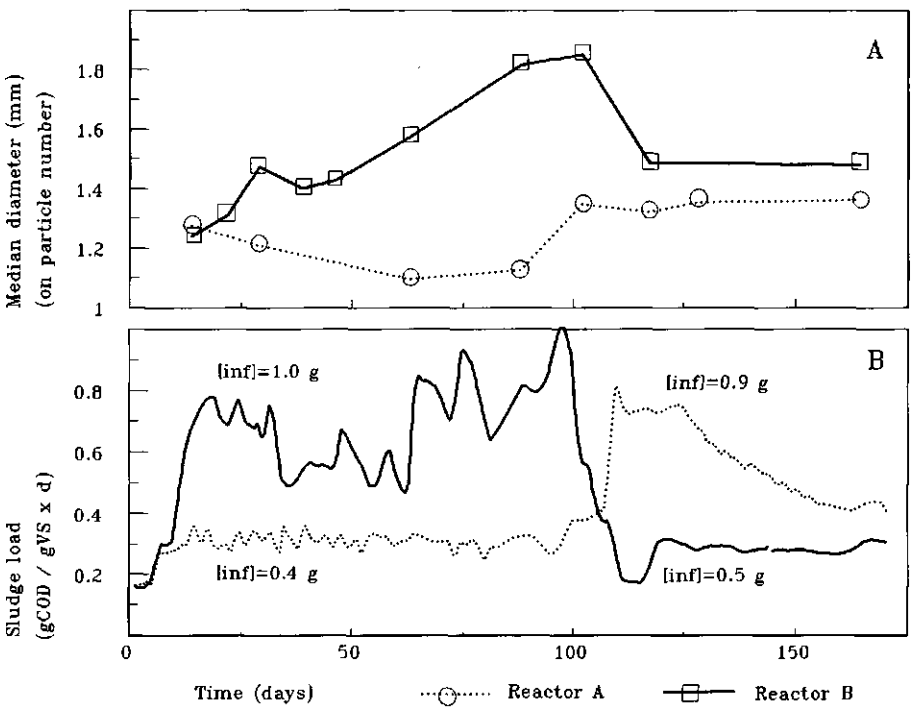


Figure 1 The relation between median diameter and sludge loading rate in UASB experiments applying constant HRT (3.2 litre reactors A and B).

Also when different loading rates were applied by the use of different HRT at a constant influent concentration, higher sludge loading rates correspond with larger granule diameters (figure 2).

After a change in the loading rate applied, within thirty days a new size-distribution was established (figure 1).

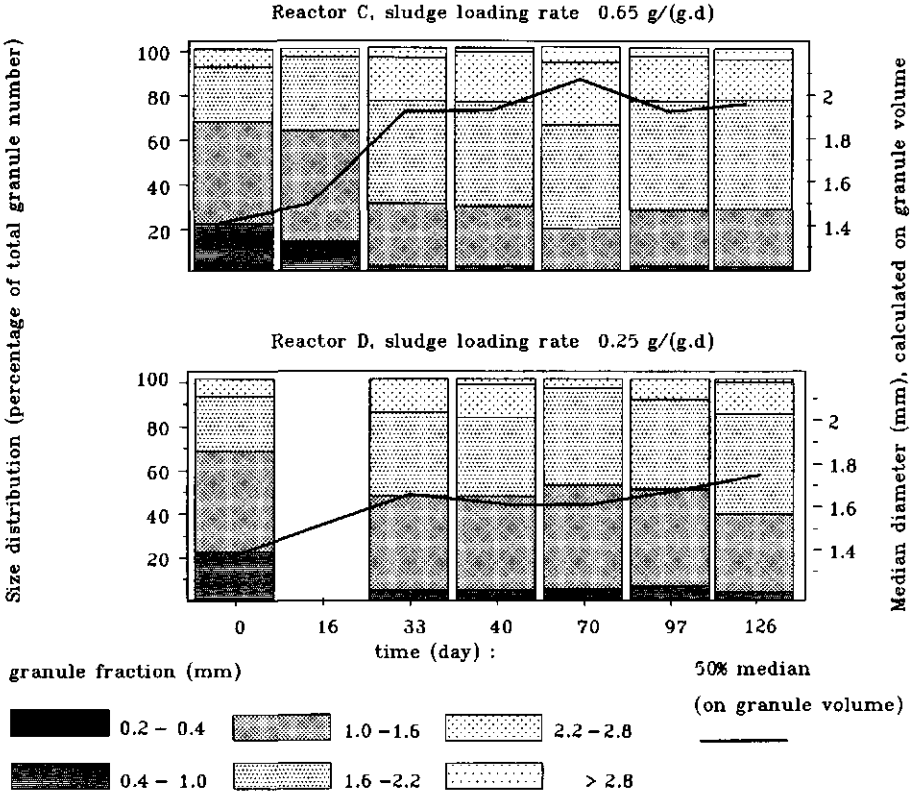


Figure 2 The granule size distribution and the median granule size related to the applied loading rate in the 3.2 litre UASB reactors. Top: Reactor C (loading rate 0.65 gCOD.(gVSS.d)$^{-1}$, influent concentration 1.6 gCOD.l^{-1}), bottom: Reactor D (loading rate 0.25 gCOD.(gVSS.d)$^{-1}$, influent concentration 1.5 gCOD.l^{-1}). The size distribution and the median are expressed as percentage of the total granule volume.

Although the granule size distribution clearly indicates the influence of loading rate changes on granule quality, the reactor performance was not affected.

Additional experiments were carried out to assess whether the size distribution changes could be indicative of changes in granule stability during reactor start-up. Granular sludge from reactor C and D was used as seed material for three 1.2 litre UASB reactors (C_a, C_b and D_a). In the first period after the sludge transfer, low sludge loading rates were applied in all these reactors. The process conditions in reactor D_a were designed to be similar to those in reactor D in order to examine the influence of the change from the 3.2 litre UASB to the 1.2 litre UASB on the granular sludge size distribution. After 56 days the loading rate of reactor C_a and C_b was increased; according to the results of reactor A and B, a new size distribution equilibrium should be established within this period.

Again, the granule size distribution could be related to the loading rate applied. However, the loading rate increase resulted in an increased number of small particles in the sludge bed. The granule size distribution as a percentage of the total particle number is given in figure 3. Optical observations of the sludge made before and after the imposed loading rate changes indicated that a serious granule disintegration occurred (figure 4). As a consequence, the fraction flocculant sludge of the sludge bed, quantified by repeated decanting of sludge samples increased significantly (table 2).

Table 2 The increase of the percentage flocculent material in the reactor.

	percentage flocculent matter		
Time (day)	0	8	78
Reactor C_a	7%	13%	35%
Reactor C_b	7%	10%	26%
Reactor D_a	--	1%	3%

An elevated flocculent sludge percentage was not observed in reactor D_a, which was operated at constant loading rate (and with a loading rate as applied in the original reactor of the sludge, reactor D). From this we can conclude that the change from the 3.2 litre reactor to the 1.2 litre reactor does not play an important role the increase of the flocculent sludge fraction as observed in reactor C_a and C_b. That increase consequently is related to the loading rate changes applied, possibly enhanced by an effect of the decrease in influent concentration.

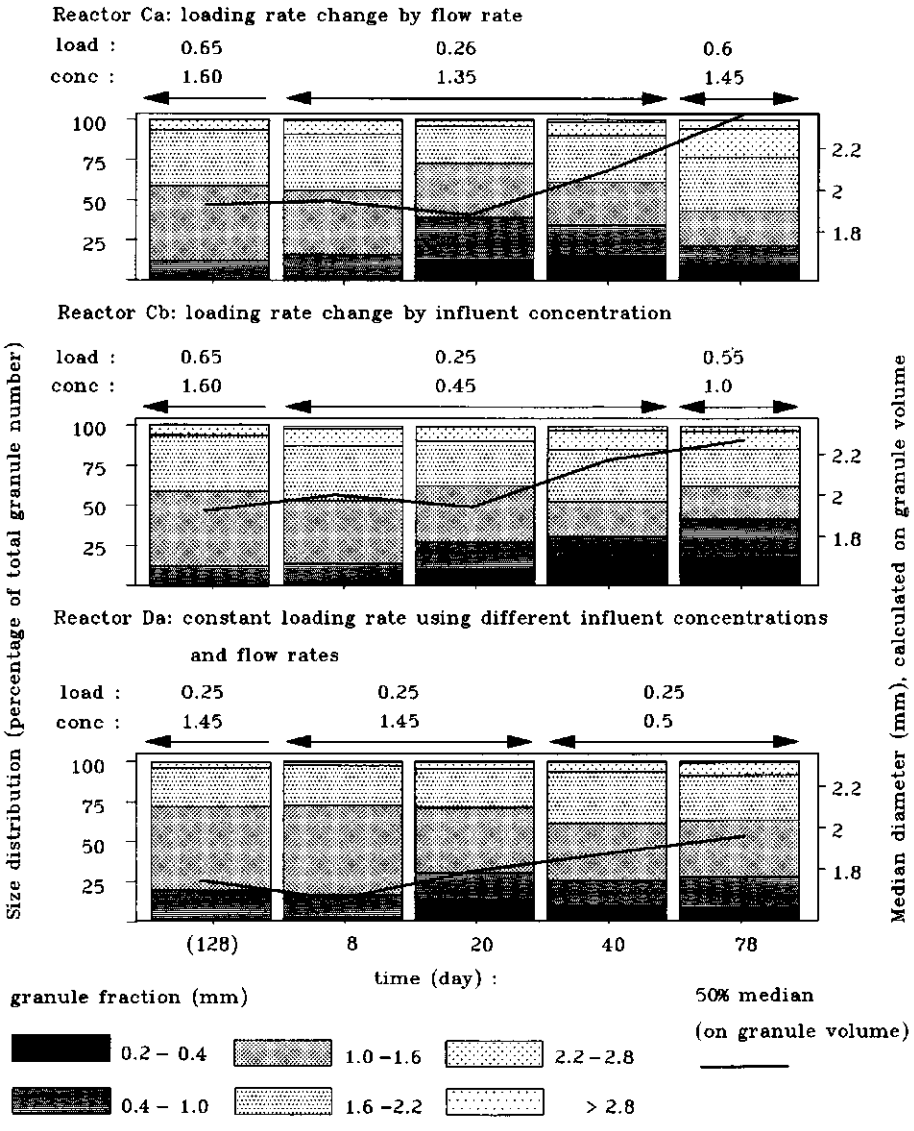
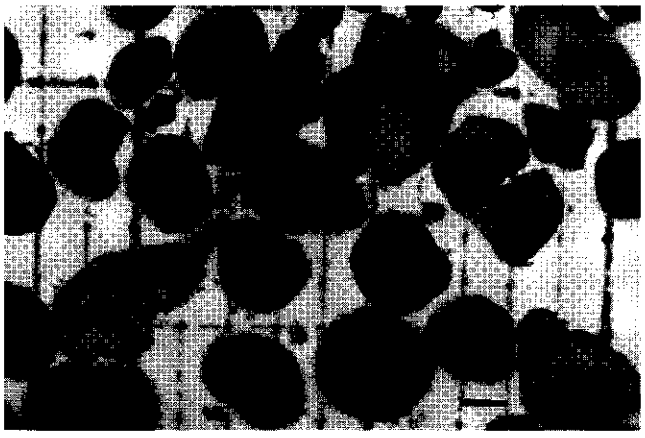


Figure 3 Granule size distribution and median granule size related to the applied loading rate changes in the 1.2 litre reactors. The distribution on the left (day 128) presents the size distribution of the seed sludge in reactor C and D respectively. The size distribution bars present the particle number of the fraction as percentage of the total number of granules, the median granule size is calculated as a percentage of the total volume of the granules present.

Figure 4
A: Granular sludge from a gelatin fed reactor, loading rate 0.65 gCOD.(gVSS.d)$^{-1}$ (reactor C). The grid indicate 1 mm.



B: Granular sludge after subsequent loading rates of 0.65, 0.26 and 0.6 gCOD.(gVSS.d)$^{-1}$ (reactor C_2). The flocculant sludge was removed before making the picture. The grid indicate 1 mm.

Both the size distribution data and the flocculent sludge increase seem to indicate the influence of loading rate decrease on the granule quality. The granular strength indeed is seriously affected by the loading rate changes applied (figure 5). The strength of the granular sludge in the reactor in which a constant loading rate was applied was not affected by the reactor change nor by the storage of 12 days. The strength of the granular sludge in this reactor even increased when, applying a constant loading rate, the influent concentration was decreased (figure 5).

The aim of the experiments was to investigate the effect of loading rate changes which may occur during start-up operations, on the granular sludge characteristics. A decreasing granule quality might negatively affect the reactor performance. However

in these experiments the efficiency is not affected by the changes applied. The COD removal was approx. 83 and 94 percent in the "high" and "low" loading rate regimens respectively.

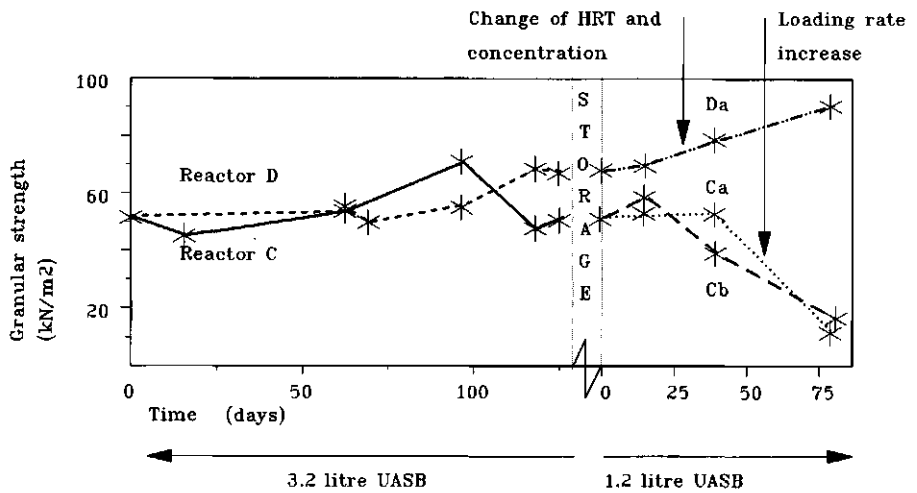


Figure 5 The granular strength related to the applied loading rate, the changes of the loading rate applied (Reactor C, C_a and C_b) and to the changes in reactor and of the influent concentration decrease (reactor D and D_a).

DISCUSSION

Frequent monitoring of the granular sludge quality may be of great importance for the wastewater treatment practice. Among the various sludge characteristics, the granular sludge size distribution is a relatively simple parameter to follow.

The size of a sludge granule depends on growth of the bacterial population present in the granule, on the surface shear and on granule deterioration. The granule size distribution of the sludge bed is determined by a combination of these factors and the excess-sludge discharge and sludge wash-out.

High loading rates stimulate both granule growth and granule deterioration. Growth is basically related to substrate degradation, while deterioration is primarily related to biogas production, which causes both external (shear) forces and internal forces. The present study reveals that high loading rates correlate with larger granule sizes, as also found by Morvai et al. (1990). According to Grotenhuis et al. (1991a) the influent concentration is the major granule size controlling factor.

In most situations it will be difficult to discriminate between influent concentration and sludge loading rate effects. Granule size distribution data from numerous lab-scale experiments carried out at our laboratory reveal the existence of correlations between influent concentration and the sludge loading rate. However we were unable to deduce a clear overall relation from these date, which points to the extreme complexity of these phenomena.

Our experiments reveal that abrupt loading rate reductions induce a decrease in granular strength. High sludge loading rates usually are related to higher substrate levels throughout the reactor. At low loading rates and/or influent concentrations the average substrate concentration at the granule surface is lower and consequently the substrate penetration depth in the granule will be reduced. For the larger granules this will induce substrate limitation in the granule core. Generally this will result in lysis of the substrate limited bacteria. As the stability of the sludge granules is related to the viable bacteria present in the granule, bacterial decay will induce a stability decrease. This phenomenon was also found by Kosaric et al. (1990b).

On the other hand, all forces related to gas production are also reduced at low loading rates. According to Christensen et al. (1989), Liu and Pfeffer (1991) and Pavlostathis and Giraldo-Gomez (1991), the internal gas production is a major factor reducing biofilm thickness. Comprehensive research on the anaerobic treatment of cold diluted municipal sewage wastewater conducted in our laboratory (van der Last 1992) revealed the importance of the internal forces due to gas production for granule characteristics. Extremely large (up to 6 mm), hollow granules developed in the Expanded Granular Sludge Bed (EGSB) reactors (chapters 3 and 4). In these experiments with unheated sewage, the superficial gas velocity was low as a result of the low methanogenic activity and the relatively high solubility of CH_4 in water. As a consequence the internal stress due to gas bubble growth in the granule was almost absent. Despite the low mechanical strength of the granules which developed, the treatment process itself was stable and looked feasible.

Clear evidence has been obtained that granule disintegration is induced by abrupt loading rate decrease and subsequently increase, as generally occur during reactor start-up using granular sludge. Due to the reduced stress on the granules when low loading rates are applied, generally no serious granule disintegration will take place despite the decreased granule stability. After a certain period a new stable granule equilibrium is established. However when the loading rate raises before a new steady state is reached, the forces related to (internal) gas production increase. As a result deterioration of the weakened granules will occur.

Regarding the excellent resistance of methanogenic granular sludge to storage, as reported by Hulshoff Pol (1989) the observed granule deterioration is very likely correlated to the decay of the acidogenic population in the granule (chapters 7 and 8).

In practice the observed relation between applied loading rate and granule diameter might be different. The relation is dependent on the reactor configuration and the shear forces because of turbulence. As turbulence, shear forces and (due to the hight of full-scale reactors) the internal forces in the granules are much more powerful in full-scale reactors than in lab scale reactors, the effect of decreasing granule strength will be much more serious.

Many start-up problems of full scale reactors using granular seed sludge presumably must be attributed to granule quality reduction due to loading rate changes. Generally, full scale reactors are started-up cautiously, in order to achieve adaptation of the seed material to the new situation. During this period the mechanical strength of the granules may decrease by substrate limitation in the granule core. When after several weeks the design load is applied, these weakened granules might disintegrate. We are in the opinion that in many situations it is profitable to apply the design load immediately from start-up. The relatively high initial wash-out of biomass is more acceptable than the total biomass loss after some weeks in case of a too cautious start-up procedure.

7 EFFECTS OF SUSPENDED ACIDOGENIC BACTERIA ON THE PERFORMANCE OF THE METHANOGENIC UASB REACTOR OF A TWO-STEP WASTEWATER TREATMENT SYSTEM.

This chapter consists of a modified edition of:
Effects of suspended acidogenic bacteria on the performance of the methanogenic UASB reactor of a two-step wastewater treatment system.
P. Arne Alphenaar, Ron Sleyster and Gatze Lettinga
submitted for publication

7 EFFECTS OF SUSPENDED ACIDOGENIC BACTERIA ON THE PERFORMANCE OF THE METHANOGENIC UASB REACTOR OF A TWO-STEP WASTEWATER TREATMENT SYSTEM.

ABSTRACT

Suspended matter present in the influent of UASB reactors often causes problems with regard to the reactor performance. In this study we investigated the possible effect(s) of suspended acidogenic bacteria, which represent one of the most common type of suspended solid present in UASB reactor influent, since they are present in most pre-acidified wastewaters. The results obtained in the present study reveal that acidogenic bacterial biomass accumulation in UASB reactors hardly take place. Despite that, the suspended acidogenic bacteria induce flotation of the methanogenic granular sludge when present in concentrations in the influent exceeding 0.3 gCOD.l^{-1}. The experiments reveal that the flotation of granular sludge apparently cannot be attributed to the suspended acidogenic bacteria themselves. No difference was observed in the effects of intact or ultrasonically disintegrated acidogenic bacteria. The flotation phenomenon probably is induced by a degradation product of the bacterial biomass. The results reveal that two-step anaerobic treatment systems gave serious drawbacks with regard to the process stability of the methanogenic reactor, unless the acidogenic bacteria are removed from the effluent of the acidogenic reactor.

INTRODUCTION

The presence of suspended matter in the UASB reactor influent may constitute a major cause of problems for anaerobic wastewater treatment in full scale UASB reactors (Hulshoff Pol 1989; Lettinga et al. 1985; Lin en Yang 1991; Sayed et al. 1988). In most cases these problems are related to accumulation in the sludge bed, or to incorporation in the sludge granules. It may cause insufficient growth of new granular sludge, poor biomass retention and also inferior granule characteristics with respect to strength, methanogenic activity and settleability.

Many different types of SS (suspended solids) can be distinguished, which may all cause their specific problems, e.g.:

- The entrapment of inorganic material such as clay and sand particles in the reactor will replace the active biomass in the system (Lettinga and Hulshoff Pol 1991; Rozzi and Verstraete 1981; van Wambeke et al. 1990).

- The incorporation of non biodegradable organic matter (fibres) in sludge granules will "dilute" the active biomass.
- Attachment of suspended methanogens on poorly settleable organic particles will cause a serious decrease in granular growth.
- The adsorption of (biodegradable) particles such as fats or proteins on the granule surface may cause substrate limitation of the active biomass when the degradation of this material proceeds too slowly. The adsorbed material may also hamper the gas release because of the formation of poorly penetrable surface layers.
- The degradation of adsorbed material may induce growth of hydrolysing and acidogenic organisms which occurs strictly at the surface and therefore may alter the granule surface characteristics.
- The adsorption of low density dispersed organic matter may induce granule washout (Sayed et al. 1988; Rinzema et al. 1989) and/or induce the development of a layered granule structure which is more sensitive to mechanical forces.

A typical and important category of degradable suspended solids are acidogenic bacteria. These bacteria can develop in the sewer system, in the influent storage tank or in a first (acidifying) step in the case of a two-step anaerobic treatment system. For many years the possible benefits of pre-acidification in anaerobic wastewater treatment have been the subject of discussion.

An important argument for recommending the use of a two-step system for non-acidified wastewaters is the significantly higher specific methanogenic activity of the sludge developing in the methanogenic reactor of a two-step system as compared to the sludge developing in a one step process (Cohen et al. 1979; 1985). Another reason is the presumed lower sensitivity to toxic compounds in the wastewater (Dinopoulou and Lester 1989; Komatsu et al. 1991). However, several researchers point to the concomitant low sludge yield in the methanogenic reactor and the significant higher investment and operation costs as serious disadvantages of applying a complete pre-acidification step (Hulshoff Pol 1989; Lettinga and Hulshoff Pol 1991; Sam-Soon et al. 1988; Vanderhaegen et al. 1992; de Zeeuw 1984).

Accumulation of SAB (suspended acidogenic bacteria) in the sludge bed of UASB reactors or incorporation of SAB in the individual sludge granules may affect the granular sludge characteristics. Therefore the objective of the present study was to assess the effects of SAB present in pre-acidified influent on the quality of the granular sludge, the behaviour of the sludge bed and on the effect on the performance of the methanogenic UASB reactors.

MATERIALS AND METHODS

Reactors

For assessment of any possible effects of suspended SAB on reactor performance, 3.2 litre liquid volume plexiglass UASB reactors (height approx. 420 mm, internal diameter 95 mm) with an external phase separator (chapter 2) were used as methanogenic reactors.

These UASB reactors were used in several experiments:

A In experiments where they were operated in series with a five litre acidogenic CSTR. The SAB concentration in these experiments was controlled by varying the sucrose / VFA ratio of the CSTR (Continuous Stirred Tank Reactor) influent.

B In experiments where they were fed with the (pre-treated) effluent of a 20 litre acidogenic CSTR (see below). The effluent of the CSTR was stored at 4°C in a refrigerator.

C In recirculated batch-fed experiments, where they were fed with concentrated SAB suspensions or with ultrasonically disintegrated SAB solutions. The recirculation flow applied was 20 $l.d^{-1}$.

A 20 litre acidogenic CSTR fed with a sucrose solution was employed to produce the suspended acidogenic bacteria (SAB) for the methanogenic UASB reactor experiments A and C. The process conditions applied for the SAB production in this reactor are given in table 1. The pH of the CSTR reactor was controlled at pH 5.8 in order to obtain the most stable acidification pattern according to the results of Zoetemeyer (1982). In some experiments, the CSTR effluent was separated in a soluble and a suspended fraction using a centrifuge. The CSTR effluent and the two separated fractions were stored at 4°C maximally for one week before use.

Media

Nutrients and trace elements were always supplied in sufficient amounts to the influent of the acidifying reactor and to the substrates consisting of concentrated SAB suspensions. The applied concentrations are given in chapter 2. Also 0.5 $g.gCOD^{-1}$ $NaHCO_3$ was supplied to the acidifying reactor influent. All chemicals were of analytical grade (Merck AG, Darmstadt Germany) except the sucrose (food quality, CSM, the Netherlands) and the yeast extract (Oxoid, Unipath Ltd, Basingstroke, England). The media were prepared in tap water.

Table 1 The main process conditions and performance data of the 20 litre acidogenic CSTR reactor used for the production of suspended acidogenic bacteria.

Influent composition				Process conditions		
Sucrose		± 5.5	$COD.l^{-1}$	Flow	20	$l.d^{-1}$
$NaHCO_b$		2.5	$g.l^{-1}$	HRT	1	d
				pH	5.8	
				Temperature	30	°C
CSTR Effluent composition						
COD_{tot}		± 5	g			
COD_{sol}	VFA[a]	51	%	(8% C_1, 7% C_2, 5% C_3, 80% C_4)		
	Lactate	32	%			
	Alcohols[b]	2	%			
		+ -------				
		85	%			
COD_{SS}	(%)	15	%			
Suspended matter		0.96	$gVSS.gTSS^{-1}$			
		0.8	$gVSS.gCOD^{-1}$			

[a] : C_1 = formic acid, C_2 = acetic acid, C_3 = propionic acid, C_4 = Butyric acid.
[b] : methanol and ethanol

Biomass

Granular sludge from a full-scale reactor treating wastewater of an alcohol industry (Nedalco, Bergen op Zoom, The Netherlands) was used as seeding sludge for the methanogenic reactors. Before use, the sludge was elutriated using an upward velocity of 15 $m.hr^{-1}$. Sludge samples were stored for approx. 1 week at 4°C before analysis. No seeding was applied in the acidogenic reactors.

Methods

To obtain a concentrate of SAB, acidogenic bacteria present in the CSTR effluent were separated from the effluent using a semi-continuous centrifuge (super-centrifuge MVF 16-21 5p1. Sharples, Rueil, France).

Suspended acidogenic bacteria were disintegrated in batches of approx. 200 ml using a 150 W ultrasonic disintegrator (MSE, Leicester, England). A disintegration time was chosen in which the maximum disintegration, measured as soluble COD was obtained. All other methods are described in chapter 2.

RESULTS

Sludge flotation in two-step systems

Accumulation of suspended matter in the sludge bed of UASB reactors or adsorption/incorporation of those materials in/on the individual sludge granules may seriously affect the granular sludge characteristics.

Preliminary UASB experiments conducted with pre-acidified sucrose revealed that SAB containing feeds cause heavy sludge flotation within a relatively short period. Since sludge flotation in conventionally designed UASB-systems may result in a high wash-out of active biomass, an improved understanding of the reasons of the sludge flotation as induced by SAB present in the influent is of crucial importance with respect to the proper application of two-step anaerobic treatment systems. For this reason in the present study we investigated the influence of various SAB concentrations in the UASB reactor feed in more detail using laboratory scale two-step systems. The SAB concentration in completely acidified UASB influent was controlled by varying the sucrose concentration of the influent of the acidogenic CSTR reactor (table 2).

The sludge loading rate applied in the methanogenic UASB reactor in these experiments was kept constant at 0.9 $gCOD.(gVSS.d)^{-1}$. Employing the two-step configuration, serious sludge flotation was observed in the UASB reactor within 55 days of operation at a SAB influent concentration of 0.38 $g.COD_{SS}.l^{-1}$. When using influent SAB concentrations of 0.56 and 0.75 $g.COD_{SS}.l^{-1}$ sludge flotation already occurred within 30 days of operation, but no sludge flotation was observed with SAB concentrations below 0.19 $g.COD_{SS}.l^{-1}$ in the influent within a period of 90 days (table 2).

Table 2 The reactor systems and the main process conditions applied in the two-step system experiments.

Volume	CSTR	5	l	(acidogenic reactor)
	UASB	3.2	l	(methanogenic reactor)
Flow		7.5	$l.d^{-1}$	
COD		7.5	$g.l^{-1}$	
$NaHCO_3$		4	$g.l^{-1}$	
granular sludge load		0.9	$gCOD.(gVSS.d)^{-1}$	

		System	1	2	3	4	5
CSTR Infl. composition	sucrose	%COD	0	25	50	75	100
	VFA[a]	%COD	100	75	50	25	0
SS CSTR effluent		$mgCOD.l^{-1}$	0	190	380	560	750
Flotation in UASB		days of operation	>90	>90	55	30	30

[a] The composition of the synthetic substrate is similar to the composition of the soluble fraction of acidified sucrose, except for the alcohols (table 1).

Sludge flotation related to suspended acidogenic bacteria

Since the sludge flotation observed in the methanogenic UASB reactor of the two-step systems possibly can be attributed to a soluble component formed from the acidified sucrose, supplementary experiments were conducted in which UASB reactors were fed with feeds consisting of:

- A synthetic VFA substrate of similar composition to the soluble fraction of the CSTR effluent;
- The soluble fraction of acidified sucrose;
- The untreated CSTR effluent containing both the soluble and the suspended fraction (SAB) of acidified sucrose;
- A mixture of SAB concentrate and a synthetic VFA substrate of similar composition to the soluble fraction of the CSTR effluent.

The effluent composition of the acidogenic CSTR and the main process conditions applied in this reactor are summarized in table 1. The main process conditions applied in the UASB reactors in this experiment are summarized in table 3a.

In neither the reactor fed with soluble synthetic substrate nor the reactor fed with the soluble fraction of acidified sucrose was any sludge flotation observed within a period of 63 days. However, when feeding the UASB reactor with the total CSTR effluent, which contained 4.5 g of soluble COD per litre and 0.7 g of suspended COD per litre, serious flotation of granular sludge occurred within a period of 26 days. At that time over 60% of the sludge volume was present in the floating layer (table 3b).

At higher SAB concentrations, viz. in experiments conducted at a concentrated SAB suspension of 3.5 $gCOD_{SS}.l^{-1}$ (COD_{SS}:$COD_{synthetic}$ = 1), heavy sludge flotation in the UASB reactor occurs already within 10 days of operation. The granular sludge characteristics determined in these experiments are summarized in table 3b.

Table 3a Experimental set-up of continuous flow UASB reactor experiments fed with suspended acidogenic (sucrose acidifying) bacteria.

substrate		synthetic substrate	soluble fraction CSTR effluent	untreated CSTR effluent	concentrated SAB + synthetic substrate
sludge load	$gCOD.(gVSS.d)^{-1}$	0.7	0.7	0.7	1.0
concentration	$gCOD.l^{-1}$	4.5	4.5	5.2	7.5
soluble	%	100	100	86	50
suspended	%	0	0	14	50
HRT	hr	7.5	8.0	7.5	13

Table 3b The effect of suspended (sucrose acidifying) bacteria on granular sludge flotation and sludge characteristics, assessed at day 63.

start of flotation	day	--	--	26	10
granule yield	$gVSS.gCOD^{-1}$	0.022	0.034	0.039	--[a]
methanogenic activity	$gCOD.(gVSS.d)^{-1}$	1.51	1.65	1.78	1.84
specific gravity	$kg.m^{-3}$	1037	1037	1030	--[a]
granule strength	% start	136	129	95	--[a]
Ash content	% TSS	10	9.6	9.2	10.8

[a] Not determined

The results clearly reveal the decisive role of SAB in the sludge flotation. The specific gravity, the mechanical strength and the ash content of the granules fed with SAB-containing influent are low relative to the granules from reactors fed with the soluble substrates, which possibly indicates a slight build-in of relatively fluffy packed SAB matter in the granules. The sludge yield values indicate a positive effect of both the total SAB containing acidified sucrose feed and the soluble fraction of the acidified sucrose relative to the synthetic substrate. However, the values found for the sludge yield do not clearly indicate however accumulation of SAB or growth of acidogenic bacteria in the granular sludge (table 3b).

The methanogenic activity of both the sludges from the reactors fed with SAB and with the soluble fraction of acidified sucrose were high relative to the activity of the sludge from the reactor fed with synthetic substrate, which contradicts the relative decrease of the methanogenic population as a result of non-methanogenic biomass build-in. Also visual observations of the sludge did not indicate any significant SAB accumulation.

Assessment of SAB degradation

In order to further elucidate the mechanism of the sludge flotation, four UASB reactors were operated in a batch-fed recirculation mode and fed with different substrates:

a one reactor was used to assess the accumulation of SAB in the sludge bed. This reactor was fed with a concentrated SAB suspension in a mineral medium.

b one reactor fed with a soluble, synthetic substrate, similar in composition to the soluble fraction of acidified sucrose was used as control.

c on reactor was fed with a solution of ultrasonically disintegrated SAB in order to assess any difference between the effects of intact and destructed SAB.

d one reactor was fed with a concentrated SAB suspension in water. This reactor was started using granular seed sludge which had previously been exposed to a concentrated SAB feed and during that time had already experienced floatation. In this reactor an attempt was made to assess the persistence of the floating tendency of the granular sludge as induced by SAB.

The experimental set-up is summarized in table 4, the feed strategy can be deduced from figure 1. A schematic view of the reactors used is given in chapter 2.

Table 4 The experimental set-up of the batch-fed recirculation UASB experiment in order to clear up the relation between SAB degradation and sludge flotation.

Total volume	3.9	*l*
	0.8	*l* (hydraulic buffer effluent recirculation)
Recirculation	20	$l.d^{-1}$
HRT	60	d
Feed	± 200	ml per 3 days
	± 38	$gCOD_{ss}.l^{-1}$

Reactor	Sludge source	Sludge amount (g)	Substrate type
a	Nedalco	19	concentrated SAB
b	Nedalco	19	synthetic substrate[b]
c	Nedalco	19	disintegrated concentrated SAB[a]
d	SAB fed reactor[c]	26	concentrated SAB

[a] 65% of the COD_{ss} was, after ultrasonically disintegration, detected as COD_{sol}.

[b] composition as soluble fraction in table 1, except the alcohols.

[c] The floating sludge used in this experiment was obtained from a previous experiment (table 2, reactor fed with concentrated SAB). The sludge was stored at 4°C for 40 days before the recirculated batch reactor experiment was started.

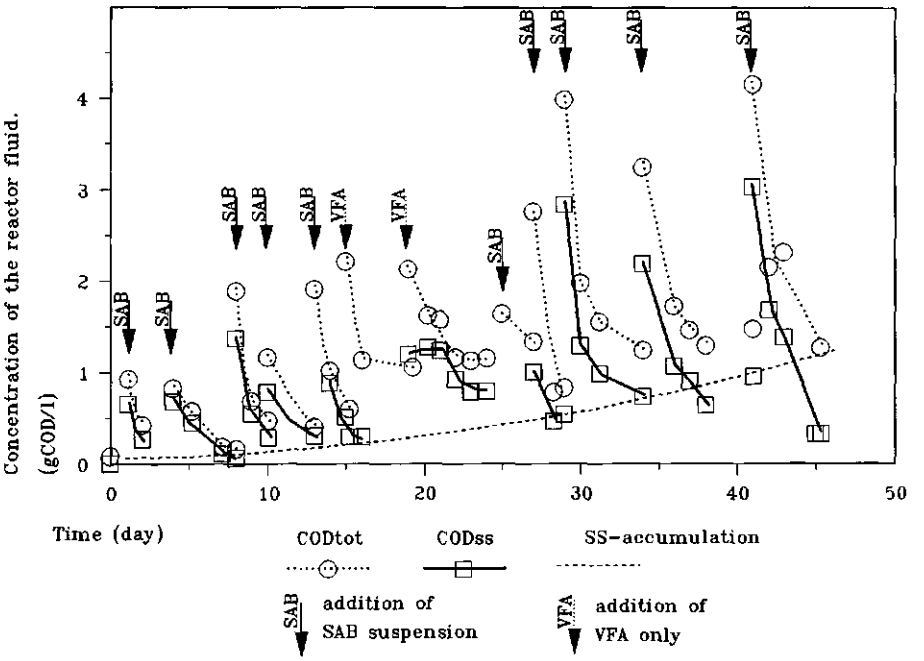


Figure 1 The feed strategy and the substrate concentration versus time of the batch-fed UASB reactor seeded with the exposed previously to SAB, floating sludge. The cumulative COD input over 45 days is 27 $gCOD_{tot}.l^{-1}$ and 16 $gCOD_{SS}.l^{-1}$. From the SS-accumulation it is concluded that at least 90% of the SAB is degraded in the system.

Absolutely no flotation of the sludge was found in the reactor fed with the synthetic feed (figure 2b). However with a feed consisting of concentrated SAB, sludge flotation occurred within a period of 40 days (figure 2a). The experiment conducted with a suspension of ultrasonically destructed SAB (figure 2c) gave very similar results to the experiment conducted with the intact SAB (figure 2a). The granular sludge which already had been exposed to a concentrated SAB feed gave a massive sludge flotation within five days of operation when exposed again to a feed containing a high SAB concentration, despite the fact that the sludge was stored for 40 days at 4°C (figure 2d).

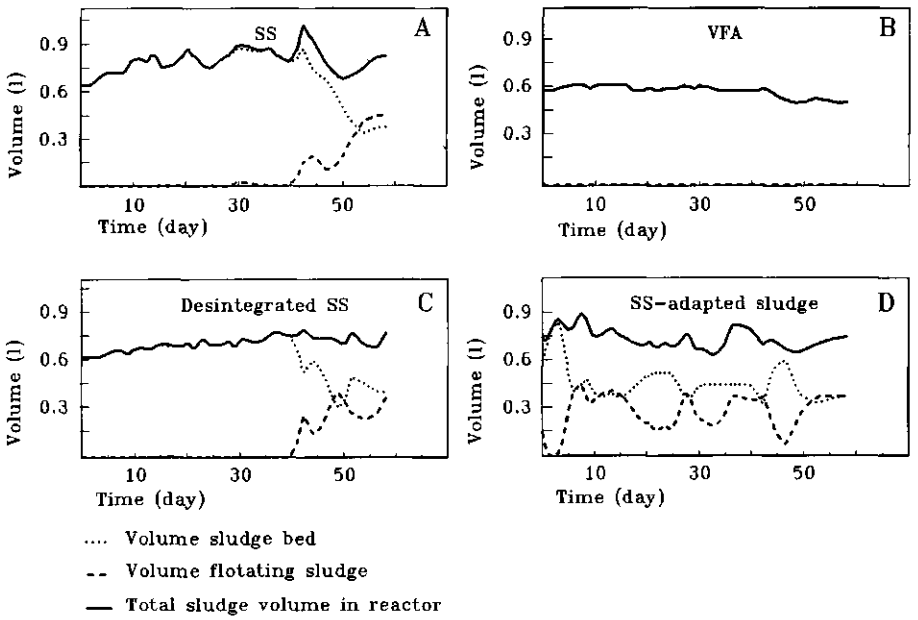


Figure 2 The sludge flotation in time related to the substrate type in relation to the substrate type in fed-batch recirculated UASB reactors.

Visual observations revealed that aggregation of individual granules, followed by entrapment of biogas in the aggregates, very likely can be considered as the direct cause for sludge flotation (figure 3a). These granule aggregates can be disrupted easily by shaking as shown in figure 3b. The sludge granules set free from these aggregates are visually unchanged and showed a good settleability. At higher magnifications, the floating granules show a "hairy" surface structure (figure 4). Morphologically, these surface 'hairs' appear similar to *Methanothrix*.

The apparent absence of accumulation of SAB in the UASB reactor presumably can be attributed to the rapid degradation of SAB. The COD balances over the methanogenic reactors reveal that approx. 30-40% of the SAB present in the feed are degraded in the UASB reactors, while the rest leaves the reactors with the effluent. In the recirculated batch-fed UASB systems, the SAB degradation to methane even is approx. 95% within a period of 3 days. From the results presented in figure 1 it can be deduced that the non-degradable fraction of the concentrated SAB suspension remains present in the recirculated liquid. The accumulation of SAB in the sludge bed of the recirculated batch-fed UASB systems is negligible.

The results of all experiments clearly reveal a relation between SAB in the feed and the flotation of granular sludge. However, it is difficult to discriminate between the influence of SAB concentration in the influent, the SAB load (input per biomass in the reactor) and the SAB amount degraded per amount of biomass present in the UASB. Table 5 summarizes the available information about the SAB input in the different experiments and the period at which sludge flotation occurred

Table 5 The relation between SAB in the influent and the time in which sludge flotation is observed. The SAB input per gram granular sludge, the SAB degraded per gram granular sludge and the SAB concentration are given.

Reactor + feed	SAB Load[a]	SAB[a] degradation rate	SAB[a] Concentration	Time when flotation first observed
	$gSS_{in}.(gVSS.d)^{-1}$	$gSS_{degr}.(gVSS.d)^{-1}$	$gSS.l^{-1}$	day
continuous UASB reactor fed with CSTR effluent (table 2)	0.067	0.043	0.75	26
continuous UASB reactor fed with concentrated SAB (table 2)	0.3	0.076	3.7	10
Two step systems fed with sucrose (table 3)	0.19	0.05	1.20	30
	0.11	0.03	0.94	30
	0.08	0.025	0.66	55
	0.03	0.01	0.40	>90
Recirculation experiments fed with SAB (table 4)	0.055	0.050	0.5-3.0	32

[a] SS expressed as SS-COD

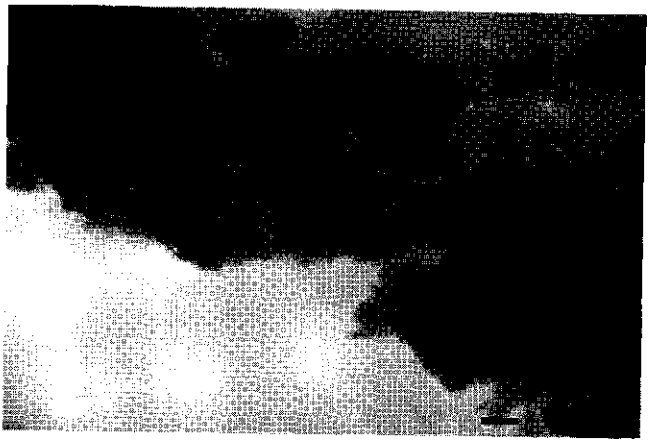
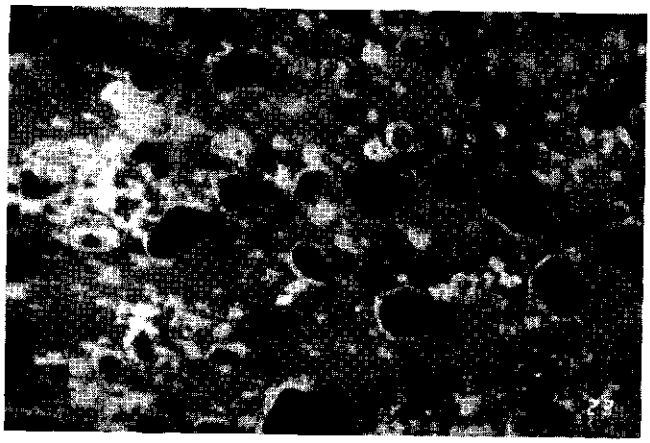


Figure 3
Example of the floating sludge nature. Sludge sampled from the recirculated SAB-fed reactor seeded with fresh granular sludge after 40 days of operation.

a: Granule agglomerate sampled from the recirculated reactor fed with SAB. The bar indicates 1 mm



b: The same agglomerate after shaking the sample. Clearly is can be seen that the agglomerate consists of individual granules. The bar indicates 1 mm

Figure 4
Surface structure of a granule after 40 days of operation in the recirculated fed-batch reactor fed with SAB. The bar indicates 10 μm.

DISCUSSION

From the results of the various experiments conducted in this study it is clear that the presence of suspended solids in the form of SAB in the influent can represent a serious limitation for performance and the loading potentials of anaerobic wastewater treatment systems like the UASB process. Despite the fact that we found little if any accumulation of dispersed SAB in the UASB reactor, the presence of SAB in the UASB influent had a serious negative impact on the system due to the strong sludge flotation induced by the SAB. Since this flotation cannot be attributed to a massive accumulation of SAB in the sludge bed nor to the incorporation of SAB in the sludge granules, it must be caused by very small amounts of some component(s) which affect the granule surface characteristics. From the fact that the floating character of the sludge still persists after 40 days of storage at 4°C, it can be concluded that the changes of the granule surface are relatively stable.

The component(s) referred to above might originate from (lysis) material(s) released during the degradation of SAB. Mulder (1990) and Mulder et al. (1989) found in experiments with anaerobic airlift loop reactors, that aggregate formation of *Selenomonas ruminantium* in these reactors is stimulated by the lysate of *S. ruminantium*. Presumably the lysate of bacterial matter, such as the SAB in the present experiments, contains a sticking component which enhances the agglomeration of individual sludge granules. However, the cell content of the SAB does not however directly initiate sludge flotation. Sludge flotation occurred in the same time period in the recirculated batch system fed with intact SAB as in the system fed with disintegrated SAB. This indicates that it is not the SAB lysate itself, as set free by ultrasonical disintegration of the SAB cells, but some biological conversed product(s) of SAB which affect(s) the granule surface characteristics and initiates the sludge flotation.

Alternatively, agglomeration of the granule might be caused by surface-oriented growth of SAB material degrading bacteria which affect the granule surface characteristics. From our observations that the penetration of suspended material and/or large molecules into the sludge granules is very limited (chapter 3), it can be concluded that the growth of SAB material degrading organisms principally must be restricted to the external regions of the granule; the substrate of these organisms simply does not reach the internal granule area. SEM microscopical examination of the floating sludge granules reveals an unusual, hairy, surface. Possibly these "hairs" are SAB degrading bacteria which "bridge" the individual granules. Morphologically these bacteria look however, very similar to *Methanothrix*.

The changes in sludge characteristics cannot explain the observed flotation phenomenon. The granule characteristics which were assessed i.e. specific gravity, ash

content, strength and activity were only slightly affected by dispersed SAB present in the UASB influent.

The sludge yield values indicate a positive effect of both the total, SAB containing, acidified sucrose feed and the soluble fraction of the acidified sucrose relative to the synthetic substrate. The values found for the sludge yield do not clearly indicate however a substantial accumulation of SAB or growth of acidogenic bacteria in the granular sludge.

Moreover, the high methanogenic activity of the sludges from the reactors fed with SAB and that of reactors fed with the soluble fraction of acidified sucrose, relative to the activity of the sludge from the reactor fed with synthetic VFA substrate, reveals the absence of any significant accumulation of non-methanogenic biomass. Such accumulation should decrease the percentage methanogens, and so decrease the specific methanogenic activity.

The relatively high methanogenic activity in the reactors fed with acidified sucrose possibly indicates that some factors present in the effluent of a sucrose acidifying CSTR stimulate the methanogenic growth. Also the more 'open' granule structure of the sludge from these reactors, as observed in the SEM micrographs, might increase the maximal activity as a result of an enhanced substrate transport.

The time interval elapsed before the first appearance of sludge flotation apparently can be correlated with the degradation of SAB by the granular sludge. From the results of both the continuous as well as the recirculated UASB experiments, we calculated that sludge flotation starts once an amount of COD_{ss} between 0.75 to 1.5 $gCOD_{ss}$ per gram granular sludge (gVSS) has been degraded. At a SAB degradation rate of approx. 0.025 $gSAB\text{-}COD_{degr}.(gVSS.d)^{-1}$, sludge flotation occurs within approx. 55 days. No flotation was observed at lower specific SAB degradation rates ($SAB_{degr}.(gVSS.d)^{-1}$). Possibly the formation of the component which causes the sludge flotation, below that degradation rate is compensated by degradation of the component by the granular sludge. Alternatively, at low SAB degradation rates, granule shear and the development of new sludge granules may counteract the adsorption of the SAB component which induces the sludge flotation.

Sludge flotation merely occurs at SAB_{COD}:COD_{total} ratios exceeding approx. 0.075. Such ratios seem to be possible especially on carbohydrate containing wastewaters because of the high acidogenic biomass yields on carbohydrates. Therefore, when two-step systems are applied to carbohydrate containing wastewaters, without removal of SAB prior to feeding to the UASB reactor, severe sludge flotation may occur which will decrease the loading rate potentials of the methanogenic reactor seriously, possibly even to values lower than those applicable in one step treatment systems.

The observed flotation of aggregates of granular sludge is related to the degradation of SAB. When the sludge flotation phenomenon indeed is caused by surface oriented degradation of SAB, a similar mechanism can be expected with various other types of biodegradable suspended solids. For this reason the removal of all the organic suspended matter from the influent prior to feeding it to the UASB reactor is highly recommended to prevent the flotation. On the other hand, also an intensive turbulence as applied in EGSB (Expanded Granular Sludge Bed) reactors may prevent the formation of large aggregates. Our experiments revealed that even a gentle agitation disrupts the aggregates formed, without affecting the individual granule characteristics.

8 THE EFFECT OF WASTEWATER PRE-ACIDIFICATION ON UASB REACTORS PERFORMANCE

This chapter consist of a modified edition of:
The effect of wastewater pre-acidification on UASB reactors performance.
P. Arne Alphenaar, Ron Sleyster and Gatze Lettinga.
Submitted for publication

8 THE EFFECT OF WASTEWATER PRE-ACIDIFICATION ON UASB REACTORS PERFORMANCE

SUMMARY

Several laboratory scale experiments were performed to investigate the influence of non-acidified and partially acidified wastewater on the process stability of UASB reactors. All reactors were seeded with methanogenic granular sludge. In addition, a model was developed to illustrate the relations between the degree of influent pre-acidification, the imposed sludge loading rate and the composition of the granular sludge present in a UASB reactor.

The experiments reveal that an influent containing non-acidified gelatin as substrate can be treated applying loading rates up to 1.2 gCOD.(gVSS.d)$^{-1}$ without any problem. An excellent granular sludge developed using this substrate.

However, non-acidified sucrose induced serious problems with regard to the process stability. The extensive growth of (chainforming) acidogenic bacteria in the reactor fluid caused an expansion of the sludge bed volume, while the sedimentation velocity of the sludge granules seriously decreased. Acidogenic biomass also covers the granular seed sludge in the sucrose-fed reactors, resulting in a decreased granular strength and gas entrapment between the (methanogenic) core, and the newly formed external layer.

The problems related to bulking sludge formation and sludge flotation in the sucrose fed reactors disappeared and the granular strength even increased when changing the substrate composition from sucrose to VFA or to gelatin. However, for the granules developed on gelatin a substrate change to VFA induced granule disintegration.

As the process stability probably is related to the growth of acidogenic bacteria, a simple model was developed to predict the composition of the organic matter of the granular sludge in relation to the degree of pre-acidification and to the sludge loading rate. The model reveals that for non-acidified sucrose a maximum sludge loading rate of approx. 0.6 gCOD.(gVSS.d)$^{-1}$ can be applied, which is in agreement with the practical results obtained.

INTRODUCTION

The upflow anaerobic sludge bed (UASB) reactor is by far the most widely applied anaerobic wastewater treatment system (Lettinga et al. 1987). Benefits of the UASB over other anaerobic systems are primarily related to autoimmobilization of bacteria into granular type of sludge (de Zeeuw 1988; Hulshoff Pol 1989; Lettinga et al. 1987; Lin and Yang 1991).

For many years the benefits and drawbacks of wastewater pre-acidification have been the subject of discussion. A high specific loading rate in the methanogenic reactor and a high process stability are reported as the main benefits for the two-step treatment (Cohen et al. 1979, 1985; Dinopoulou and Lester 1989; Komatsu et al 1991). However, previous investigations conducted in our laboratory reveal the negative effects of suspended acidogenic bacteria generated in the first reactor on the sludge retention of the second (methanogenic) reactor (chapter 7). The sludge flotation caused by these suspended acidifying bacteria strongly reduces the capacity of the second step. The poor granule formation in methanogenic reactors treating acidified wastewater (Hulshoff Pol 1989; Sam-Soon et al. 1988; Vanderhaegen et al. 1992) and the higher investment and operating costs of two-step systems (Lettinga and Hulshoff Pol 1991) can also be considered as serious drawbacks. Therefore in most situations an one-step treatment system is the preferred option.

So far little information is available about the influence of the growth of acidogenic bacteria in one phase systems on the process stability and the granule quality. Problems related to the formation of bulking sludge and sludge flotation in UASB reactors treating non-acidified sucrose were reported by Anderson et al. (1991); Hulshoff Pol et al. (1983) and Mendez-Rapin et al. (1986) at loading rates of approx. 0.6 gCOD.(gVSS.d)$^{-1}$. Hulshoff Pol et al. (1983b) and Siera Alvarez et al. (1988) described the rapid formation of a fairly weak type of methanogenic granular sludge when applying loading rates up to 0.95 gCOD.(gVSS.d)$^{-1}$. On the other hand, in experiments of Guiot et al. (1992) with UASB reactors using non-acidified sucrose, no problems were encountered when applying sludge loading rates up to 1.3 gCOD.(gVSS.d)$^{-1}$.

For non-acidified substrates, the developed biomass very likely will consist for an important fraction of acidogenic bacteria. The size of the various biomass fractions will depend on the yield and decay rates of the populations involved (table 1), and consequently on the applied loading rate and the sludge retention of the system.

The aim of the present study was to assess the effect(s) of non-acidified substrates on the performance of granular sludge containing UASB reactors. Several experiments using gelatin, sucrose and volatile fatty acids as model substrates were conducted while a calculation model was developed to illustrate the relation between substrate composition, sludge loading rate and the composition of the anaerobic sludge granules.

Table 1 Growth parameters for anaerobic bacteria under mesophilic conditions

System / population		substrate	k[a] g.(g.d)$^{-1}$	Yield[b] g.g^{-1}	Decay d^{-1}	Reference
Acid producing systems						
acid producing sludge		glucose		0.34	0.43	Eastman and Ferguson 1981[d]
" "		" "	9.5	0.40	0.79	Ghosh et al. 1975[d]
mixed culture		" "	2.5	0.54	0.87	Andrews and Pearson 1965[d]
" "		" "	179	0.17	6.1	Ghosh and Pohland 1974[c]
disperse culture	CSTR	" "	63	0.10-0.18		Zoetemeyer 1982
" "	UASB, HRT < 81 min	" "	24-33	0.12-0.17		" "
aggregates	UASB, HRT > 26 min	" "	11-19	0.06-0.08		" "
" "	Gaslift	" "	74	0.13	0.96	Beeftink 1987
disperse culture	CSTR	gelatin	81	0.085-0.14		Breure and v. Andel 1984
" "	UASB	" "	10.5	0.094		Breure et al. 1985
Methane producing systems						
Methanothrix	pure culture	acetate	3.3	0.018-0.023		Huser et al. 1982
" "	" "	" "	2.3			Zehnder et al. 1980
" "	" "	" "	2.3	0.023		Jetten et al. 1992
Sarcina	" "	Acetate	14-20	0.035		Smith & Mah 1978
" "	" "	H_2		0.136		" "
mixed culture	CSTR	acetate	5.1	0.054	0.037	Lawrence and McCarty 1969[c]
" "	" "	" "	8.5	0.04	0.036	Kugelman and Chin 1971[c]
granular sludge	UASB	" "	3.1	0.013		Dolfing 1987
mixed culture	CSTR	VFA mix	17	0.03	0.099	Lin et al. 1986
granular sludge	UASB	VFA mix		0.015-0.025	0.02	Hulshoff Pol 1989
" "	" "	" "	2.85	0.064	0.06	De Zeeuw 1984
" "	UASB	sucrose		0.12-0.15		Hulshoff Pol 1989
" "	1 step UASB	glucose	0.4			Cohen et al. 1980
" "	2 step UASB	" "	1.6-2.2	0.11		" "
		" "	0.16	0.21	0.03	Anderson and Duarte 1980[d]
" "	full-scale UASB	brewery Papermill sugar beet	0.45-1.9 0.2-0.6 1.2			Hulshoff Pol 1989
thermophilic granular sludge at 30°C					0.011	Wiegant 1986

[a] gCOD.(gVSS.d)$^{-1}$

[b] gVSS.gCOD^{-1}

[c] Sited by Pavlostathis et al. 1990

[d] Sited by Henze and Harremoës 1982

MODEL TO ESTIMATE THE COMPOSITION OF THE GRANULE POPULATION

Assumptions and limiting conditions

To quantify the effects of the sludge loading rate and the wastewater composition on the composition of the biomass (and consequently on the maximum methanogenic activity) of the granular sludge in UASB wastewater treatment systems, we used a simple model. The following assumptions and restrictions were made:

- The reactor is fed with (a mixture) two soluble substrate types; non-acidified substrate (S_a) and acidified substrate (S_m), where $S_{tot} = S_a + S_m$;
- The substrates are consumed at the maximum specific degradation rate;
- Only two populations (acidogenic bacteria and methanogenic bacteria) are involved, the sludge consists of three organic fractions; acidogenic biomass (X_a), methanogenic biomass (X_m) and an inactive organic fraction (X_i) (consisting of dead methanogenic bacteria only), where $X_{tot} = X_a + X_m + X_i$;
- The organic matter is expressed in units of COD_{VSS}. To simplify the notations, in the equations below 'VSS' is used instead of the more correct notation 'COD_{VSS}'. This implicates that in the formulas 1 gVSS ≡ 1 gCOD;
- The amount of organic material in the reactor (the VSS hold-up) is constant. Consequently, the amount of sludge discharge and sludge wash-out is equal to the sludge growth;
- The sludge bed is perfectly mixed, i.e. the composition of the excess (discharge and wash-out) granular sludge is equal to the granular sludge in the reactor;
- Suspended growing bacteria are presumed to leave the system instantaneously;
- The ratio "immobilized growth" : "suspended growth" of each of the involved population only depends on the characteristics of that population and is independent on sludge composition or process conditions.
- Lysed bacteria are considered to be non-acidified substrate which will be degraded by the acidogenic bacteria. Since organic material amounts are expressed in units of COD, the lysis rate of bacterial matter (k_d) can be expressed in the same units as a sludge loading rate (L): $gVSS.(gVSS.d)^{-1} \equiv gCOD.(gVSS.d)^{-1}$.

Calculations

The increase or decrease of the biomass fraction consisting of acidogenic bacteria, relative to the (constant) total amount of organic material present, can be calculated according equation 1. The substrate supply of the acidogenic population is determined by the amount of non-acidified substrate present in the influent and the COD amount released by lysis of bacteria present in the reactor. The increase or decrease of the biomass fraction consisting of the methanogenic population is calculated using equation 2. The substrate supply of the methanogenic population is determined by the amount of methanogenic substrate present in the reactor influent and the amount of acidified (methanogenic) substrate produced by the acidogenic bacteria.

$$\frac{\Delta X_a'}{\Delta t} = I_a . Y_a . L_a - k_{d,a} . X_a' \qquad (1)$$

where:

t	= *time*	(d)
X_a'	= X_a / X_{tot} = *acidogenic biomass fraction in the sludge*	$(gVSS_a / gVSS_{tot})$
X_i'	= X_i / X_{tot} = *inactive organic fraction in the sludge*	$(gVSS_i / gVSS_{tot})$
Y_a	= *Yield of acidogenic bacteria*	$(gVSS_a / gCOD_a)$
$k_{d,a}$	= *decay rate of acidogenic bacteria*	$(1/d)$
I_a	= *immobilised fraction of total acidogenic growth*	$(gVSS_{a,I} / gVSS_{a,tot})$
L_a	= *specific loading rate of acidogenic population*	
	= *minimum of acidogenic substrate supply and acidogenic capacity*	
	= *minimum of* $(L . \frac{S_a}{S_{tot}} + k_{d,a} . X_a' + k_{d,m} . X_i')$ *and* $(\hat{k}_a . X_a')$	$gCOD_a / (gVSS_{tot} . d))$
L	= *sludge loading rate* = $S_{tot} . Q / X_{tot}$	$(gCOD_{tot} / (gVSS_{tot} . d))$
$\hat{k}_a$	= *maximal specific activity acidogenic bacteria*	$(gCOD_a / (gVSS_a . d))$
S_{tot}	= *substrate concentration influent* = $S_a + S_m$	$(gCOD / m^3)$
$k_{d,m}$	= *decay rate methanogenic bacteria* = $k_{d,i}$	$(1/d)$
S_a	= *acidogenic substrate concentration influent*	$(gCOD_a / m^3)$
S_m	= *methanogenic substrate concentration influent*	$(gCOD_m / m^3)$
Q	= *flow rate influent*	(m^3 / d)

$$\frac{\Delta X_m'}{\Delta t} = I_m . Y_m . L_m - k_{D,m} . X_m' \qquad (2)$$

where:

X_m'	= X_m / X_{tot} = *methanogenic biomass fraction in the sludge*	$(gVSS_m / gVSS_{tot})$
Y_m	= *Yield methanogenic bacteria*	$(gVSS_m / gCOD_m)$
$k_{D,m}$	= *death rate methanogenic bacteria*	$(1/d)$
I_m	= *immobilised fraction of growth methanogenic bacteria*	$(gVSS_{m,I} / gVSS_{m,tot})$
L_m	= *specific loading rate of methanogenic population*	
	= *minimum of methanogenic substrate supply and methanogenic capacity*	
	= *minimum of* $(L.(\frac{S_m}{S_{tot}} + (1 - Y_a).\frac{S_a}{S_{tot}}))$ *and* $(\hat{k}_m . X_m')$	$(gCOD_m / (gVSS_{tot} . d))$
$\hat{k}_m$	= *maximal specific activity methanogenic bacteria*	$(gCOD_m / (gVSS_m . d))$

Changes in the inactive organic fraction of the sludge are determined by the death rate of the methanogenic bacteria ($k_{D,m}$, defined as the rate in which the activity of the methanogenic population decreases), and the decay rate of the methanogens ($k_{d,m}$, defined as the lysis rate of the methanogenic and inactive biomass). When $k_{D,m} > k_{d,m}$, inactive organic material will accumulate in the sludge granules. In view of the high lysis rate of the acidogenic bacteria (figure 1), and their almost complete degradation as was observed in previous experiments (chapter 7), accumulation of dead acidogenic bacteria in the sludge granules is neglected. The relative increase or decrease of the inactive organic fraction in the sludge therefor can be calculated according equation 3:

$$\frac{\Delta X_i'}{\Delta t} = X_m' . k_{D,m} - X_i' . k_{d,m} \qquad (3)$$

The relative increase or decrease of the organic fractions involved as an effect of variations in loading rate or substrate composition are estimated using iterative calculation of equations 1, 2 and 3. Changes in the fraction acidogenic biomass are calculated according to equation 4. The relative increase or decrease of the other sludge fractions are calculated in the same way.

$$X_{a,(t+\Delta t)}' = \frac{X_{a,(t)}' + \Delta X_a'}{1 + \Delta X_a' + \Delta X_m' + \Delta X_i'} \qquad (4)$$

In a steady state situation the composition of the sludge is constant. In the calculations a steady state situation is defined as: $\Delta X_a : \Delta X_m : \Delta X_i = X_a : X_m : X_i$

Since the sludge hold-up of the system is supposed to be constant, the sludge age (Θ) can be expressed as a function of the increase and decrease of the various (organic) fractions involved:

$$\theta = 1/\frac{\Delta X_a' + \Delta X_m' + \Delta X_i'}{\Delta t} \quad (5)$$

The maximum specific methanogenic activity of the sludge (A_m) can be calculated using:

$$A_m = \hat{k}_m \cdot X_m' \quad (6)$$

The maximum possible sludge loading rate is defined as the maximum COD supply at which all available methanogenic substrate (as present in the influent and produced by the acidogenic bacteria) is converted by the methanogens present in the sludge:

$$L.(\frac{S_m}{S_{tot}} + (1 - Y_a).\frac{S_a}{S_{tot}}) = \hat{k}_m \cdot X_m' \quad (7)$$

Finally, the efficiency of the system (E), as the percentage methane (COD) produced per gram COD fed to the system, can be calculated:

$$E = L_m \cdot (1 - Y_m) / L \cdot 100 \quad (8)$$

The simple model used in our calculations only provides a rough indication of the effects of sludge loading rate and substrate composition (changes) on the granular sludge in UASB reactors precisely. Nevertheless, the model is useful to illustrate the trends in the relation between wastewater pre-acidification, sludge loading rate, granular sludge growth and treatment efficiency, as is shown in figure 2.

MATERIALS AND METHODS

Reactors

Plexiglass UASB reactors (3.2 litre liquid volume) with an external phase separator were used. Three separate influent flows were applied; the organic substrate stock solution, the nutrient/trace element solution and the tap water were mixed just before the reactor inlet (chapter 2, figure 1a). The experiments were conducted in a temperature controlled room (30 $\pm$ 1°C), the organic substrate was held at 4°C to prevent pre-acidification.

Biomass

The reactors were seeded with granular sludge originating from a full-scale reactor processing wastewater of an alcohol producing industry (Nedalco, Bergen op Zoom, The Netherlands). The sludge was elutriated (upward velocity 15 $m.hr^{-1}$) before use. The methanogenic activity decline during unfed storage was assessed using granular sludge originating from a full scale reactor treating wastewater of a starch factory (Latenstein, Nijmegen, The Netherlands).

Media

The mineral medium (trace elements and nutrients) used is given in chapter 2. Sucrose and gelatin were used as non-acidified substrates. Pre-acidification was simulated by adding a mixture of acetate, propionate and butyrate (1:1:1, based on COD-values) to these substrates.

Methods

The decay rate of the acidogenic bacteria at 30°C, defined as the lysis rate or mineralization rate, was determined using 2.5 litre, intermittently stirred batch reactors (30 s pulse, 180 s pause), temperature controlled at 30°C and using concentrates of suspended acidifying bacteria present in the effluent of a sucrose acidifying CSTR reactor (chapter 7). The decay rate was calculated from the daily monitored increase of soluble COD in the reactor fluid. Similar tests were performed in the presence of granular sludge (approx. 2 $gVSS.l^{-1}$). In these tests the decay rate was calculated from the increase of soluble COD and the decrease of suspended COD in the reactor fluid and the methane production rate.

The death rate of the methanogenic population in granular sludge (defined as the irreversible loss of methanogenic activity) was calculated from the decrease of methanogenic activity during unfed storage. A number of one litre serumflasks containing approx. 2 $gVSS.l^{-1}$ and the nutrient/trace element solution applied in the activity tests (chapter 2) were stored at 30°C. The methanogenic activity of the stored sludge was assessed within intervals by adding two subsequent VFA feedings to one of the bottles, and measuring the methane production rate (see activity tests, chapter 2). The fed bottles were not used in further tests.

Set-up of the UASB experiments

This chapter gives an overview of the results of lab-scale UASB experiments with regard to the influence of (partly)acidified and non-acidified substrates on the quality and the functioning of granular sludge. In all experiments the same reactors and the same methanogenic granular seed sludge was used. Several individual reactor

experiments and experimental series were performed. The main characteristics of these were the application of:

- various ratios of gelatin / sucrose in the influent applying constant loading rates and influent concentrations;
- various ratios of sucrose / VFA in the influent in order to simulate the effect pre-acidification;
- various sludge loading rates and hydraulic retention times were applied in reactors fed with non-acidified gelatin or sucrose;
- changes in substrate composition from gelatin to sucrose or VFA and from sucrose to gelatin or VFA were applied at constant loading rate and constant HRT.

The presentation of the relevant data with respect to the experimental set-up is coupled to the results to avoid indistinctness.

RESULTS

Experimentally assessed death- and decay rates

The results of several lab-scale studies at our laboratory, and experiences with full scale UASB reactor treatment systems, reveal that growth and decay of acidogenic bacteria significantly affects the granular sludge quality and the reactor performance. In order to be able to predict the influence effect of (changes in) process conditions on the granule quality, particulary also adequate knowledge of the decay rates of all relevant populations present in the granule is essential. However, so far only little reliable data are available in the literature (table 1). For that reason, in this study we assessed the decay rate of suspended, sucrose acidifying bacteria, and the death rate of methanogenic bacteria present in the granular sludge. The decay rate assessed for acidifying bacteria ranged from 0.07 d^{-1} to 0.25 d^{-1} possibly related to the process conditions in the CSTR. Figure 1 presents the most typical values, approx. 0.1 d^{-1} - 0.15 d^{-1}, which is in the same order of magnitude as reported in the literature (table 1). As illustrated in figure 1a and 1b, no big differences are observed for the decay rate measured as CH_4 production rate (in presence of granular sludge) or the decay rate determined as the increase of soluble COD (in absence of granular sludge).

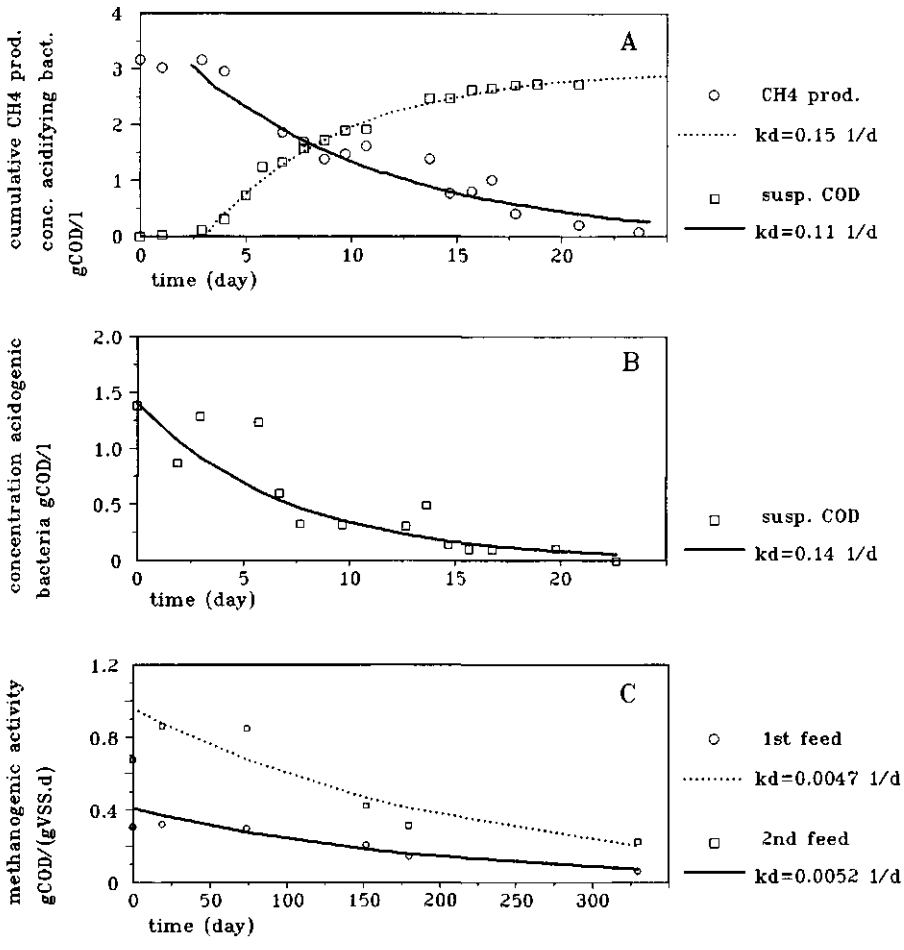


Figure 1 The results of batch assays to estimate the decay rate of suspended acidogenic bacteria and the death rate of the methanogenic bacteria in granular sludge from a full-scale UASB reactor (symbols). The lines are calculated according to the equation $\ln (X_t / X) = -k_d.t$, in which X is activity or concentration.

a: The lysis rate of the suspended acidifying bacteria measured both as methane production (gCH_4-COD.$(l.d)^{-1}$) and as suspended COD decreases (gCOD.$(l.d)^{-1}$) in stirred batch experiments seeded with approx. 2 g VSS.l^{-1} granular sludge. The suspended bacteria used were developed in a acidifying CSTR reactor, as discussed in chapter 7).

b: The lysis rate of the suspended acidifying bacteria was measured as the decrease in suspended COD (gCOD.$(l.d)^{-1}$) in stirred batch experiments without granular sludge.

c: Methanogenic death rate measured as methanogenic activity decrease during unfed storage at 30°C.

The death rate of the methanogenic population in anaerobic granular sludge was assessed from the decline in methanogenic activity during non-fed storage at 30°C (figure 1c). A death rate value of approx. 0.005 d^{-1} was calculated, which is one order of magnitude lower than the values reported in literature. However, this low death rate is in agreement with the high mechanical stability and the slow decrease of methanogenic activity of granular sludge for periods up to several years (Hulshoff Pol (Hulshoff Pol et al. 1983; Hulshoff Pol 1989; Wu et al. (1985). According to de Zeeuw (1984), the decay rate of the methanogenic population (defined as the lysis rate of the biomass) in the granular sludge can be presumed to be 1/5 of their death rate (defined as the rate at which the activity of the population decrease). Due to this discrepancy in death rate and decay rate, inactive biomass will accumulate in the granules when low loading rates are applied.

Calculation of the composition of the biomass in the granule

The fraction acidogenic bacteria in the granules may represent one of the main factors affecting reactor performance and granular sludge quality. In order to illustrate the influence of the sludge loading rate and extent of wastewater pre-acidification applied on the fraction of the acidogenic population in the granular sludge, the simple model presented above in this chapter can be used. Figure 2 presents the relation between the reactor performance and the granule composition for a reactor fed with a non-acidified substrate at a steady state situation. The data used in the calculations are presumed to be typical for the degradation of a non-acidified sucrose containing wastewater in a UASB reactor (table 2).

Table 2 Assumptions used in the model presented before to estimate the relations between the sludge loading rate, the degree of wastewater pre-acidification and the composition of the granular sludge.

Yield	$gVSS_{COD}.gCOD^{-1}$	0.18	acidogens
		0.03	methanogens
immobilized growth	%	100	both populations
specific activity	$gCOD.(gVSS_{COD}.d)^{-1}$	75	acidogens
		2.3	methanogens
decay rate	d^{-1}	0.1	acidogens
		0.001	methanogens
death rate	d^{-1}	0.005	methanogens

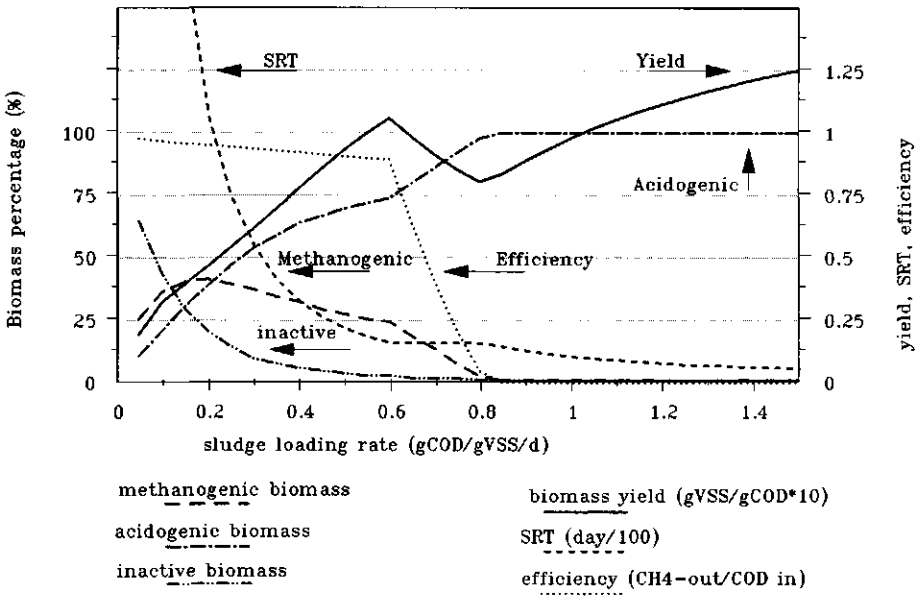


Figure 2 The estimated relations between the imposed sludge loading rate, the sludge retention time, the total granule yield and the composition of the granular biomass, for a UASB reactor treating non-acidified (sucrose containing) wastewater, using the assumptions given in table 2.

When a methanogenic granular seed sludge is fed with non-acidified substrate, the specific methanogenic activity of the sludge will decrease due to an increase of the fraction acidogenic bacteria in the total granule biomass. As shown in figure 3 the methanogenic sludge activity in a lab-scale reactor seeded with granular sludge from a full-scale UASB significantly decreases when the reactor is fed with non-acidified sucrose. The assessed methanogenic activity decrease correlated quite well with the calculations concerning the increase of the acidogenic population in this reactor.

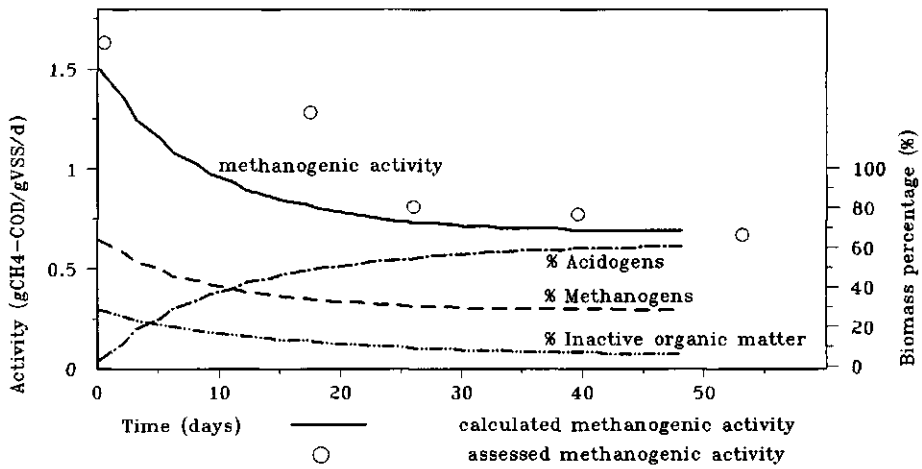


Figure 3 The maximum methanogenic activity decrease of granular sludge from a full-scale UASB reactor used as seed material of a lab scale UASB reactor fed with non-acidified sucrose. (HRT = 1.5 hr, sludge loading rate = 0.4 gCOD.(gVSS.d)$^{-1}$, influent concentration = 400 mgCOD.l^{-1}). In the calculations, the granule composition at the start was chosen as 65% methanogens, 5% acidifying bacteria and 30% inactive organic material. The lines are calculated according the models developed using the assumptions presumed to be typical for a non-acidified sucrose wastewater treating UASB reactor (table 2).

Estimation of the maximum sludge loading rate

The fraction acidogenic biomass in the sludge granules positively correlates with both the imposed sludge loading rate and the percentage non-acidified substrate present in the wastewater. The specific methanogenic activity, and consequently the maximum possible sludge loading rate, of an one-phase system so depends on the degree of wastewater pre-acidification. The relation between the degree of wastewater pre-acidification and the maximum possible sludge loading rate is illustrated in figure 4.

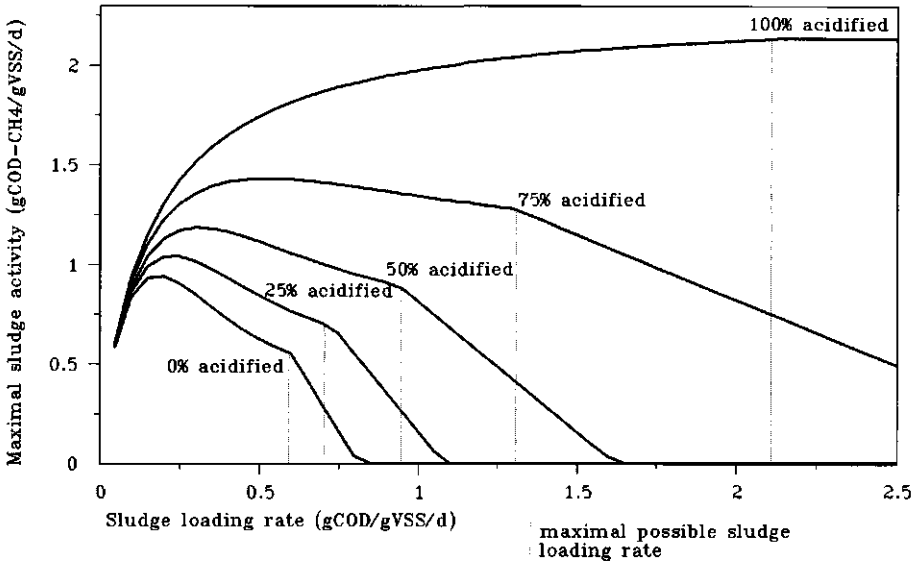


Figure 4 The calculated maximum possible loading rates and the relation between loading rate and the specific methanogenic sludge activity for various degrees of wastewater pre-acidification. The lines are calculated according the formulas described before, using the data given in table 2.

Assessed granule growth

The results of several lab-scale UASB experiments reveal that the composition of the non-acidified substrate affects the growth and the functioning of granular sludge. The assessed yield of granular sludge on non-acidified sucrose appears to be significantly higher than the yield on non-acidified gelatin, which in turn is higher than on VFA. The average sludge growth data over the experiments are summarized in table 3.

Table 3 Average granular sludge yield in UASB reactors using different substrates under conditions of moderate sludge loading rate (0.4 - 0.6 gCOD.(gVSS.d)$^{-1}$), HRT: 2.2 - 4 hr.

COD-source	100% VFA	100% Sucrose	100% gelatin	25 - 30% sucrose 70 - 75% VFA
granule yield ($gVSS.(gCOD_{degraded}.d)^{-1}$)	0.03	0.11	0.06	0.05

Granule quality

An excellent type of granular sludge developed in a reactor fed with gelatin as substrate. The granules grown on gelatin were homogeneous in structure, and both the strength and methanogenic activity of the granules remained unaffected or even improved relative to the seed sludge. This good performance with respect of granular sludge growth was contrary to the observations of Breure (personal communication) using the same substrate, but similar to the results found by Schulze et al. (1988).

Feeding granular sludge with non-acidified sucrose clearly reduces the granular sludge quality with regard to granular strength, settleability and methanogenic activity. Also in experiments in which the reactors were fed with mixtures of sucrose and gelatin, it was found that sucrose was detrimental for the granular sludge quality (table 4, figure 5). As illustrated in figure 6, fluffy attached layers (of sucrose acidifying bacteria) cover the original seed granules. As a consequence, the granular strength dropped to 5% of the initial value. In some cases entrapment of gas between the external layer and the (methanogenic) interior initiated granule flotation figure 6c).

Table 4 Assessed values of the granular sludge strength and the methanogenic activity for granular sludge grown for 55 days in UASB reactors on feeds containing different fractions of gelatin and sucrose, applying moderate loading rates (0.4 - 0.6 gCOD.(gVSS.d)$^{-1}$, influent concentration 1.1 - 2.5 gCOD.l^{-1}, HRT 2.5 - 5 hr).

Substrate	100% S	90% S, 10% G	50% S, 50% G	10% S, 90% G	100% G
activity[b]	0.05 - 0.8[c]	0.6	0.7		1.4
strength[d]	<5	37	85	65	100

[a] S = sucrose, G = gelatine (in % of COD total)

[b] Methanogenic activity of the granular seed sludge: 1.0 gCOD.(gVSS.d)$^{-1}$

[c] In most experiments values of approx. 0.7 were found. The low value is probably due to a is serious underestimation of the sludge loading rate applied in the reactor due to sludge wash-out and poor mixing.

[d] Granular strength in percentage of start

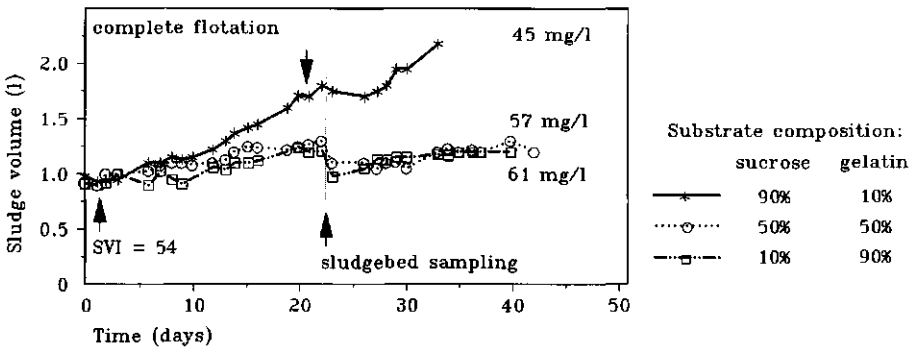


Figure 5 Sludge bed volume increase due to granule growth and bed expansion (expressed as the sludge density, mgVSS.ml^{-1}) related to the substrate composition. Sludge loading rate approx. 0.5 gCOD.(gVSS.d)$^{-1}$, HRT = 4.4 hr. influent concentration approx. 2.3 gCOD.l^{-1}

Reactor performance

UASB reactors using gelatin as substrate could be operated without any problem for several months, applying influent concentrations between 0.5 and 4.6 gCOD.l^{-1} and hydraulic retention times between 10 hr and 2.2 hr. Loading rates up to 1.2 gCOD.(gVSS.d)$^{-1}$ could be applied within three weeks after the start-up using granular sludge as seeding material.

Contrary to the non-acidified gelatin, serious problems manifested when UASB reactors were fed with non-acidified sucrose. In these reactors a massive sludge flotation and a pronounced sludge-bed expansion occurred. Dispersed and fluffy attached growth of chain forming acidogenic bacteria seriously hamper granule sedimentation, resulting in a low sludge density (a high sludge volume index, SVI). This effect clearly was related to the non-acidified sucrose in the influent (figure 5). In addition, also the gas transport through the sludge bed was seriously hindered, which led to the formation of large gas bubbles in the sludge bed which dragged the sludge to the top of the reactor. In some of the experiments the major part of the biomass was present in floating sludge layers. The extent of that sludge flotation is illustrated in figure 7.

Figure 6

a: Example of the external layer developed when granular sludge was fed with 30% non-acidified sucrose during 30 days. Sludge loading rate: 0.5 gCOD.(gVSS.d)$^{-1}$, HRT 5.5 hr. Bar indicates 1 mm.

b: Granular sludge fed with 25% non acidified sucrose during 55 days. Sludge loading rate 1.2 gCOD.(gVSS.d)$^{-1}$, HRT 9.4 hr. Bar indicates 1 mm.

c: Sludge washed-out from a papermill wastewater treating UASB reactor. Gas entrapment in methanogenic granules covered with newly formed (acidogenic) biomass. Bar indicates 1 mm.

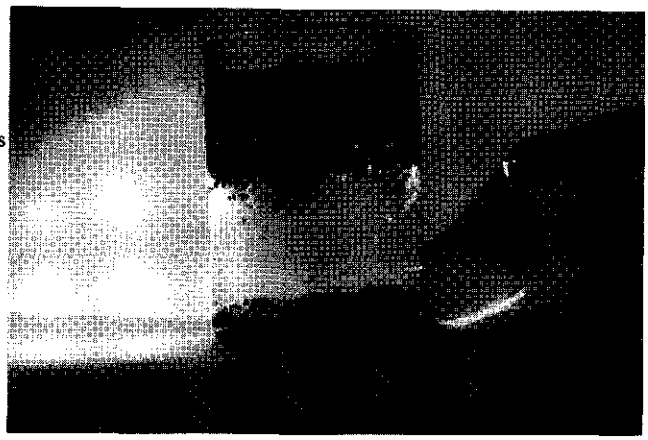


Figure 7
Sludge flotation as occurring in a sludge sample from a reactor fed with 30% non-acidified sucrose, five minutes after shaking. Sludge load of the reactor: 0.55 gCOD.(gVSS.d)$^{-1}$, HRT 4.2 hr.

The problems related with sludge flotation in the sucrose fed reactors were observed at imposed sludge loading rates of 0.5 gCOD.(gVSS.d)$^{-1}$ and higher, and at $COD_{sucrose}$: COD_{total} ratios exceeding 0.3. Although no clear relation was found, the problems became more serious at higher loading rates and sucrose percentages for all experiments conducted at hydraulic retention times exceeding 2.5 hour. In experiments conducted at a HRT as short as 1.5 hr, no sludge flotation was observed, even when a loading rate of 0.8 gCOD.(gVSS.d)$^{-1}$ of non-acidified sucrose was imposed.

The effect of changes in substrate composition

It is obvious that sudden changes in process conditions and/or in wastewater composition, as generally occur during start-up of reactors seeded with granular sludge, will alter the functioning of the granular sludge. To assess the effect of a change in the substrate composition some experiments were carried out.

A change of the substrate composition from sucrose to VFA or to gelatin affects the sludge bed behaviour. The sludge flotation and sludge bed expansion vanish within two days and the sludge density increased from approx. 0.045 gVSS.ml^{-1} with sucrose as feed to approx. 0.070 gVSS.ml^{-1} when the reactor was fed with a VFA mixture. The change of the substrate composition also resulted is a significant wash-out of dispersed biomass. The biomass content in the reactor declined approx. 25% within a few days after the change. The granular strength remained unaffected or even improved.

In the reactors originally fed with gelatin, a change of the substrate composition to sucrose or to VFA did not induce a wash-out of biomass at the short term. However, on longer term a completely acidified substrate induces granule deterioration, as was observed after a sudden loading rate decrease (chapter 6).

DISCUSSION

Irrespective of the occurrence of sludge flotation and other problems related to biomass retention, the maximum possible loading rate of an one-step anaerobic wastewater treatment reactor depends on the percentage of methanogenic biomass present. Both calculations as well experimental results reveal the possibility of treating non-acidified wastewater in one-step UASB reactor systems. However, when the yield of the involved acidogens is high relative to the methanogenic population present, a successful wastewater treatment is feasibly only at moderate sludge loading rates.

Besides the net growth of the acidogenic bacteria, as a result of yield and decay rate, the fraction of immobilized growth will affect the contribution of the relevant populations. The attached growth of acidogenic bacteria directly affects the maximum loading rate of one-step reactors treating non-acidified wastewater. The calculation of the maximum possible sludge loading rate is based on the ratio methanogenic biomass / total biomass in the system. With respect to this, the model does not discriminate between granular biomass and (acidogenic) biomass which is immobilized in the space between the granules.

The performance of the laboratory-scale reactors however was significantly affected by the way in which the acidogenic biomass was fixed in the system. Even when sufficient methanogenic activity was present, the growth of non-granular (suspended or loosely attached) acidogenic bacteria in the reactor hampers the reactor performance strongly. When short HRTs were applied to reduce the retention of suspended or loosely attached acidogenic bacteria, sludge flotation did not occur. The yield of granular sludge in these experiments increased relative to experiments using a longer HRT. Possibly the short HRTs stimulate the granular growth of the acidogenic bacteria, and prevent abundant suspended growth. The latter observations correlate with those of Guiot et al. (1992) and with the earlier results obtained in our laboratory (de Zeeuw, 1984, Hulshoff Pol, 1992) were a positive effect of very high upward velocities was found.

In many experiments using sucrose containing influents the quantity of sludge in the reactor could be assessed only roughly due to sludge bed expansion. Probably because of the resulting inaccuracy in the calculated sludge loading rate, only a qualitative relation between the sludge loading rate and the occurrence of flotation was found.

When the substrate composition is changed, in particular the acidifying population will decline. Consequently, granular sludge consisting of a relatively high percentage of acidogens will be sensitive to changes in substrate type and loading rate. The

different effects of a change in substrate composition for gelatin fed and sucrose fed granules possibly are explained by the differences in granule structure.

The granules in the sucrose fed reactors show a strong segregation in an internal and an exterior granule region. A change in substrate composition will merely affect the exterior shell of (acidogenic) biomass. However, the methanogenic granule cores, and so the methanogenic capacity of the reactor, remain unaffected. The gelatin acidifying population possibly is more homogeneously distributed over the granule than the sucrose acidifying population. Deterioration of the acidifying population thus does not directly induce a rapid wash-out of an outer-shell. However, on longer term the change in substrate composition will affect the granule stability more seriously.

For reactor start-up, granular sludge with a minimal content of acidifying bacteria is preferred because of the higher stability against substrate composition changes.

9 THE EFFECT OF LIQUID UPWARD VELOCITY AND HYDRAULIC RETENTION TIME ON GRANULATION IN UASB REACTORS TREATING WASTEWATER WITH A HIGH SULPHATE CONTENT

This chapter consists of a modified edition of:

The effect of liquid upward velocity and hydraulic retention time on granulation in UASB reactors treating wastewater with a high sulphate content
P. Arne Alphenaar, André Visser and Gatze Lettinga.
Bioresource technology 43:(1993) 249-258

9 THE EFFECT OF LIQUID UPWARD VELOCITY AND HYDRAULIC RETENTION TIME ON GRANULATION IN UASB REACTORS TREATING WASTEWATER WITH A HIGH SULPHATE CONTENT

ABSTRACT

The effect of hydraulic retention time and liquid upward velocity on the granulation process and the competition between sulphate-reducing and methane-producing bacteria during anaerobic treatment of sulphate-containing wastewater was studied.

The results showed that hydrogen, generated during the anaerobic mineralization process, was completely oxidized by sulphate-reducing bacteria. Acetate was oxidized by both sulphate-reducing and methane-producing bacteria. The fraction of acetate used by sulphate reducers relative to methanogens increased with time, resulting in a predominance of sulphate-reducing bacteria, especially at relative long hydraulic retention time (40 hr).

The granulation process was favoured by the combination of high upward velocity and short hydraulic retention time. Very thin filaments (possibly sulphate reducers) may serve as primary nuclei for the attachment of *Methanothrix*, which starts the granulation process. No difference between attachment capacity of sulphate-reducing and methanogenic bacteria was found.

INTRODUCTION

During the past two decades anaerobic treatment systems have been developed for the purification of large wastewater streams, e.g. the fluidized bed reactor (FB), anaerobic filter (AF) and the upflow anaerobic sludge blanket (UASB) reactor, (e.g. van den Berg 1984; Lettinga et al. 1984, 1987; de Zeeuw 1988).

In these types of reactors anaerobic bacteria are immobilized on inert solid particles such as sand, plastic, etc. (FB, AF), or by a process of spontaneous aggregation of the bacteria to dense compact granules with high activity and good settling properties (Lettinga et al. 1984; de Zeeuw 1988). Owing to the immobilization of the anaerobic biomass within the system biomass concentrations are high and, consequently, high loading-rates can be applied in anaerobic treatment systems. Several industrial wastewaters have high concentrations of sulphate, sulphite or other S-compounds, e.g. fermentation industry-, edible oil industry- and paper industry-wastewaters (Rinzema and Lettinga 1988).

In the anaerobic degradation of these wastewaters both sulphate reduction and methanogenesis can be the final step in the anaerobic degradation pathway. Sulphate-reducing bacteria (SRB) as well as methane-producing bacteria (MPB) are capable of using acetate and hydrogen as substrates, resulting in a competition between SRB and MPB for hydrogen and acetate.

Table 1 lists thermodynamic and kinetic data of SRB and MPB for the oxidation of acetate and hydrogen. It is clear from table 1 that,based on thermodynamic and kinetic data, for both acetate and hydrogen SRB should be able to out-compete MPB. This prediction has been confirmed by several studies on marine and freshwater sediments (Winfrey and Zeikus 1977; Banat 1981; Smith and Klug 1981; Zaiss 1981; Lovley et al. 1982; Lovley and Klug 1986). Also, research results from anaerobic digesters show that SRB will outcompete MPB for hydrogen (Mulder 1984; Rinzema et al. 1986). For acetate the situation is less clear. Several researches have observed that acetate can be completely converted into methane (Hoeks et al. 1984; Mulder 1984; Rinzema and Lettinga 1988), whereas other results showed that SRB can compete with MPB for acetate (Rinzema and Schultz 1987).

For anaerobic treatment systems, a low HRT/SRT ratio (hydraulic retention times/solid retention times), and therefore biomass immobilization is essential. In these high-rate reactors, beside thermodynamic and kinetics parameters the occurrence of immobilization will control the competition between SRB and MPB. A selective wash-out of SRB was suggested as a selection mechanism for acetotrophic methanogens (Isa et al. 1986a, 1986b). According to Yoda et al. (1988) acetotrophic SRB growing in a biofilm have a lower growth-rate and K_s-value than MPB. Therefore, at low and high acetate-concentrations a predominance of SRB and MPB, respectively might be expected. Other kinetic data from the literature (table 1) however, do not confirm these findings.

The possibility of controlling the competition between SRB and MPB could be very important in anaerobic treatment of sulphate containing wastewater. For instance, sulphide production must be minimized since the presence of the toxic (hydrogen) sulphide can cause potential problems (corrosion, malodour, toxicity). On the other hand, sulphate reduction followed by conversion of sulphide into elemental sulphur can be used to remove sulphate or other S-compounds from wastewater.

The aim of the present study was to investigate the influence of different process-technological conditions in UASB-reactors on biomass immobilization and competition between MPB and SRB.

Table 1: Thermodynamic and kinetic data of sulphate-reducing bacteria (SRB) and methane-producing bacteria (MPB) for oxidation of hydrogen and acetate

Thermodynamics		ΔG^o	Ref.
$4\ H_2 + SO_4^{2-} + H^+$	$\rightarrow HS^- + 4\ H_2O$	− 38	Thauer et al. (1977)
$4\ H_2 + HCO_3^- + H^+$	$\rightarrow CH_4 + 3\ H_2O$	− 32.7	Thauer et al. (1977)
$CH_3COO^- + H_2O$	$\rightarrow CH_4 + HCO_3^-$	− 28.2	Thauer et al. (1977)
$CH_3COO^- + SO_4^{2-}$	$\rightarrow HS^- + 2\ HCO_3^-$	− 39.5	Thauer et al. (1977)

Kinetics, hydrogen	K_s (μM)	μ_m (d^{-1})	Y ($gVSS.mol^{-1}$)	pH	T (°C)	Ref.
SRB						
Desulfovibrio Vulgaris		5.52	1.0–1.25	7.2	35	Badziong and Thauer (1978)
	4		1.1	7.0	30	Lupton and Zeikus (1984)
Desulfovibrio sp.	3.3	1.37	0.85	6.7	37	Robinson and Tiedje (1984)
Desulfovibrio gigas		1.37	1.75–2.0	7.0	35	Brandis and Thauer (1981)
MPB						
Methanobacter formicicum	2	2.0	0.8			Schauer and Ferry (1980)
Methanobacter Hungatei	6.6	1.2	0.2	6.7	37	Robinson and Tiedje (1984)
Methanobacterium sp.	14		0.6	7.0	30	Lupton and Zeikus (1984)

Kinetics, acetate	K_s mM	μ_m (d^{-1})	Y ($gVSS.mol^{-1}$)	pH	T (°C)	Ref.
SRB						
Desulfobacter Postagei	0.23	1.03	2.56		28	Brandis-Heep et al (1983)
Desulfotomaculum acetoxidans		0.55	5.52	7.1	36	Widdel and Pfennig (1977)
Desulfonema limicola		0.55		7.6	30	Widdel (1980)
mixed culture	0.10	0.51	3.72		31	Middleton and Lawrence (1977)
MPB						
Methanothrix soehgenii	0.44	0.11	1.47	7.6	37	Huser (1980)
Methanosarcina Barkeri	4.02	0.21			37	Wandrey and Aivasisdis (1983)
mixed culture	6.39	0.24	3.24		30	Lawrence and Mc Carty (1969)

METHODS.

Reactors

Three PVC UASB reactors with a liquid volume of 1.1 litres (height 40 cm, internal diameter 6 cm) were used (figure 1c, chapter 2). To increase the HRT, one reactor was placed in series with a continuously-stirred, 5 litre, PVC, tank reactor (figure 2c, chapter 2). Effluent recycling was applied in the UASB/CSTR (continuous stirred tank reactor) system as well as in one of the single UASB systems (flow 40 $l.d^{-1}$), in order to increase the upflow velocity. In table 2 the general data for the three experimental arrangements are summarized.

The reactors were placed in a temperature controlled room (30 ± 1 °C), the influent was stored at 4 °C to prevent premature degradation. Methane production was measured by a wet gas meter (Meterfabriek Dordrecht, Dordrecht, the Netherlands.) placed after a 3% NaOH solution and a column of soda-lime pellets to remove CO_2 and H_2S.

Table 2 General Data for reactors 1-3.

Reactor type	UASB	UASB	UASB + CSTR
Recirculation factor[a]	0	10	10
Hydraulic retention time (h)	7.5	6.9	40.2
Upwards velocity ($m.hr^{-1}$)	0.05	0.65	0.65

[a] recirculation flow / influent flow

Biomass

Granular sludge, crushed under anaerobic conditions by frequent pressing through a syringe needle, was used to inoculate the reactors. Twenty per cent of the sludge was adapted to a mixture of acetate, propionate, butyrate and sulphate (COD 2.5 $g.l^{-1}$, SO_4^{2-} 1.25 $g.l^{-1}$, C2:C3:C4 = 5:3:2 on COD-values), while 80% of the sludge originated from a full scale UASB treating distillery wastewater (Nedalco, Bergen op Zoom, The Netherlands).

Media

The reactors were fed with a medium containing nutrients and trace elements as described in chapter 2. 5000 $mg.l^{-1}$ SO_4^{2-} was added as Na_2SO_4. All chemicals were of analytical grade and provided by Merck AG (Darmstadt, Germany), except the Oxoid yeast extract which was supplied by Unipath Ltd (Basingstoke, England). The organic

substrate concentration was 2500 mgCOD.l^{-1}; (acetic acid (50%), propionic acid (40%) and sucrose (10%)). The influent had pH 6.8. The COD to sulphate ratio in the influent ratio was 0.5. Tap water was used for all media.

Analysis

All analysis are described in chapter 2.

Calculations

From the data obtained, the amounts of organic-COD and the amount of acetate and hydrogen used by SRB and MPB were calculated. The substrate used consisted of acetate, propionate, sucrose and sulphate. The various degradation reactions for the organic substrates present in the feed are shown in table 3.

Table 3 Anaerobic degradation reactions

Reaction	*reaction number*
$C_{12}H_{22}O_{11} + 5H_2O \rightarrow 4CH_3COOH + 4CO_2 + 8H_2$	(1)
$CH_3CH_2COO^- + 2H_2O \rightarrow CH_3COO^- + HCO_3^- + H^+ + 3H_2$	(2)
$CH_3CH_2COO^- + 0.75SO_4^{2-} \rightarrow CH_3COO^- + HCO_3^- + 0.75HS^- + 0.25H^+$	(3)
$CH_3CH_2COO^- + 1.75SO_4^{2-} \rightarrow 3HCO_3^- + 1.75HS^- + 0.5H^+ + 0.25OH^-$	(4)
$CH_3COO^- + SO_4^{2-} \rightarrow 2HCO_3^- + HS^-$	(5)
$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$	(6)
$4H_2 + SO_4^{2-} + H^+ \rightarrow HS^- + 4H_2O$	(7)
$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 4H_2O$	(8)

Oxidation of propionate can proceed via acetogenic oxidation (reaction 2) or a direct oxidation of propionate by SRB (reactions 3 and 4). Using only mass balances no distinction between these reactions can be made. It is easily seen that an incomplete oxidation of propionate by SRB (reaction 3) will give the same balance as the acetogenic oxidation of propionate followed by hydrogen oxidation by SRB (reactions 2 and 7, respectively). By analogy, a complete oxidation of propionate by SRB (reaction 4) will give the same balance as the acetogenic oxidation of propionate, followed by acetate and hydrogen oxidation by SRB (reactions 2, 5 and 7, respectively). In the rest of this paper we therefore will speak only of acetate or hydrogen oxidation by SRB,

which can be either a direct oxidation (reaction 5 and 7) or an indirect oxidation (reactions 3 and 4).

The equations used to calculate the amount of organic-COD, acetate-COD and hydrogen-COD used by either SRB and MPB are shown in the appendix. To calculate the amount of organic-COD, acetate-COD and hydrogen-COD degraded by either SRB and MPB, we made the following assumptions:

- The biological conversion reactions describing the system are according to table 3.
- SRB primarily use hydrogen as substrate. If the amount of sulphate reduced exceeds the amount of hydrogen converted the difference is accounted for as acetate oxidation by SRB.
- MPB primarily use acetate as substrate. If the amount of methane produced exceeds the amount of acetate converted, the difference is accounted for as hydrogen oxidation by MPB.

The last 2 assumptions are based on literature studies (Mulder 1984; Rinzema and Lettinga 1988; Rinzema and Schultz 1987). It is generally found that in anaerobic treatment of wastewater containing excess sulphate all hydrogen is used by SRB, whereas acetate will be partly or totally oxidized by MPB.

The sludge amounts in the reactor between the samplings, and the total sludge yield were calculated according to equation 1 and 2:

$$Y_{tot} = \frac{X_{t_2} - X_{t_1} + \sum_{t_1}^{t_2} X_{discharge} + \sum_{t_1}^{t_2} (VSS_{effl}\ \phi)}{\sum_{t_1}^{t_2} [(COD_{infl} - COD_{effl})\phi]}\ 100 \qquad (1)$$

$$X_t = X_{t_1} - \sum_{t_1}^{t} [VSS_{effl}\ \phi] - \sum_{t_1}^{t} X_{waste} + \sum_{t_1}^{t} [(COD_{infl} - COD_{effl})\ \phi\ \frac{Y_{tot}}{100}] \qquad (2)$$

where:

Y_{tot}	=	*Net total Sludgeyield between* t_1 *and* t_2	(%)
X_t, X_{t1}, X_{t2}	=	*Calculated sludge biomass in reactor at time t,* t_1 *and* t_2	(*g VSS*)
$X_{discharge}$	=	*sludge biomass discharged*	(*g VSS*)
X_{waste}	=	*sludge biomass washout*	($g.VSS$)
VSS_{effl}	=	*Volatile suspended solid concentration in effluent*	($g.l^{-1}$)

RESULTS

During the start-up period (days 0-50) the sludge loading rates in the three reactors were gradually increased from 0.25 to approx. 1 gCOD.(gVSS.d)$^{-1}$. During this period reactor 1 became accidently overloaded (days 45-49, loading rate approx. 2 gCOD (gVSS.d)$^{-1}$. Average performance values for the reactors from day 50 onwards are summarized in table 4. The ratios of the calculated values of organic-COD converted by SRB relative to organic-COD concerted by MPB for reactors 1, 2 and 3 are shown in figure 1.

Table 4 Performance data for the reactors, average values over day 50-150.

Sludge loading rate gCOD.(gVSS.d)$^{-1}$	1.06	0.98	1.01
Organic-COD-removal rate gCOD.(gVSS.d)$^{-1}$	0.79	0.73	0.79
Acetate removal rate[a] gC2-COD.(gVSS.d)$^{-1}$	0.66	0.61	0.67

[a] Hydrogen and acetate consumption calculated according equation 5 and 6 of the appendix

The calculated ratio of organic-COD converted by SRB relative to that converted by MPB in the sludge activity tests differs from the ratios observed during the reactor running. For all reactors, the ratios of organic-COD and acetate-COD degraded by SRB relative to MPB observed at the end of the experiment are shown in figure 2a and 2b, respectively.

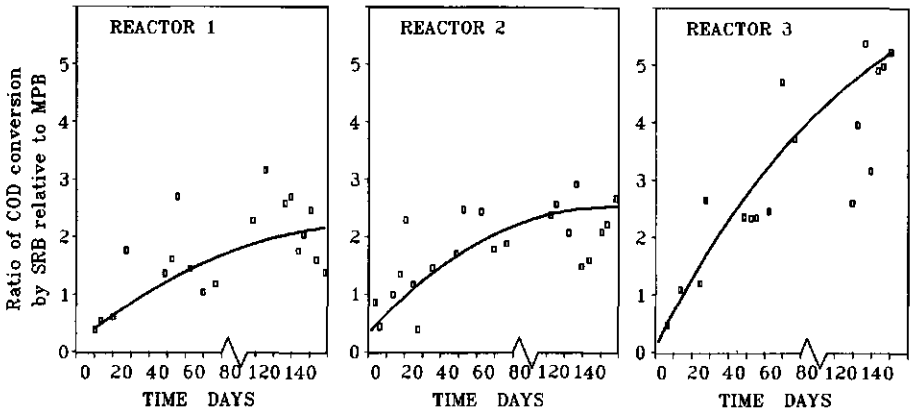


Figure 1 The ratio of organic-COD converted by sulphate-reducing bacteria (SRB) relative to COD conversion by methane-producing bacteria (MPB) for reactors 1-3.

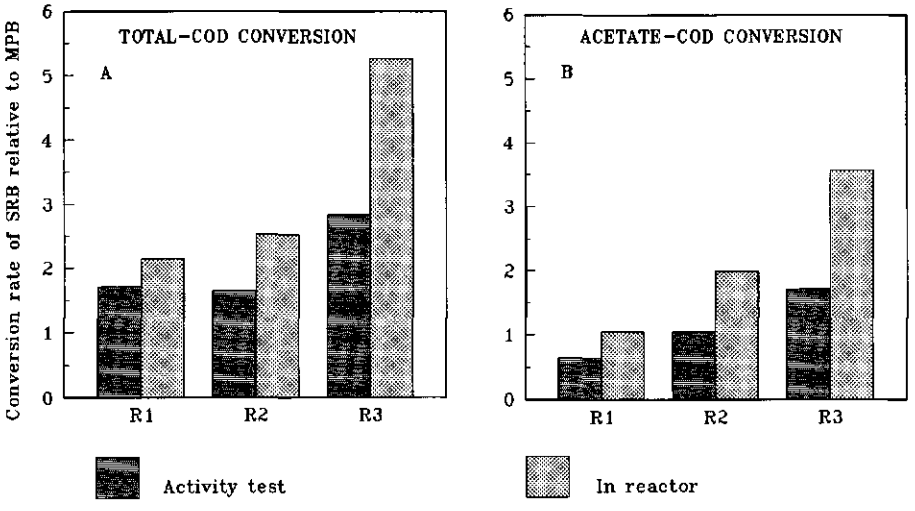


Figure 2 The ratio of organic- and acetate-COD converted by sulphate-reducing bacteria (SRB) relative to methane-producing bacteria (MPB) in the activity test and the UASB-reactors at the termination of the experiment.

Sludge taken from the reactors at the end of the experiment was fractionated into granular and flocculant sludges in order to assess the distribution of MPB and SRB activity by means of elutriation (V_{upw} 15 $m.hr^{-1}$). No clear differences were found between the granular and more flocculant parts of the sludge.

Granule development was followed during the experiment by measurement of the particle-size distribution of the total amount of sludge present. The granule development is demonstrated in figure 3 by changes in the 50% median, calculated from granule diameter and from the calculated biomass volume of the particles. The results obtained for the samples at day 123 are summarized in table 5 and figure 4.

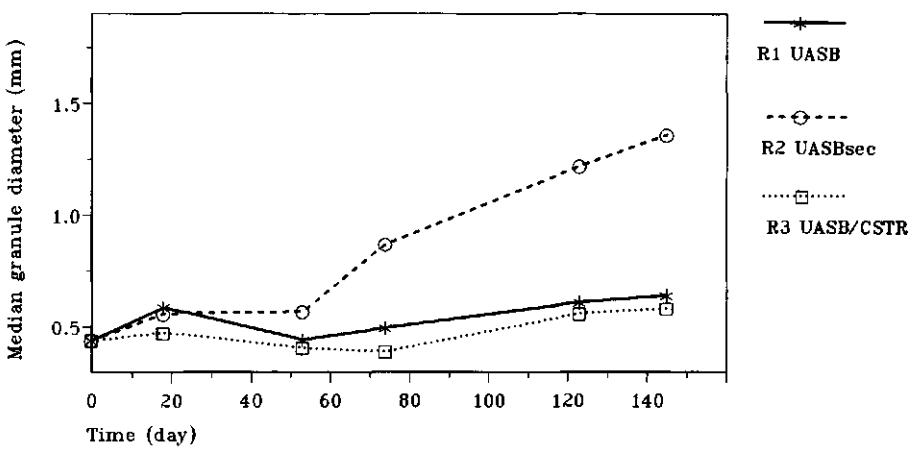


Figure 3 The granule size development with time, expressed as the median diameter related to the biomass volume represented by the granules.

Table 5 Size distribution at the end of the experiments.

	Start			Reactor 1[a]			Reactor 2[a]			Reactor 3[a]		
	30%	50%	80%	30%	50%	80%	30%	50%	80%	30%	50%	80%
Number[b]	0.16	0.19	0.35	0.13	0.19	0.35	0.13	0.20	0.40	0.11	0.16	0.31
Volume[c]	0.43	0.61	0.99	0.43	0.61	0.99	0.95	1.22	1.57	0.46	0.56	0.89

[a] At day 123 (after 100 days of performance)

[b] Fraction of particles. 30%, 50% and 80% of the granules were smaller than the indicated diameter.

[c] Fraction of granule volume. 30%, 50% and 80% of the biomass volume was present in granules smaller than the indicated diameter.

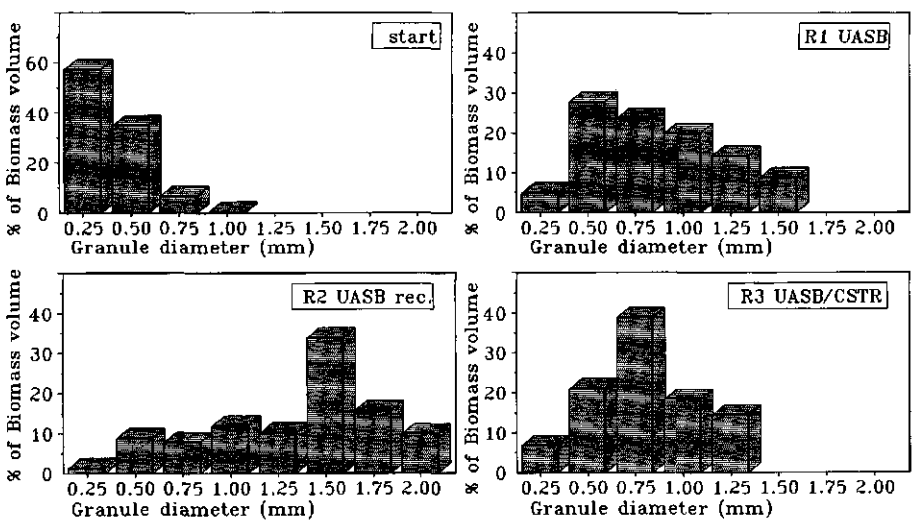


Figure 4 The particle size distribution at the start of the experiments and at day 123, expressed in percentage of the biomass volume represented by the granules.

By means of SEM the morphology of the sludge present in the three systems was examined. figures 5a and 5b represent the observed morphology extremes. The thin filaments of figure 5a were more frequently observed in the flocs than in the granules and were most frequent in the reactor 3 sludge (flocs). The *Methanothrix*-like thick filamentous organisms (figure 5b) seemed dominant in the granules of reactors 1 and 2. Surprisingly, the thin filaments seemed to be also more frequently present in the centres of the granules than on the surfaces.

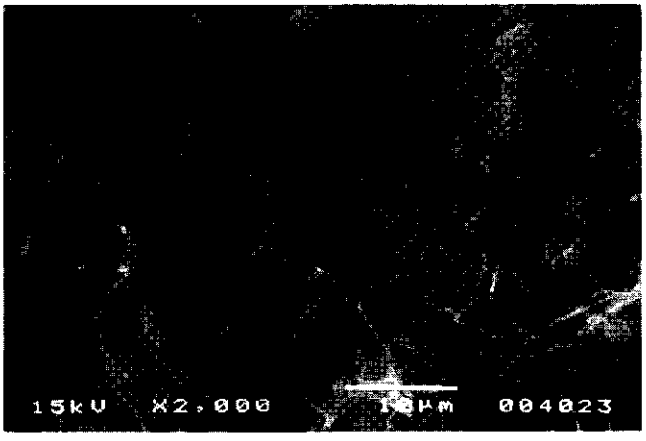
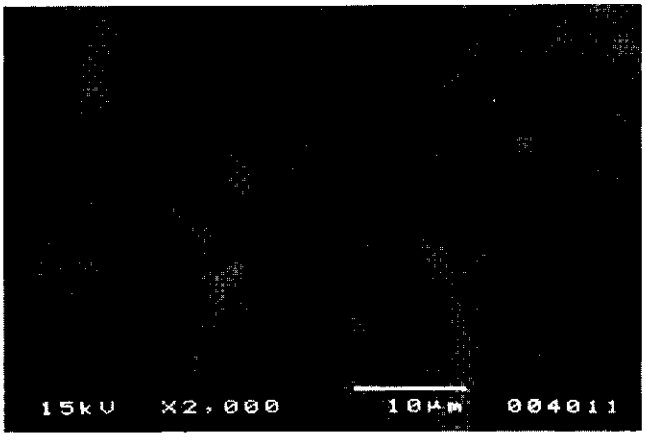


Figure 5

Scanning electron microscopy pictures of a characteristic sludge sample present in the reactors.
A: Sludge floc (sedimentation velocity < 15 m.hr^{-1}) from reactor 3, day 123).



B: Surface of a granule (sedimentation velocity $>$ 15m.hr^{-1}) from reactor 1, day 140. Bars indicate 10 µm.

DISCUSSION

In all the reactors operated, the hydrogen produced in the anaerobic degradation of the substrates was completely oxidized by SRB. Therefore, in agreement with the observations made in earlier research in our department (Mulder 1984; Rinzema et al. 1986; Rinzema and Lettinga 1988) we concluded that SRB will out-compete hydrogenotrophic MPB. On the basis of the substrate degradation and the assumptions mentioned before with regard to the primarily use of hydrogen and acetate by SRB and MPB respectively, it can be concluded that acetate is used by both MPB and SRB in all reactors (figure 2). Contrary to the results of several other studies (Hoeks et al. 1984;

Mulder 1984; Isa et al. 1986a,b) the present study revealed that acetate oxidation by SRB can occur in UASB-reactors. Complementary sludge activity measurements confirmed this (figure 2). The results in figure 1 also show that the amount of total-organic-COD oxidized by SRB increases with time for all reactor sludges. As already stated, all the hydrogen was oxidized by SRB during the whole experiment. The increase in organic-COD oxidation by SRB relative to MPB (figure 1) should, therefore, be a result of an increase in acetate utilization by SRB relative to MPB. At the termination of the experiment, no steady-state with respect to acetate-utilization by SRB and MPB had been established in any of the systems. Although acetotrophic SRB tended to predominate in the sludge of the reactors, it cannot be decided whether or not SRB will out-compete MPB completely with respect to acetate utilization in the long term. More experiments are needed to quantify long-term competition between SRB and MPB.

Considering the results in figure 1, it is clear that in the UASB/CSTR combination (reactor 3) SRB are most competitive. This finding is confirmed by the results of the sludge activity measurements (figure 2). The total-organic-COD removal by SRB is more important in sludge samples from the reactor 3 (UASB/CSTR combination) than in sludge samples from reactors 1 and 2 (figure 2). Apparently, a relatively longer HRT is favourable for SRB with respect to acetate competition between SRB and MPB.

An explanation for the observed higher extent of organic-COD removal by SRB in the UASB/CSTR-reactor could be that in a reactor-system operated at a longer HRT, dispersed-growing SRB contribute to the total- and acetate-COD removal. Growth rates and doubling times of acetotrophic SRB are favourable for dispersed growth relative to MPB. From table 1 it is seen that the maximum growth rate of acetotrophic freshwater SRB is approx. 0.5 d^{-1} (doubling time 33 hr) while it is 0.11 and 0.24 d^{-1} (doubling times 150 and 70 hr) for *Methanothrix* spp. and *Methanosarcina* spp., respectively. Since the CSTR/UASB combination was operated at a HRT of 40 hr, dispersed growth of SRB in this reactor combination was possible, whereas dispersed-growing MPB would be washed out from such a system. The UASB-reactors were both operated at a HRT of 7-7.5 hr. Under these conditions wash-out of both suspended-growing SRB and MPB will occur. However, in activity tests a higher removal of acetate by SRB in the sludge from the CSTR/UASB-combination than in the sludge of the UASB-reactors was found (figure 2). Therefore, operation of anaerobic reactors at a longer HRT is favourable for SRB, irrespective of whether they grow in dispersed or in immobilized form.

No important differences were found in the ratios of the organic-COD removed by SRB relative to MPB in the UASB reactors 1 and 2. Relatively more acetate was removed by SRB in the recirculated UASB (reactor 2) than in the non-recirculated UASB (reactor 1). This finding was confirmed in the sludge activity experiments (figure

2). The difference between the two single UASB reactor systems could be related to the hydraulic regime. Because of the more complete mixing regime in the recirculated reactor the substrate concentrations would be relatively low throughout the sludge bed in that reactor, whereas in the non-recirculated UASB-reactor a concentration gradient would prevail ranging from high concentration levels at the inlet point to low concentration levels in the upper part of the sludge bed. This difference in hydraulic regime could have resulted in a different selection for microorganisms related to the μ_{max} and Ks values of the bacteria involved. From table 1 it can be seen that at low acetate concentrations SRB have a higher growth rate than MPB. A more completely mixed system, like the recirculated UASB-reactor in these experiments, will, therefore, be more favourable than a plug-flow system (the non-recirculated UASB-reactor) for the development of SRB. Comparing the results obtained, it appears that relatively more acetate was used by SRB in UASB reactor conditions than in the sludge activity measurements (figure 3). These discrepancies cannot be satisfactory explained. Possibly, diffusion limitation of substrate as well as of sulphate in the (non-stirred) batches could have played a role. Also the differences in substrate levels between both systems might be a reason for the observed discrepancy; in the batch assays the substrate level was significantly higher than in the continuous reactors.

After termination of the experiments, the sludge of the three systems was separated into a granular and a flocculant part by means of elutriation. Sludge activity measurement revealed that there was not any clear difference between the granular part and flocculant part of the sludge with respect to sludge activities and the fraction of the substrate used by SRB and MPB. No clear difference in immobilization ability between MPB or SRB could be detected.

The granulation process proceeded best in the recirculated UASB-reactor. Small particles were more abundant in the CSTR/UASB system (table 5). A shift toward granules was found especially in the recirculated UASB (figure 3). A slight tendency to granule formation was observed in the non-recirculated UASB, whereas in the CSTR/UASB-combination this was not clearly the case (figure 4). A long HRT is less favourable for the granulation process, which is in agreement with the results of Hulshoff Pol (1989). A short HRT, especially in combination with a high upward velocity clearly favours granulation.

In all reactors the average sludge growth, calculated according to equation 1, was 4.1%. For the recirculated UASB the biomass increase was mainly in the form of large granules up to 2 mm diameter. For the non-recirculated UASB reactor (1), the biomass increase resulted in an increase of the average diameter of the small particles (figure 4). No clear granulation was observed in the UASB/CSTR combination during the experimental period. In the examination of the sludges, particles smaller in size than 0.1 mm were neglected in the size-distribution calculations.

Although bacterial granulation is a complex process and is related to many factors, e.g. environmental conditions, process conditions applied and type of seed sludge used, we are convinced that a selection pressure imposed on the sludge is one of the crucial factors in the granulation process. The selection process is based on differences in settling properties of dispersed and granular sludges. In this study we used HRT and upward velocity as selection pressures. The results obtained clearly reveal the importance of the HRT and the upward velocity in the granulation process. A long HRT may allow dispersed bacterial growth and be less favourable for granulation. A short HRT, especially if combined with a high upflow velocity, could cause wash-out of dispersed bacterial matter and promote granulation, even in sulphate containing wastewater. The results obtained from the activity tests performed with sludge samples of the reactors (figure 2), clearly reveal that both MPB and SRB, including acetotrophic SRB, developed in both the flocculent and granular parts of the sludge. No difference between the sludge fractions with respect to sludge activity and fraction of substrate used by SRB and MPB could be detected. Also no clear differences with respect to attachment ability between SRB (including acetotrophic SRB) and MPB was observed. The predominance of acetotrophic SRB over MPB in all reactors, including the granular part of the sludge, demonstrated that SRB are able to compete against MPB for acetate in UASB-reactors or other reactor-systems based on immobilization of biomass.

Besides sludge activity measurements the sludge in the three systems was also characterized by means of SEM. A relative dominance of a thin filamentous organism (a quarter of the diameter of *Methanothrix*) was found in the flocculent (very small particles, separated by elutriation) part of the sludge of all reactors (figure 5). These organisms were also more abundant in sludge from the UASB/CSTR-combination than in the sludge from the UASB-reactors. Surprisingly, for the UASB-reactors these thin filaments seem to be orientated in the centre of the granules, while *Methanothrix* like organisms were more frequent at the surface (figure 5). Very small particles of these thin filaments may function as primary nuclei on which *Methanothrix* bacteria will attach, initiating the granulation process. The thin filaments were also frequently observed in a later experiment in which methanogenic growth was suppressed by addition of chloroform, resulting in a sulphidogenic system. It is therefore likely that these bacteria are sulphate-reducers.

Appendix

Equations for calculation substrate utilization by sulphate-reducing bacteria (SRB) and methane -producing bacteria (MPB)

Calculation of the loading rate

$$COD_{load} = \phi\ COD_{infl} \tag{1}$$

Calculation of the organic COD conversion

$$COD_{conv} = \phi(COD_{infl} - COD_{effl}) \tag{2}$$

$$COD_{conv,srb} = 2\phi(SO_{4,infl}\ \ SO_{4,effl}) \tag{3}$$

$$COD_{conv,mpb} = \phi_g f + g\phi \tag{4}$$

Calculation of the acetate, hydrogen conversion

$$C_{2,conv} = \phi[(C_{2,infl} - C_{2,effl}) + 0.57\ (C_{3,infl} - C_{3,effl}) + 0.7(SUC_{infl} - SUC_{effl})] \tag{5}$$

$$H_{2,conv} = \phi[0.43\ (C_{3,infl} - C_{3,effl}) + 0.3(SUC_{infl} - SUC_{effl})] \tag{6}$$

Calculation of the acetate used by MPB, SRB

$$\begin{aligned} C_{2,conv,mpb} &= COD_{conv,mpb} \quad for\ COD_{conv,mpb} \le C_{2,conv} \\ C_{2,conv,mpb} &= C_{2,conv} \quad\ \ for\ COD_{conv,mpb} > C_{2,conv} \end{aligned} \tag{7}$$

$$\begin{aligned} C_{2,conv,mpb} &= C_{2,conv} \quad\ \ for\ COD_{conv,mpb} > C_{2,conv} \\ C_{2,conv,srb} &= COD_{conv,srb} - H_{2,conv} \quad for\ COD_{conv,srb} > H_{2,conv} \\ C_{2,conv,srb} &= 0 \quad\ \ for\ COD_{conv,srb} \le H_{2,conv} \end{aligned} \tag{8}$$

Calculation of the hydrogen used by MPB, SRB

$$\begin{aligned} H_{2,conv,mpb} &= COD_{conv,mpb} - C_{2,conv} \quad for\ COD_{conv,mpb} > C_{2,conv} \quad (9) \\ H_{2,conv,mpb} &= 0 \quad\ \ for\ COD_{conv,mpb} \le C_{2,conv} \end{aligned} \tag{9}$$

$$\begin{aligned} H_{2,conv,srb} &= COD_{conv,srb} \quad for\ COD_{conv,srb} \le H_{2,conv} \\ H_{2,conv,srb} &= H_{2,conv} \quad\ \ for\ COD_{conv,srb} > H_{2,conv} \end{aligned} \tag{10}$$

Where:

$COD_{infl,\,effl}$	=	*Chemical oxigen demand in influent, effluent*	$(g.l^{-1})$
COD_{load}	=	*Organic COD loading rate*	$(gCOD.d^{-1})$
$(^{t}COD,\ C_2,\ H_3)_{conv}$	=	*(COD, Acetate, Hydrogen) converted*	$(gCOD.d^{-1}$
C_2, C_3	=	*Acetate, Propionate*	$(gCOD.l^{-1})$
SO_4	=	*Sulphate concentration*	$(gSO_4\text{-}S.l^{-1})$
Suc	=	*Sucrose concentration*	$(gCOD.l^{-1})$
mpb	=	*by methane producing bacteria*	
srb	=	*by sulphate reducing bacteria*	
ϕ	=	*Influent flow rate*	$(l.d^{-1})$
ϕ_g	=	*gas flow rate* CH_4	$(l.d^{-1})$
f	=	*COD methane gas (at* $30\,^{\circ}C$*)*	$(2.56\ gCOD.l^{-1})$
g	=	*Solubility* CH_4 *in Water (at*$30\,^{\circ}C$*)*	$(0.08\ gCOD.l^{-1})$

10 GRANULATION AND IMMOBILIZATION OF METHANOGENIC AND SULPHATE REDUCING BACTERIA IN HIGH RATE ANAEROBIC REACTORS

This chapter consists of a modified edition of:
Granulation and immobilization of methanogenic and sulphate reducing bacteria in high rate anaerobic reactors.
A. Visser, P.A. Alphenaar, Y. Gao, G. van Rossum and G. Lettinga
In press, Applied Microbiology and Technology

10 GRANULATION AND IMMOBILIZATION OF METHANOGENIC AND SULPHATE REDUCING BACTERIA IN HIGH RATE ANAEROBIC REACTORS

ABSTRACT

The formation of anaerobic granular sludge on a sulphate containing wastewater was studied in upflow anaerobic sludge blanket reactors. Three systems were examined: a sulphidogenic system, a methanogenic system and a mixed sulphidogenic / methanogenic system. No significant granulation was observed in the sulphidogenic system. For the methanogenic and the mixed methanogenic / sulphidogenic system granulation proceeded well, and no significant difference in the granule diameter could be detected. In the three systems studied, different types of sludge developed. A (mainly) methanogenic granular sludge was developed in the methanogenic system, a (more) sulphate reducing granular sludge was developed in the mixed methanogenic / sulphidogenic system, and a flocculant sulphate reducing sludge was developed in the sulphidogenic system.

INTRODUCTION

Immobilization of bacteria is a widely used process in anaerobic digestion. The bacteria are immobilized e.g. by the formation of a biofilm on inert solid particles or by a spontaneous formation of bacterial aggregates (sludge granules).

So far, most research concerning immobilization and granulation of anaerobic bacteria has been focused on methanogenic systems and the role of methanogenic bacteria (MPB). Several researches have discussed the crucial role of *Methanothrix* in the granulation process (Hulshoff Pol 1989; Grotenhuis 1992). Contrary to the methanogenic systems only little is known about the immobilization in sulphidogenic systems. Although pure cultures of sulphate reducing bacteria (SRB) cultivated in the laboratory often aggregate in clumps or stick to surfaces (Widdel 1988), the ability of SRB to form a biofilm or a sludge granule in anaerobic reactors is still not clear.
Several industrial wastewaters, like wastewater from the edible oil industry, fermentation industry and the paper and pulping factories, have high concentrations of sulphate or other sulphur compounds. During the anaerobic treatment of these wastewaters SRB and MPB will compete for hydrogen and acetate. The outcome of this competition will determine the end product of the anaerobic mineralization process: methane or sulphide. On the basis of kinetic data it can be explained that SRB are able to out-compete MPB for hydrogen (Robinson and Tiedje 1984). This has been verified

by several researches both for sediments and for anaerobic reactors (Winfrey and Zeikus 1977; chapter 9, Alphenaar et al. 1993b). With respect to the competition in anaerobic reactors between SRB and MPB for acetate, the situation is less clear. Both a complete conversion of acetate into methane (Hoeks et al. 1984; Mulder 1984) and a pre-dominance of acetotrophic SRB (Rinzema 1988; chapter 9, Alphenaar et al. 1993) have been reported.

The goal of the present study is to investigate the ability of SRB and/or MPB to form sludge granules in a methanogenic, a mixed methanogenic / sulphidogenic and a sulphidogenic system, and to study the role of the immobilization process in the competition between MPB and SRB.

MATERIALS AND METHODS

Reactors

Three UASB reactors made of polyvinylchloride, with a liquid volume of 1.1 litre (height 400 mm, internal diameter 60 mm) were used.

The reactors were placed in a temperature controlled room (30°C ± 1). The methane gas production of the reactors was measured using a wet gas meter (meterfabriek, Dordrecht, The Netherlands) after scrubbing CO_2 and H_2S from the biogas using a 3% NaOH solution and a column of soda-lime pellets. Effluent recirculation was applied in order to improve mixing, to limit the occurrence of concentration gradients in the reactor and to increase the upward liquid velocity to approx. 0.35 $m.h^{-1}$.

The reactors were inoculated with a blend of two types of granular sludge: approx. 20% of the Volatile suspended solids (VSS) originated from a lab scale UASB reactor adapted to a mixture of acetate, propionate, butyrate and sulphate, while the rest of the sludge originated from a full scale UASB reactor treating distillery wastewater (Nedalco, Bergen op Zoom, The Netherlands). Before seeding the reactor, the sludges were mixed and crushed under anaerobic conditions by pressing the sludge through a syringe needle (Microlance 21g1½ 0.8x40).

The reactors were fed with a medium described in chapter 2. The organic substrate had a chemical oxygen demand (COD) concentration of 2500 $mg.l^{-1}$ and consisted of acetic acid, propionic acid, butyric acid and sucrose (COD = 3:3:3:1)., neutralized with a 30% NaOH solution to pH 6.8. Sulphate was added as Na_2SO_4 until a concentration of 5000 $mgSO_4^{2-}.l^{-1}$ in two of the three reactors. All chemicals used were of analytical grade and provided by Merck AG, Darmstadt, Germany, except for the Oxoid yeast extract which was supplied by Unipath Ltd (Basingstoke, Hampshire, England).

The reactor fed without sulphate shall in the rest of the text be indicated as the methanogenic system. In one of the two reactors fed with sulphate, 5 mg.l^{-1} chloroform was added during days 1-5 in order to terminate the methanogenic activity of the sludge. In the rest of the text this reactor will be indicated as the sulphidogenic system. The other sulphate-fed reactor will be indicated as the mixed sulphidogenic / methanogenic system.

Sludge Activity measurements

The methanogenic and the sulphidogenic activity of the sludge was measured using a mixture of acetate, propionate and butyrate, and described in more detail in chapter 2. At the start 2 gCOD.l^{-1} of substrate, and for the assays in the presence of sulphate, 4 $gSO_4^{2-}.l^{-1}$ was added to the serum bottles. The sludge concentration during the activity assay was 1.5 gVSS.l^{-1}. The sludge used was fresh sludge from the reactors, which was added to serum bottles directly after it was removed from the reactors.

The serum bottles were incubated at 30°C and pH 7. During the incubation the sludge activity was measured by monitoring substrate concentrations, sulphate concentrations, sulphide concentrations and the methane production. The activity was measured for the total sludge sample and for separate fractions of the sludge sample: sludge particles < 0.5 mm and sludge particles ≥ 0.5 mm.

Calculation of the organic-COD (used by SRB and MPB)

In the anaerobic digestion of organic matter in the presence of sulphate, the substrate electrons (in terms of COD) are partitioned between the SRB and MPB. The electron flow can be calculated using the following equations:

For the methane bacteria:

$$4\,H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3\,H_2O$$
$$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$$

For the sulphate reducing bacteria:

$$4\,H_2 + H^+ + SO_4^{2-} \rightarrow HS^- + 4\,H_2O$$
$$CH_3COO^- + SO_4^{2-} \rightarrow HS^- + 2\,HCO_3^-$$

Since 1 mole of CH_4 produced = 2 mole of COD = 64 gCOD, and
1 mole of SO_4^{2-} reduced ≡ 1 mole of H_2S produced = 2 mole of COD = 64 gCOD,

COD_{org} = organic-COD
A = COD_{org} used by MPB = moles of methane produced x 64 (gCOD)
B = COD_{org} used by SRB = moles of sulphate S reduced x 64 (gCOD)

The percentage of COD_{org} used by MPB is given by : $A / (A+B) \times 100$ (%)
The percentage of COD_{org} used by SRB is given by : $B / (A+B) \times 100$ (%)

RESULTS

Performance of the UASB-reactors

In the sulphidogenic system no methane production was detected during the whole experiment. The substrate degradation was completely sulphidogenic of nature. During the course of the experiment the organic-COD removal rate in the reactor increased with time (figure 1a).

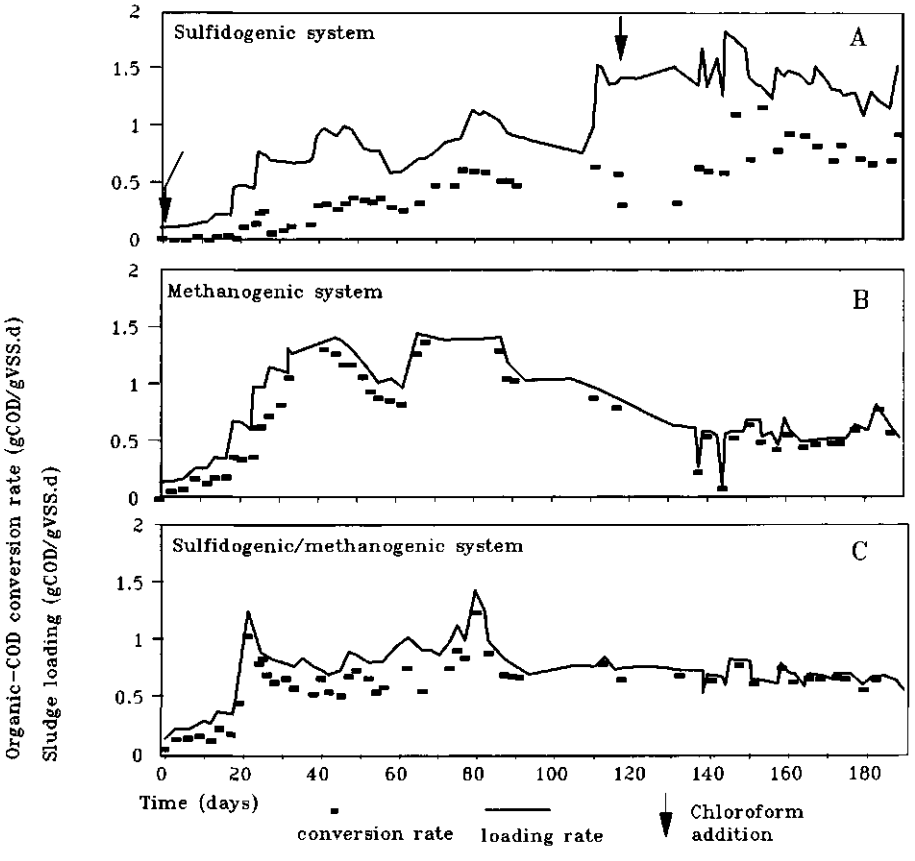


Figure 1 Organic loading rate (gCOD.(gVSS.d)$^{-1}$) and Organic-COD conversion rate (gCOD.(gVSS.d)$^{-1}$) in the sulphidogenic system (figure 1a), the methanogenic system (figure 1b) and the mixed sulphidogenic / methanogenic system (figure 1c). The arrows in figure 1a indicates the days at which a chloroform dose of 5 mg.l^{-1} was imposed on the system for about 5 days.

This was mainly due to an increase in the acetate removal rate; propionate and butyrate were almost completely degraded during all stages of the experiment. During days 115-118 a second chloroform dose of 5 mg.l^{-1} was imposed on the sulphidogenic system. This resulted in an inhibition of the organic COD removal rate (figure 1a). Particularly the oxidation of acetate was inhibited strongly. The breakdown of propionate and butyrate was only slightly affected. Recovery of the substrate degradation after the chloroform dose proceeded fairly quickly. At the end of the experiment a removal rate of about 0.9-1.0 $gCOD_{org}.(gVSS.d)^{-1}$ was obtained.

In the methanogenic and the mixed sulphidogenic / methanogenic system a very good removal of the organic substrate was obtained (figure 1b,c). In the mixed sulphidogenic / methanogenic system a shift in the percentage of organic-COD degraded by SRB and MPB was found, resulting in a pre-dominance of the organic-COD removal by SRB (figure 2). At the end of the experiment the ratio of the organic-COD removed by SRB relative to MPB was about 4. In all the reactors an average net sludge yield of about 0.04 $gVSS.gCOD_{org}^{-1}$ degraded was found.

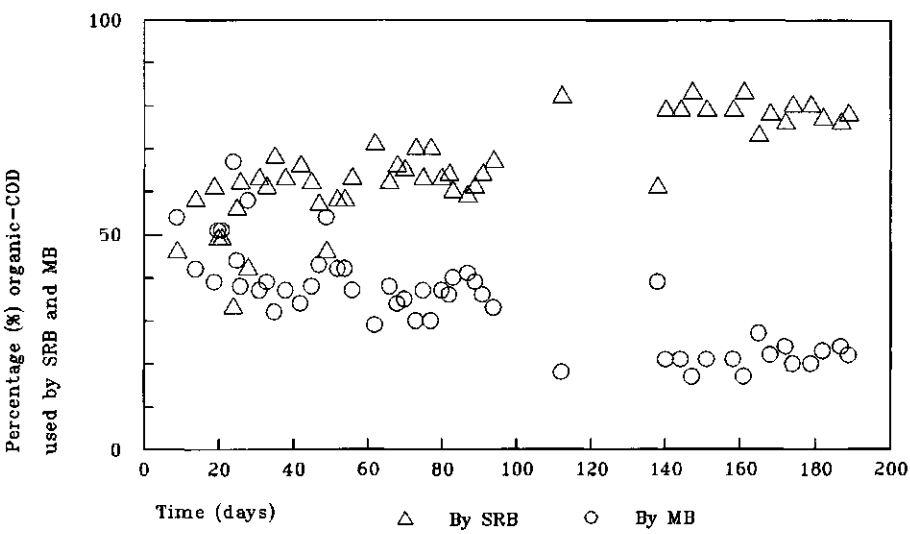


Figure 2 Percentage of organic-COD used by SRB and MPB in the mixed sulphidogenic / methanogenic system.

Sludge characterization

The characteristics of the sludge was followed by means of sludge activity tests, the sludge size distribution, and the granular strength of the sludge at the end of the experiment. The sludge activity in the presence of sulphate at the end of the experiment was about the same for the sludges from the different systems (table 1).

Table 1 The total-, methanogenic- and sulphidogenic sludge activity ($gCOD_{org}.(gVSS.d)^{-1}$) in the presence and the absence of sulphate for sludge samples of the sulphidogenic, methanogenic and sulphidogenic / methanogenic system at the end of the experiment

system	Total	Methanogenic	Sulphidogenic
sulphidogenic			
+ sulphate	0.95	0	0.85
- sulphate	0	0	
methanogenic			
+ sulphate	1.15	0.85	0.35
- sulphate	1.35	1.15	
sulphidogenic / methanogenic			
+ sulphate	1.05	0.25	0.85
- sulphate	0.40	0.32	

In activity assays with sludge samples from the sulphidogenic system in the absence of sulphate, no degradation of the organic substrate was found (table 1).

In activity assays with sludge samples from the methanogenic system in the presence of sulphate, with time a decrease in the percentage of organic-COD used by SRB was found (figure 3a). This indicates that the number of SRB decreased. Activity assays with different sludge fractions showed no significant difference between the fractions (table 2). This indicates that the composition of the more granular part resembled the more flocculant part of the sludge from the methanogenic system.

The activity of the sludge of the mixed methanogenic / sulphidogenic system in the presence of sulphate was significant higher than in the absence of sulphate (table 1). The difference was mainly due to a lack in the degradation of propionate and butyrate in the absence of sulphate. In the activity assays in the presence of sulphate, an increase in the percentage of organic-COD degraded by SRB was found (figure 3b). This indicates that the SRB became the dominant species. Activity assays done with different sludge fractions of sludge samples from the mixed methanogenic / sulphidogenic system showed no significant difference between the fractions (table 2). This indicates that the composition of the granular part was similar to that of the flocculant part of the sludge.

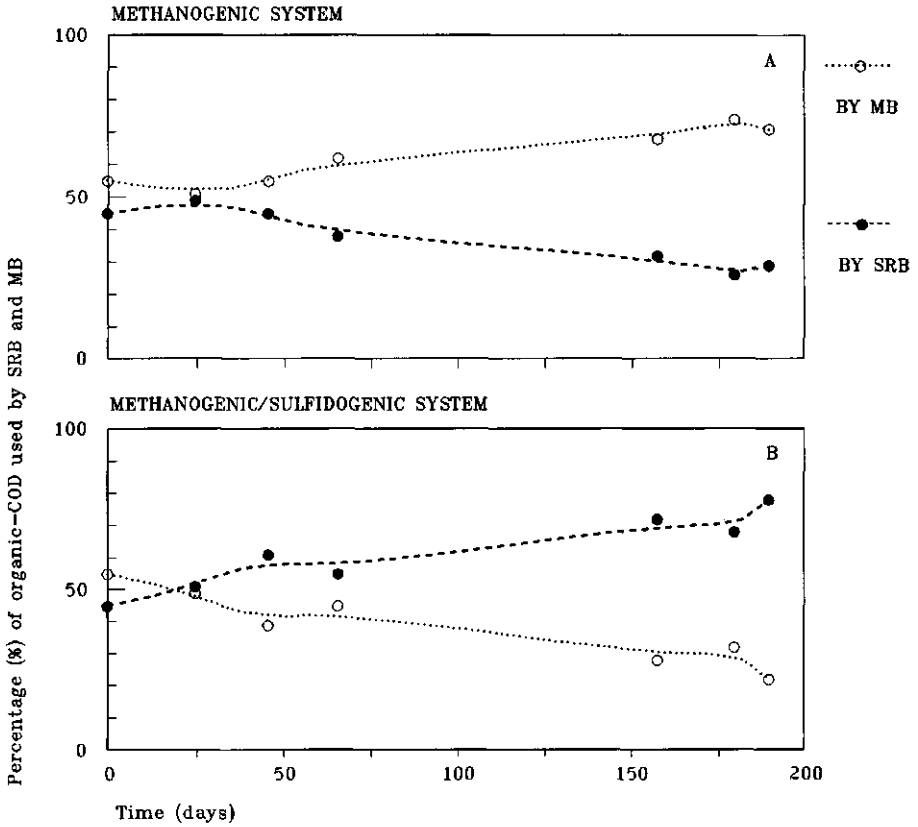


Figure 3 Percentage of organic-COD used by SRB and MPB for sludge samples of the methanogenic system (figure 3a) and the mixed sulphidogenic / methanogenic system (figure 3b) in the activity assays in the presence of sulphate.

Table 2 The percentage of organic-COD used by SRB and MPB in different sludge fractions for sludge from the methanogenic and mixed methanogenic / sulphidogenic system at the end of the experiment.

Sludge type and sludge fraction	Percentage (%) of organic-COD used by	
	SRB	MPB
Methanogenic system		
Fraction with diameter < 0.5 mm	25	75
Fraction with diameter ≥ 0.5 mm	20	80
Total sludge	28	72
Mixed methanogenic / sulphidogenic system		
Fraction with diameter < 0.5 mm	86	14
Fraction with diameter ≥ 0.5 mm	84	16
Total sludge	80	20

For the sulphidogenic system no significant increase in the sludge diameter was found (figure 4a). This indicates that no granulation in the system occurred. At the end of the experiment, however, a (slight) tendency towards an increase in the average sludge diameter was observed, indicating that on long term the formation of sulphidogenic sludge granules might be possible.

For the methanogenic and the mixed methanogenic / sulphidogenic system granulation proceeded well (figure 4b,c). With respect to the sludge diameter no significant difference was found. In both systems the average granule diameter increased from about 0.5 at the start to 1.5 mm at the end of the experiment.

Since granulation was observed in the methanogenic and mixed methanogenic / sulphidogenic system only, the granular strength was measured for sludge samples of these two systems only. At the end of the experiment the granular strength of the sludge from the methanogenic and the mixed methanogenic / sulphidogenic system were about $6.5\ 10^4$ and $2.5\ 10^4$ $N.m^{-2}$, respectively. It is clear that the granules formed under pure methanogenic conditions are more stable than the granules formed in the presence of sulphate reduction.

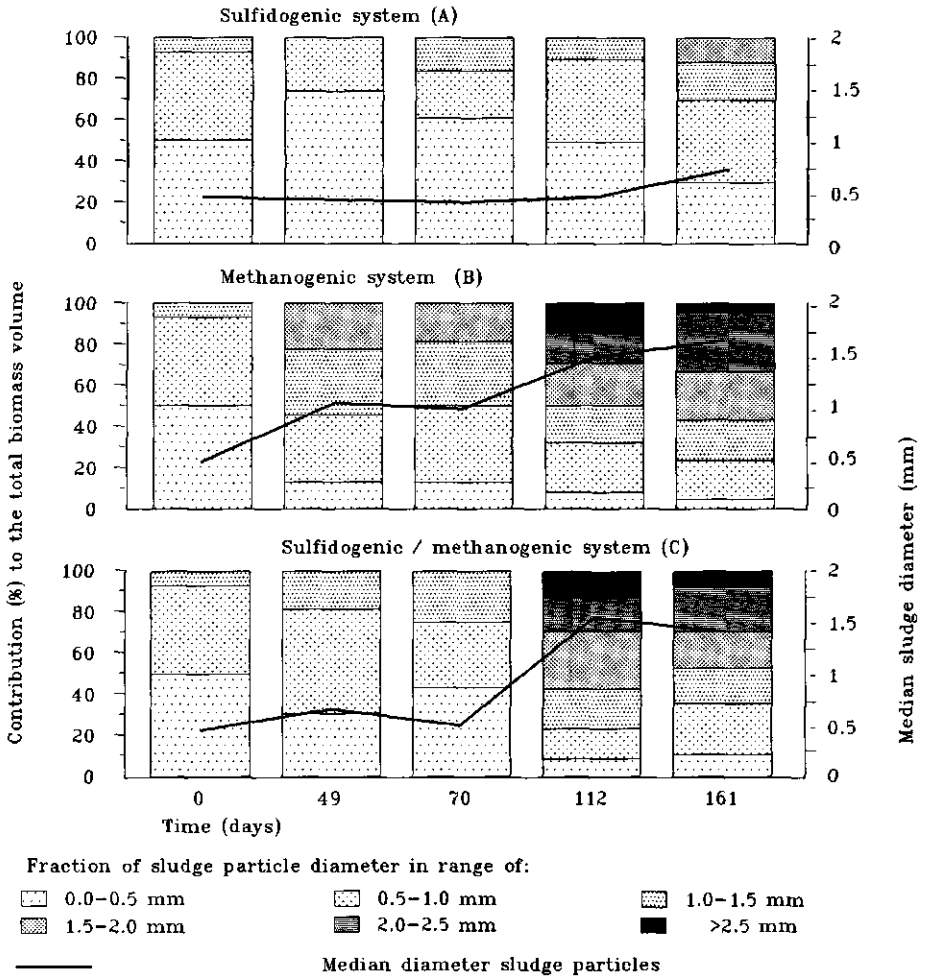


Figure 4 The development of the granulation process in the sulphidogenic system (figure 4a), the methanogenic system (figure 4b) and the mixed methanogenic / sulphidogenic system (figure 4c). For each of the systems the percentage of the different sludge fractions based on their contribution to the total biomass volume, and the average granule diameter is shown at different sampling days.

DISCUSSION

This study showed that in the mixed sulphidogenic / methanogenic system SRB became the pre dominant species and were very effectively able to out compete the MPB.

Using mass balances it was calculated that the reducing equivalents formed in the oxidation of propionate and butyrate to acetate, in the following termed as "hydrogen", were completely oxidized by SRB. The oxidation of "hydrogen" by the SRB can be a oxidation of molecular hydrogen by hydrogenotrophic SRB during a acetogenic oxidation of propionate and butyrate coupled to sulphate reduction, or a direct incomplete oxidation of propionate and butyrate by SRB. However, using mass balances as was done in this study, no distinction between these two reactions is possible. In the rest of the text we will therefore use the generalized term "hydrogen" oxidation by SRB. The pre domination of SRB for the oxidation of "hydrogen" found in this study, is in agreement with earlier observations showing that in anaerobic reactors operated at an excess of sulphate, all "hydrogen" will be used by SRB (Mulder 1984; Rinzema et al. 1986; chapter 9).

Using mass balances it was calculated that in the mixed sulphidogenic / methanogenic system besides "hydrogen" also the major fraction of acetate was oxidized by the SRB. With respect to the competition between acetotrophic MPB and SRB in anaerobic reactors, the literature shows inconsistent results. Both a pre-dominance of SRB (Rinzema 1988; chapter 9), as found in this study, and a complete conversion of acetate into methane (Hoeks et al. 1984; Mulder 1984; Rinzema et al. 1986) have been reported. Competition between SRB and MPB for acetate in anaerobic reactors is likely to be governed by the kinetic growth- and immobilization properties of the bacteria. A better immobilization capacity of MPB relative to SRB, resulting in a selective washout of SRB, has been mentioned to explain for the apparent successful competition of acetotrophic MPB against SRB as observed in some studies (Isa et al. 1986 a,b). However, in this study such a superior immobilization of the MPB was not observed. In the sludge of the mixed sulphidogenic / methanogenic system no significant difference in the number of SRB and MPB in the granular and flocculant part of the sludge was observed. SRB were the pre dominant species in both sludge fractions. These results strongly indicate that it is more feasible to assume a comparable colonization capacity for acetotrophic SRB and MPB, rather than a major difference. Consequently, the kinetic growth properties of SRB and MPB for acetate likely are the key factors in the competition. Based on the kinetic data of acetotrophic SRB and MPB published thus far, a pre-dominance of SRB is expected (chapter 9; Rinzema and Lettinga 1988; Schönheit et al. 1982; Widdel 1988). However, this study clearly show that a long time will be needed for the acetate degrading SRB to out compete the MPB

from the sludge. In the mixed sulphidogenic / methanogenic system it took about 150 days to accomplish an increase in the percentage of the organic-COD degraded by SRB from about 50% at the start, until about 80% at the end of the experiment. The reason for this slow shift in the number of acetotrophic MPB and SRB is due to the long biomass retention time in the UASB reactors as used in this study. In anaerobic reactors like the UASB reactor the biomass retention time can be as high of ½-1 year (Hulshoff Poll 1989). Since the time needed for the SRB to replace the MPB in the sludge is strongly related to the biomass retention time, in UASB reactors a very long time can be needed for the SRB to pre dominate. This will especially become true if at the start of the experiment the number of SRB is very low in comparison to the MPB.

In the methanogenic system a decrease in the number of SRB was found. At the start of the experiment both acetate- and "hydrogen" degrading SRB were present in the sludge. Using mass balances it was calculated that in the activity assays performed at the end of the experiment, all acetate was converted into methane whereas all "hydrogen" was used by SRB. Apparently, under methanogenic conditions acetate degrading SRB were expelled, whereas "hydrogen" degrading SRB remained present in the sludge. This finding can be explained by earlier observations showing that SRB can, in the absence of sulphate, grow as an acetogen. SRB capable of a syntropic oxidation of lactate (Bryant et al. 1977; Mc Inerney and Bryant 1981; Traore et al. 1983) and propionate (Wu et al. 1991) have been reported. For acetate degrading SRB, however, such acetogenic properties have not yet been reported. Our results suggest that acetate degrading SRB do not posses this ability.

With respect to the coarse of the granulation process in the different systems the results showed that MPB are well able to form sludge granules on short term. SRB also are capable to attach and grow in granules when cultivated simultaneously with MPB. However, in the absence of methanogenesis, SRB lacked the ability to form sludge granules on short term. Apparently, an active methanogenic bacterial consortium is needed to initiate a rapid formation of sludge granules. Possibly, the specific morphology of the filamentous *Methanothrix* (Wiegant 1988), or the specific hydrophobic properties of *Methanothrix* (Van Loosdrecht et al. 1987,1990) might be the key factors in initializing the granulation process. Several other researches have postulated the important role of *Methanothrix* in the granulation process (Alibhai and Forster 1986 a,b; Wiegant and de Man 1986; Yoda et al. 1989). Another explanation for the need of methanogenic bacteria to get a rapid granulation originates from the importance of the sludge selection pressure during the granulation process in anaerobic reactors (Hulshoff Pol 1989). The imposed selection pressure results in the washout of dispersed (growing) bacterial matter whereas bacterial (growing) aggregates are retained within the system. The hydraulic loading rate (or upward liquid velocity) and the gas

loading rate, originating from the methane production, play an important role during this process (Wiegant and Lettinga 1985; Wiegant 1988). In a (pure) sulphidogenic system such a gas loading rate is lacking, resulting in conditions which are unfavourable for a rapid formation of sludge granules.

The lower granular strength observed in the mixed methanogenic / sulphidogenic system in comparison with the methanogenic system might be due to the much lower methane production rate in the mixed system. Under methanogenic conditions methane gas bubbles are formed in the sludge granules. These gas bubbles induce an internal force which can cause the granule to deteriorate (Kosaric et al. 1990a; Liu and Pfeffer 1991). Furthermore, external shear forces on sludge granules and biofilms are mainly caused by the gas production (Christensen et al. 1989). The sludge granule formed under methanogenic conditions therefore must possess enough strength to remain a stable aggregate. Under mixed sulphidogenic / methanogenic conditions there is (much) less methane production and the granules formed can be "weaker" and still remain stable aggregates.

11 IMMOBILIZATION OF SULPHATE REDUCING BACTERIA IN ANAEROBIC REACTORS

A modified edition of this chapter is submitted for publication:
Immobilization of sulphate reducing bacteria in anaerobic reactors
André Visser, P. Arne Alphenaar, and Gatze Lettinga

11 IMMOBILIZATION OF SULPHATE REDUCING BACTERIA IN ANAEROBIC REACTORS

ABSTRACT

The immobilization of sulphate reducing bacteria on inert solid particles (pumice) and on granular sludge was studied in upflow anaerobic sludge blanket reactors. The sludge characteristics were followed during the experiment by means of sludge activity tests, scanning electron microscopy and by measurement of the strength of the sludge granules. Sulphate reducing bacteria were found to be able to form a stable biofilm on the pumice carrier. The matrix of the biofilm was formed by filamentous sulphate reducing bacteria. Granular sludge, in which methanogenesis was totally inhibited, was also a suitable carrier for the attachment of sulphate reducing bacteria, resulting in the formation of sulphidogenic sludge granules. However, the strength of these granules decreased in time. Both the biofilm on pumice and the granular sludge showed a specific surface structure which was formed by domes of precipitates (partly) covering the bacteria.

INTRODUCTION

At this moment almost all anaerobic treatment systems used for large wastewater streams are based on biomass immobilization and biomass retention. In these systems the bacteria are immobilized by the formation of a biofilm on inert solid particles (anaerobic filter, fluidized bed reactor) or by a spontaneous formation of sludge granules (upflow anaerobic sludge blanket (UASB) reactor).

Sofar, most research concerning immobilization of anaerobic bacteria has been focused on methanogenic systems. Immobilization of methanogenic producing bacteria (MPB) has already been discussed extensively by others (Dolfing 1987; Hulshoff Pol 1989; Grotenhuis 1992) and will not be discussed in detail here. Compared with MPB, relatively little is known about the immobilization of sulphate reducing bacteria (SRB) in anaerobic reactors. During the anaerobic treatment of wastewaters containing high levels of sulphate, SRB and MPB will compete for the available substrates: acetate and hydrogen. The ability of the SRB and MPB to immobilize could influence the competition between the two bacteria. According to Isa et al. (1986a,b) a better attachment capacity of the MPB, resulting in a selective wash-out of the SRB, enables MPB to successfully compete with the SRB. However, other researchers showed that SRB are well able to effectively attach on or grow in sludge granules, enabling the SRB to out-compete MPB both for hydrogen and acetate (chapter 9, 10). The control of the

competition between the SRB and the MPB will provide ways to treat sulphate containing wastewater more efficiently. A better understanding of the immobilization capacity of the bacteria therefore is important. Furthermore, there is a growing interest in developing wastewater treatment systems based on the sulphur cycle. For the anaerobic (sulphate reducing) part of such a system a reactor based on immobilized biomass and biomass retention is to be preferred over a system based on a suspended biomass.

The goal of this research was to study the immobilization of the SRB cultivated in the absence of methanogenesis on inert solid particles and on granular sludge as the supporting material.

MATERIAL AND METHODS

Reactors

Three UASB reactors with a liquid volume of 1.1 litre were used. The reactors were placed in a temperature controlled room of 30 ± 1°C. In all reactors effluent recirculation was applied in order to improve mixing, to limit the occurrence of concentration gradients over the reactor and to increase the liquid upward velocity. The reactors were operated at a dilution rate of 5 d^{-1}. The organic substrate had a chemical oxygen demand (COD) of 2500mg.l^{-1} and consisted of acetic acid (750 mgCOD.l^{-1}), propionic acid (750 mgCOD.l^{-1}), butyric acid (750 mgCOD.l^{-1}) and sucrose (250 mgCOD.l^{-1}), neutralized with a 30% NaOH solution to pH 6.8. Sulphate was added as Na_2SO_4 until a concentration of 5000 mg SO_4^{2-}.l^{-1}. The mineral medium used was described previously by Huser 1981 (see chapter 2).

Operation and experimental set-up

Reactor 1 was seeded with a suspended sludge which was adapted to a mixture of volatile fatty acids, sucrose and sulphate. The seed sludge showed no methanogenic activity and was sulphidogenic of nature. In the rest of the text this reactor will be indicated as the sulphidogenic biofilm reactor. To this reactor 300 ml of pumice was added as an inert carrier material. In order to achieve an expansion of the pumice a high recirculation flow of 800 $l.d^{-1}$ was used at the start of the experiment, which resulted in an expansion of the pumice from 300 to 500 ml of volume.

Reactor 2 and 3 were seeded with a blend of two types of granular sludges. Approx. 20% of the volatile suspended solids (VSS) originated from a lab-scale UASB reactor adapted to a mixture of acetate, propionate, butyrate and sulphate, while the rest of the sludge originated from a full scale UASB reactor treating distillery wastewater

(Nedalco, Bergen op Zoom, The Netherlands). Before seeding the reactors the two sludges were mixed. In both reactor 2 and 3 a recirculation flow of 50 $l.d^{-1}$ was used.

In reactor 2 about 5 mg.l^{-1} chloroform was added to the feed during the first five days in order to terminate the methanogenic activity. In the rest of the text this reactor will be indicated as the sulphidogenic UASB reactor. Reactor 3 will be indicated as the mixed methanogenic / sulphidogenic UASB reactor.

Methods

The methanogenic and the sulphidogenic activity of the sludge was measured using a mixture of acetate, propionate and butyrate (1:1:1, based on COD-values). At the start 2 gCOD.l^{-1} of substrate and 4 g $SO_4^{2-}.l^{-1}$ was added to the serum bottles. The sludge concentration during the activity assay was 1.5 gVSS.l^{-1}. The medium solution used in the activity tests is described in chapter 2. The test was performed in 500 ml serum bottles. The sludge used was added to serum bottles directly after it was removed from the reactors. The serum bottles were incubated at 30°C and pH 7. During the incubation the sludge activity was measured by monitoring substrate concentrations, sulphate concentrations, sulphide concentrations and the methane production.

The size distribution of the sludge was determined by the image analyzing technique as described in chapter 2. For sampling, the total sludge bed was removed from the reactor. Subsequently the sludge was mixed to obtain a representative sludge sample.

The granular strength was measured as the resistance against compression forces using a dynamic overload apparatus, as described by Hulshoff Pol et al. (1986).

Electron microscopy was used to study differences in the morphology of the populations present in the sludge from the reactors. Sludge sample preparation is described in chapter 2.

Analysis

Sulphate was measured using high pressure liquid chromatography, sulphide was measured colorimetrically (Trüper and Schlegel 1964), as described in chapter 2.

RESULTS

Performance of the UASB reactors

The sulphidogenic biofilm reactor (Reactor 1) was started-up applying a batch mode operation in order to support an initial attachment of the biomass to the pumice surface. After switching to continuous feeding the suspended non-attached biomass was washed out from the reactor. During the whole experiment no methane was produced so the anaerobic degradation of the substrate was completely sulphidogenic of nature. During days 1 to 50, an increase in the organic-COD removal rate was observed (figure 1a). This increase was a result of the attached growth of biomass to the pumice, resulting in a higher biomass concentration in the reactor. Due to the biofilm formation on the pumice the density of the particles and, consequently, the sedimentation velocity decreased. In order to prevent a severe biomass washout the recirculation flow had to be decreased from 800 until 400 $l.d^{-1}$ resulting in a decrease of the liquid upward velocity from 11.7 to 5.9 $m.hr^{-1}$. The expanded volume of the pumice covered with biomass expanded from 500 ml at the start until about 950 ml at the end of the experiment. From day 50 until the end of the experiment the organic-COD removal rate remained fairly constant (figure 1a). The observed removal rate at the end of the experiment was approx. 11 $gCOD.(l.d)^{-1}$, corresponding with 0.8 $gCOD.g^{-1}$ $VSS.d^{-1}$.

Table 1 Average performance of the reactors during the last 20 days of the experiment.

	Reactor 1[a]	Reactor 2[b]	Reactor 3[c]
Sludge loading rate $gCOD.(gVSS.d)^{-1}$	0.97	0.93	0.58
Organic-COD removal rate $gCOD.(gVSS.d)^{-1}$	0.79	0.55	0.55
Ratio organic-COD removed by SRB relative to MPB	∞	∞	2.72

[a] sulphidogenic biofilm reactor

[b] sulphidogenic UASB reactor

[c] mixed methanogenic / sulphidogenic UASB reactor

The sulphidogenic UASB reactor (Reactor 2), which was seeded with granular sludge, was started-up using 5 mg.l^{-1} chloroform added to the feed in order to terminate the methanogenic activity in the sludge. During the whole experiment no methane production could be detected, so in this reactor also the anaerobic degradation of the substrate was completely sulphidogenic of nature. During days 1-50 an increase in the organic-COD removal rate was observed (figure 1b). This increase was mainly a result of an increase in the degradation rate of acetate. Propionate and butyrate were almost completely converted during the whole experiment. At the end of the experiment the organic-COD removal rate was approx. 9 gCOD.(l.d)$^{-1}$, corresponding with 0.55 gCOD.(gVSS.d)$^{-1}$.

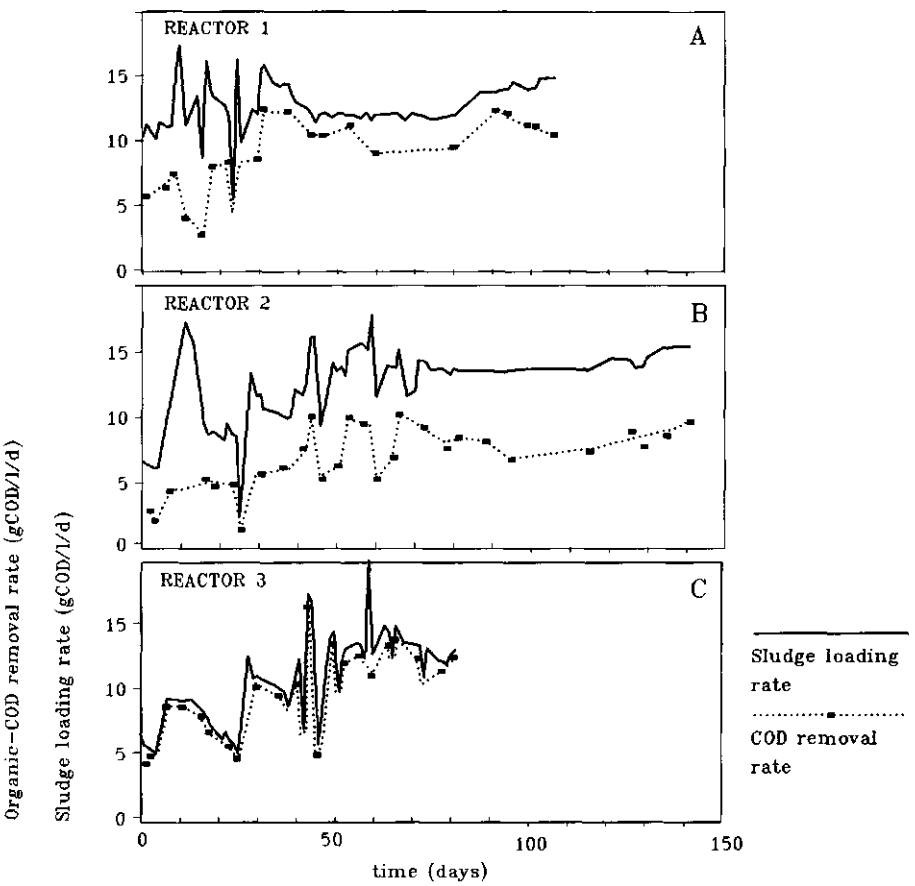


Figure 1 The sludge loading rate and the organic-COD removal rate in the sulphidogenic biofilm reactor (1a), the sulphidogenic UASB reactor (1b) and the mixed methanogenic / sulphidogenic UASB reactor (1c).

In the mixed methanogenic / sulphidogenic UASB reactor (Reactor 3), which was seeded with granular sludge, methane production and sulphate reduction occurred simultaneously. During the course of the experiment the amount of organic COD removed via sulphate reduction and methanogenesis remained fairly constant. Sulphate reduction was the pre-dominant route for the removal of the organic substrate (table 1). The ratio of the amount of organic-COD removed via methanogenesis and sulphate reduction was about 2.7. The organic-COD removal rate in the reactor was governed by the organic loading rate imposed on the reactor (figure 1c).

Sludge characterization

The development of the sludges in the different reactors was followed by means of sludge activity tests (table 2), sludge size distribution measurements (table 3 , figure 2), the granular strength of the sludge (figure 3) and scanning electron microscopy (figure 4, 5).

Sludge activities

The sludge activities of sludge samples from the sulphidogenic biofilm reactor remained fairly constant during the experiment. At the end of the experiment the activity was about 0.6 gCOD.(gVSS.d)$^{-1}$. In all the activity assays no methane production was detected. The substrate degradation was sulphidogenic of nature. The sludge activity measured in the batch experiments (table 2) was lower than the actual conversion rate in the reactor at the end of the experiment (table 1). This indicates that in the batch procedure the maximum potential conversion rate of the sludge was not measured.

The sludge activity for sludge samples of the sulphidogenic UASB reactor increased in time. At the end of the experiment the sludge activity was about 0.6 gCOD.(gVSS.d)$^{-1}$. In all the activity assays no methane production could be detected. The substrate degradation was sulphidogenic of nature. The sludge activity (table 2) was about the same as the observed organic-COD conversion rate in the reactor (table 1).

The activity of sludge samples of the mixed methanogenic / sulphidogenic UASB reactor showed an equilibrium between COD removal via sulphate reduction and methanogenesis. The ratio of organic COD removed via sulphate reduction and methane production (table 2) was lower than the ratio observed in the reactor (table 1).

Table 2 Obtained sludge activities for sludge samples from the reactors at the end of the experiment.

	Reactor 1[a]	Reactor 2[b]	Reactor 3[c]
Total activity gCOD.(gVSS.d)$^{-1}$	0.57	0.57	0.55
Ratio organic-COD removed by SRB relative to MPB	∞	∞	1.43

[a] sulphidogenic biofilm reactor,
[b] sulphidogenic UASB reactor
[c] mixed methanogenic / sulphidogenic UASB reactor

Sludge size distribution

The size distribution of the pumice at the start of the experiment and after 100 days of continuous operation clearly show the development of the sulphidogenic biofilm on the pumice in the sulphidogenic biofilm reactor (figure 2). At the end of the experiment the median diameter (based on the particle volume) of the pumice covered with biomass was approx. 0.44 mm compared with a median diameter of 0.26 mm of the raw pumice at the start of the experiment. In the mixed methanogenic / sulphidogenic reactor an increase in the median sludge diameter of the granular sludge was observed (table 3). For the sulphidogenic UASB reactor the average diameter of the granular sludge remained about the same (table 3).

Table 3 Average diameter of the sludge in the reactors

Average diameter (mm) at time t (days)	Reactor 1[a]	Reactor 2[b]	Reactor 3[c]
t = 0	0.26	1.24	1.26
t = 50	--	1.28	1.36
t = 70	--	1.50	1.91
t = 100	0.44	1.90	--

[a] sulphidogenic biofilm reactor
[b] sulphidogenic UASB reactor
[c] mixed methanogenic / sulphidogenic UASB reactor

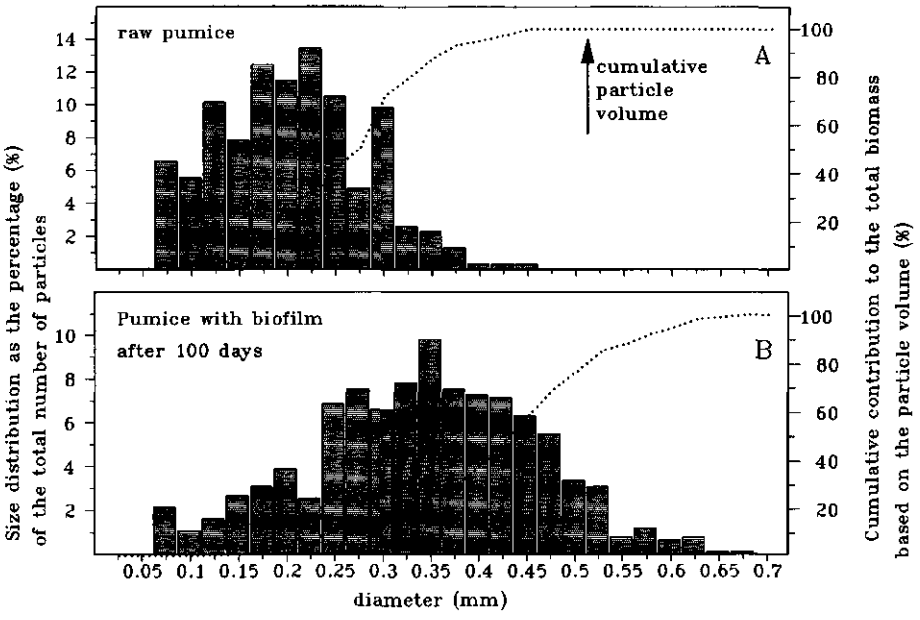


Figure 2 The size distribution of the pumice with biomass in the sulphidogenic biofilm reactor, calculated as the percentage of the particle number and the cumulative percentage of the particle volume at the start (2a) and after 100 day of continuous operation (2b).

Granular strength

The granular strength of the sludge from the mixed methanogenic / sulphidogenic UASB reactor increased during the operation of the reactor (figure 3). Contrary, in the sulphidogenic UASB reactor a decrease in the granular strength was found (figure 3). However, despite this decrease in granular stability no disintegration of the granules was observed during the experiment. The granular structure of the sludge remained intact.

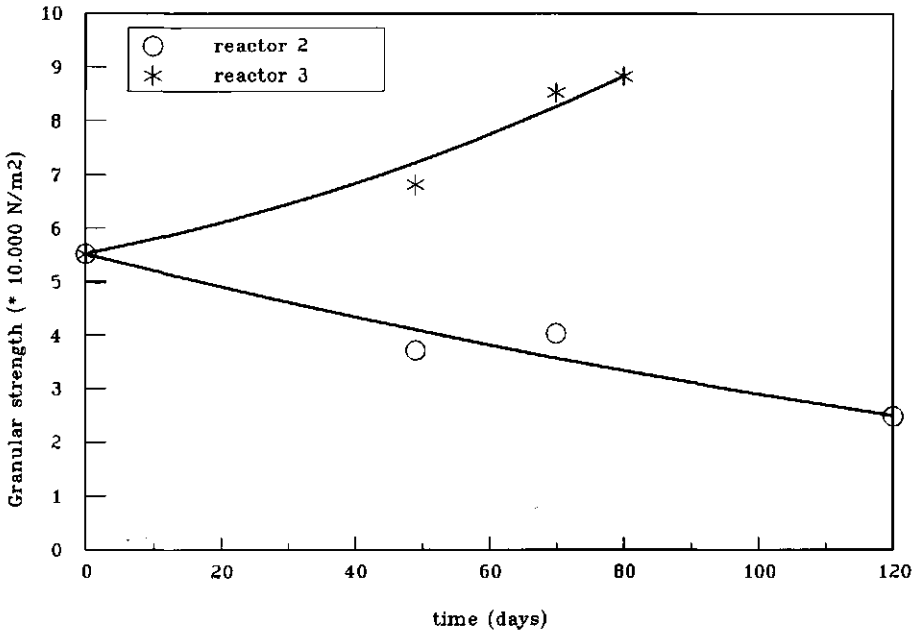


Figure 3 The granular strength of the sludge in the sulphidogenic UASB reactor and the mixed methanogenic / sulphidogenic UASB reactor.

Microscopy

Scanning electron microscopy revealed that the biofilm formation in the sulphidogenic biofilm reactor started with the attachment and growth of vibrio shaped bacteria in the holes and other disturbances on the pumice surface (figure 4a). Later on, thin (0.2 μm) filamentous bacteria attached over the whole surface of the pumice (figure 4b). Finally the growth of the filamentous bacteria resulted in the formation of the sulphidogenic biofilm (figure 4c).

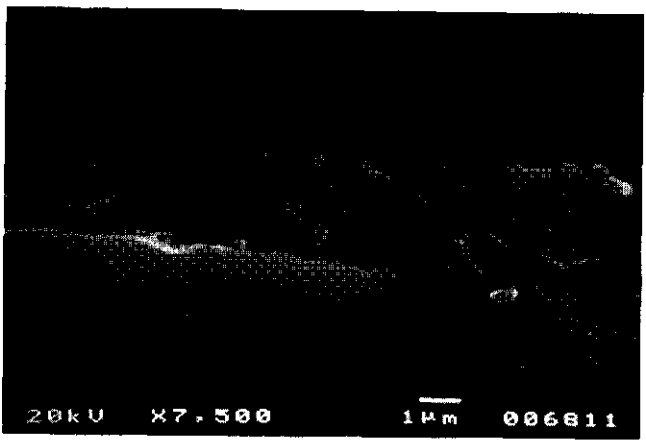
In the granular sludges of the sulphidogenic and mixed methanogenic / sulphidogenic UASB reactor a shift of *Methanothrix* like bacteria towards vibrio shaped bacteria was noticed, especially at the surface of the granular sludge. The thin filamentous bacteria which were typical for the sulphidogenic biofilm on the pumice, were also observed on the surface and, to a less extend, in the interior of the sludge granules. However, the typical filamentous structure as observed for the biofilm on the pumice was not observed in the sludge granules.

For the biomass of all the reactors a typical "dome shaped" surface structure was noticed (figure 5). These "domes" probably originated from precipitates on the biofilm or sludge granules.

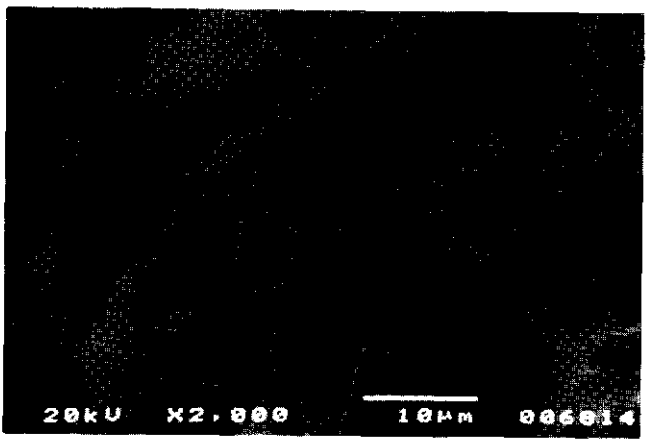
Figure 4

The surface of the pumice with biomass from the sulphidogenic biofilm reactor at different stages of the experiment.

a: the initial stage of the biofilm formation characterized by the attachment of vibrio like bacteria in the disturbances and holes of the surface. (Bar indicating 1 μm



b: Later on filamentous bacteria attach to the surface. (Bar indicating 10 μm



c: The final biofilm was formed by a matrix of the filamentous bacteria. (Bar indicating 50 μm

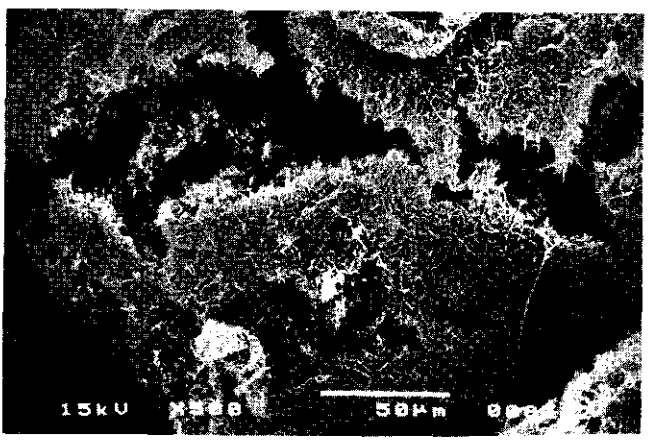
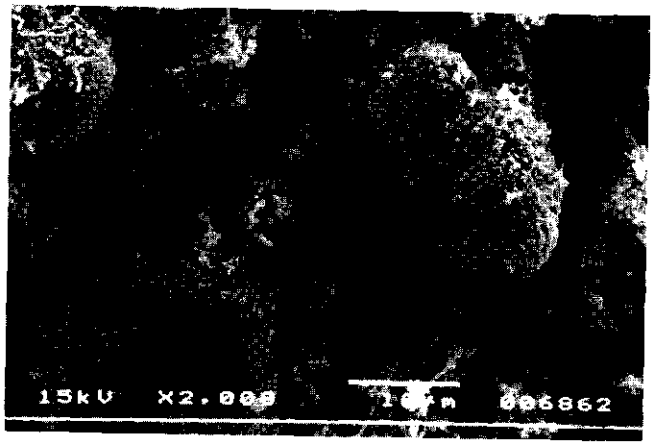


Figure 5

The typical "dome" shaped structure, probably caused by sulphide precipitates, was observed in the sludges of all reactors fed with sulphate containing influent

a: The surface of a granule from the sulphidogenic UASB reactor (bar indicates 50 μm)



b: A detail of the domes at the surface of the sulphidogenic / methanogenic UASB reactor. Individual bacteria, including the thin filaments, can be observed. (bar indicates 10 μm).

c: An example of covered domes at the surface of a granule from the mixed sulphidogenic / methanogenic reactor. The surface, including the thin filaments (F) look quit similar to the covered pumice particles (bar indicates 10 μm).

DISCUSSION

This study clearly showed that SRB were able to form a stable sulphidogenic biofilm up to 0.2 mm thickness on the pumice carrier. The formation of the biofilm resulted in a higher biomass concentration in the sulphidogenic biofilm reactor. Since the sludge activity of the biomass in the reactor remained fairly constant during the experiment, the increase in the organic-COD removal rate observed in the reactor can be attributed to the increase in the biomass concentration.

The mechanism of the early stage biofilm formation on the pumice observed in this study was related to the attachment of mainly vibrio shaped bacteria in holes and other surfaces disturbances on the pumice. This agrees well with earlier observations showing a similar initial biofilm formation on carrier material (Eighmy et al. 1983; Beeftink and Staugaard 1986). This initial stage was followed by the attachment and growth of filamentous bacteria on the surface of the pumice. The final sulphidogenic biofilm consisted of a matrix formed by the filamentous bacteria in which other bacteria attached or were entrapped.

Remarkably the organic-COD conversion rate observed in the sulphidogenic biofilm reactor was higher than the conversion rates observed in the sludge activity tests. This difference can be explained by mass transport of substrate and sulphate under both reactor and batch assays conditions. High liquid upward velocities as used in the reactor decrease the mass transfer limitation at the biofilm surface (Guiot et al. 1992a). This results in a better penetration of the biofilm and as a result the active biofilm layer will be thicker. When no external mixing is applied under batch assays conditions, as was the case in this study, a part of the biofilm will be substrate limited (Dolfing 1985). The measured sludge activity is then lower than the real potential sludge activity.

For the sulphidogenic UASB reactor with time the accomplished organic-COD removal rate and the (sulphidogenic) sludge activity of the biomass increased. These results show that the SRB, under pure sulphidogenic conditions, are well able to use the sludge granules as a substratum for attachment and immobilization. Although the stability of the granules in the sulphidogenic biofilm reactor decreased, no disintegration of the granular structure was observed. Contrary to the sulphidogenic system, an increase in the granular strength was found in the mixed methanogenic / sulphidogenic UASB reactor. The main difference between both UASB reactors was the chloroform dose during days 1 to 5 imposed on the sulphidogenic UASB reactor. Chloroform itself did not show a direct effect on the granular strength after 5 days of incubation. However, due to chloroform dose the MPB present in the sludge were killed. This death of the MPB probably negatively affected the structure and stability of the sludge granules with time. Several authors have mentioned the presence of the MPB as the most important parameter in the granular stability of granular sludge (Alibhai and

Forster 1986 a,b; Wiegant and de Man 1986; Dubourgier et al. 1988; De Zeeuw 1988; Hulshoff Pol 1989; Yoda et al. 1989; Morgan et al. 1990). Especially the specific structure formed by the filamentous *Methanothrix* can be essential for the stability of the granule (Wiegant 1988). Although the granular strength of the sulphidogenic sludge granules were lower than the mixed methanogenic / sulphidogenic sludge granules this does not mean that the sulphidogenic granules are more unstable under the conditions in the UASB reactor. Under methanogenic or mixed methanogenic / sulphidogenic reactor conditions the production of biogas is considered as the major cause for shear forces on the sludge granule (Christensen et al. 1989). Furthermore, the biogas can accumulate in the granule interior, introducing an internal force which can cause granule deterioration (Liu and Pfeffer 1991; Kosaric et al. 1990a). In order to remain stable the granule must posses enough strength. In pure sulphidogenic systems there is no significant gas production and as a result sulphidogenic granules can remain stable aggregates at much lower granular strength. This is in agreement earlier findings showing that during the granulation process the granular strength of granules formed under more sulphidogenic conditions were significant lower than for granules formed under pure methanogenic conditions (chapter 10).

In all the systems operated the surface of the biofilm or sludge granules showed a specific "dome" shaped structure. For sludges cultivated under pure methanogenic conditions such structures have not yet been reported. These structures probably originates from sulphide precipitation, e.g. FeS, on the biomass.

12 PHOSPHORUS REQUIREMENT IN HIGH-RATE ANAEROBIC WASTEWATER

This chapter consists of a modified edition of:
Phosphorus requirement in high-rate anaerobic wastewater treatment.
P.Arne Alphenaar, Ron Sleyster, Peter de Reuver, Gert-Jan Ligthart and Gatze Lettinga.
Wat. Res. (1993) 27:749-756

12 PHOSPHORUS REQUIREMENT IN HIGH-RATE ANAEROBIC WASTEWATER

ABSTRACT

In this study the effect of phosphate limitation on reactor performance and on methanogenic activity was examined. Laboratory scale UASB reactors were fed with gelatin or volatile fatty acids as COD substrate while the phosphorus content of the substrate was varied. Phosphate uptake was investigated using the granular sludge which was developed in the phosphorus deficient reactors.

Phosphorus deficiency reduces methanogenic activity in UASB reactors to 50% of the control. This reduction was found to be reversible by assessing the effect of dosage of phosphate afterwards. In batch experiments a complete recovery of the maximum methanogenic activity was observed by a dosage of 5 $mgPO_4$-$P.l^{-1}$. Also at low phosphorous concentrations (0.1 gPO_4-$P.l^{-1}$), phosphate dosage increases the methanogenic activity.

When the applied sludge loading rate was below the methanogenic activity of the sludge under phosphorus-limiting conditions, the treatment efficiency remains unaffected. The sludge growth however then is slightly lower. Overdosage of phosphate, which in practice is related to high effluent phosphate concentrations, was found to be not profitable.

INTRODUCTION

At present, anaerobic wastewater treatment is a widely applied biotechnological technique. The Upflow Anaerobic Sludge Bed Reactor (UASB) concept is, and has been for almost two decades, the most widely applied anaerobic wastewater treatment system (Lettinga et al. 1983; Lettinga and Hulshoff Pol 1991). The spontaneous immobilization of biomass into granular sludge has been an important aspect of the success of the UASB system. The production and quality of granular sludge is influenced by the wastewater composition, the reactor design and the process-technological conditions (Hulshoff Pol 1989).

The availability of nutrients is an important factor related to microbial growth (Harder and Dijkhuizen 1983; Speece 1987; Jarrell and Kalmokoff 1988). One of the most important nutrients is phosphorus. In literature various phosphorus contents of viable bacterial biomass are mentioned; Stanier et al. (1977) mentioned for bacterial matter in general a C:N:P ratio of 100:28:6 (% wt/wt), Scherer et al. (1983) reported C:P ratios for methanogens varying from 16:1 to 75:1 (% wt/wt). Speece and McCarty

(1964) gave an empirical microbial formula for the population in digesters of $C_5H_7O_2NP_{0.06}$. For many nutrient deficient industrial wastewaters a phosphorus dosage is necessary in order to make it feasible for anaerobic wastewater treatment (Hulshoff Pol et al. 1983a; Speece 1987). Also for the fermentation of some solid wastes phosphorus dosage is required (Rivard et al. 1989). Nevertheless, the optimal phosphorus dosage for anaerobic wastewater treatment systems is still largely unknown, probably due to the traditional use of phosphorus buffers in microbiological research. According to Speece (1987) the lack of adequate knowledge of the nutritional requirements of methanogens has hindered the development of the anaerobic digestion process for decades. The required phosphorus concentration in the wastewater of course is based on its biological availability. The requirement also is dependent on the COD loading and type of organic substrate (Speece 1983; Lohani and De Dios 1984).

In practice, phosphate is added to the wastewater to reach concentrations ranging from 2 to more than 50 mgP.l^{-1} without a clear correlation with the applied loading rate or the wastewater composition. Overdosage of phosphorus is an unacceptable situation both from an economical as well as from an environmental point of view. In the near future for many regions the phosphorus concentration in wastewater will be restricted by law (Britz et al. 1988; Dutch government 1990). In some cases overdosage may even cause trace element limitations by precipitation of phosphorus salts (Speece et al. 1986).

Another aspect in treatment of nutrient deficient wastes is the possibility of using nutrient addition for control of sludge growth. Uncoupling of utilization of the energy yielding substrate from growth by phosphate limitation was found for some eubacteria (Forrest 1969). Uncoupling of growth and methane formation was also reported for *Methanobacterium thermoautotrophicum* (Schönheit et al. 1980). For *Methanosarcina* cultures, Powell et al. (1983) and Archer (1985) found a reversible uncoupling of growth and substrate utilization due to phosphorus limitation. They also mentioned the possibility of exploiting these phenomena to improve sludge growth control.

The study presented here was performed to investigate the effect of different phosphorus dosage regimes on growth of granular sludge, on the methanogenic activity of the granular sludge and on the physical characteristics of granular sludge. The study aimed to reveal the phosphorus requirement of methanogenic granular sludge.

MATERIALS AND METHODS

Reactors

Different types of reactors were uses:

- 0.2 litre liquid volume glass UASB reactors with a glass marble serving as three-phase separator (chapter 2, figure 1d). Two subsequent series using four reactors were carried out. No sludge was removed (table 1).
- 3.2 litre liquid volume plexiglas UASB reactors with a traditional phase separator (chapter 2, figure 1c). Four reactors were fed with gelatin as substrate. Reactor 3, which had an excess of PO_4, acted as the control. Reactor 4 was operated analogous to reactor 2 after day 50, to breed sludge for the batch experiments (table 2).
- 2.5 litre (liquid volume), intermittently stirred plexiglas batch reactors (chapter 2, figure 2c) were used to measure the phosphate uptake velocity.
- All other batch experiments were carried out in 500 ml serum flasks unless specially mentioned as 1 litre (chapter 2, figure 2a).

All experiments were conducted in a temperature controlled room (30°C ± 1).

Table 1 Set up of the 200 ml UASB experiments fed with VFA mixture of acetic acid, propionic acid and butyric acid.

		Series 1	Series 2
Operating Strategy		P concentration (0, 1, 5, 10) mg.l^{-1} maximum loading rate	P concentration variable, Loading rate fixed
Influent	(gCOD.l^{-1})	2 - 3.5[a]	4.5[b]
Effluent	(gCOD.l^{-1})	0.2 - 0.5	up to 1.2
Flow	(l.d^{-1})	2.5	3.5
Inoculum	(gVSS)	2.5	6.5

[a] Influent concentration variable in order to obtain a stable effluent concentration

[b] Influent concentration constant

Table 2 Set up of the 3.2 litre UASB experiments fed with 2 gCOD.l^{-1} gelatin.

Parameter	Reactor 1	Reactor 2	Reactor 3
P-source	gelatin	gelatin, yeast, KH_2PO_4	gelatin, yeast, KH_2PO_4
PO_4-P influent[a] (mg.l^{-1})	0.6	3.6 → 2.1 → 1.1[b]	40
Load (gCOD.(gVSS.d)$^{-1}$)	<0.5	0.5 → 0.65[c]	0.5 → 0.9[c]
HRT (hr)	3.5 → 7.0[d]	3.5	3.5
Remark	Plug-dose of phosphate at day 88[d]	loading rate increase at day 115[c]	loading rate increase at day 115[c]

[a] Except from reactor 3, and subsequent of the P dose experiment (Reactor 1, figure 6.2) no phosphorus was detected in the effluent

[b] At day 50 the KH_2PO_4 dosage (1.5 mgPO_4-P.l^{-1}) was terminated. Yeast extract dose was lowered at day 85 from 1.5 mg till 0.5 mgP.l^{-1}. The phosphorus dosage by gelatin stays constant at 0.6 mgP.l^{-1}

[c] At day 115 sludge was removed to increase the loading rate

[d] Before investigating the effect of an intermitted P-dosage of 55 mgP.l^{-1} during 2 HRT at day 88, sludge was sampled and the HRT was raised to 7 hr

Biomass

The UASB reactors were seeded with granular sludge originating from full-scale reactors processing wastewater of a potato industry (Aviko, Steenderen, The Netherlands) and an alcohol producing industry (Nedalco, Bergen op Zoom, The Netherlands), for the 0.2 litre UASB experiments, and 3.2 litre UASB reactors respectively. The sludges were elutriated (upward velocity 15 m.hr^{-1}) before use. All sludges and samples were stored at 4°C.

Medium

The mineral medium for the UASB reactors and the batch experiments is described in chapter 2. $NaHCO_3$ was added to the UASB reactor medium relative to the COD (1 g.gCOD^{-1}). Yeast extract (18 mg.l^{-1}) and KH_2PO_4 were only added when mentioned. Phosphate was added by a separate KH_2PO_4 stock solution (28.3 g.l^{-1}). For the continuous reactor experiments and batch assays 1 ml and 0.1 ml respectively, of a trace element solution according to Huser (1980) was added per litre of medium.

The substrates used in the different experiments were:

200 ml reactors and batch tests	- VFA solution, C2:C3:C4 = 1:1.42:1.69 (COD), neutralized to pH 7.0 by NaOH
3.2 litre reactors	- Gelatin

All chemicals were of analytical grade (Merck AG, Darmstadt Germany) except the gelatin (food quality, 150 Bloom, Sanofi Brussel, Belgium) and the yeast extract (Oxoid, Unipath Ltd, Basingstroke, England). The media were prepared in demineralized water unless mentioned otherwise. Besides KH_2PO_4, also the used gelatin (0.26 $mgP.(gCOD)^{-1}$ and the yeast extract (16 $mgP.g^{-1}$) acts as phosphorus source. The phosphorus content of the tap water was 12 $\mu gP.l^{-1}$.

Methods and analysis

The phosphate concentration in the solution was determined according to Dutch Standard Methods (NEN 6479), Nederlands normalsatie instituut (1988). The phosphorus content of sludge was determined by boiling the samples after addition of 0.4 g $K_2S_2O_8$ and 1 ml H_2SO_4 (18M). Because of possible incomplete destruction of organic matter, control determinations were carried out by destruction of samples with 2.5 ml HNO_3 (16M) and 5 ml H_2SO_4 (18M), according to Dutch Standard Methods (NEN 6662) Nederlands normalisatie instituut (1988). No significant differences were observed. All liquid samples were centrifuged.

The sludge amount in the reactors was evaluated by sampling the total sludge bed, as described in chapter 2. The total yield factor (Y_{tot}) is defined as the sum of sludge bed growth, particle washout and washout of suspended matter, all per amount of COD degraded, as described in chapter 2. The particle wash out was determined in this study by filtering effluent samples of approx. 10 litre, (Schleicher & Schuell 520b½, Dassel, Germany) and measuring the wash-out after drying (105°C) by weight. The suspended wash-out is calculated from the average difference between COD-total and COD-soluble of the effluent samples. The granular sludge growth rate in the reactor was determined by periodical sampling of the sludge quantity.
The VSS/COD ratios used for the SS and granules (0.83 and 0.72 respectively) are averages of experimental values. The suspended biomass amount in the reactor is assumed to be neglectable related with the granular biomass amount present.

The maximum methanogenic activity tests were carried out in serumflask batch reactors, as described in chapter 2. Identical conditions, except for phosphate concentration, were applied to determine the activity under P-limiting conditions.

$$Y_{tot} = Y_{gran} + Y_{wash} + Y_{ss}$$

$$Y_{gran} = \frac{(X_{t_2} - X_{t_1})}{\sum_{t_1}^{t_2}(COD_i^{sol} - COD_e^{sol}) \times (Q \cdot t)}$$

$$Y_{part} = \frac{\sum_{t_1}^{t_2} X_e}{\sum_{t_1}^{t_2}(COD_i^{sol} - COD_e^{sol})} \times 0.72$$

$$Y_{ss} = \frac{\sum_{t_1}^{t_2}(COD_e^{tot} - COD_e^{sol})}{\sum_{t_1}^{t_2}(COD_i^{sol} - COD_e^{sol})} \times 0.83$$

Xt_1	=	*Biomass in reactor at time* t_1	*g VSS*
X_e	=	*Biomass concentration in reactor effluent*	*g VSS* . l^{-1}
Y	=	*Biomass growth*	*g VSS* . (*g* COD_{degr})$^{-1}$
0.72	=	*VSS content granule*	*g VSS* . (*g COD*)$^{-1}$
0.83	=	*VSS content SS*	*g VSS* . (*g COD*)$^{-1}$
Q	=	*Flow*	$l \cdot d^{-1}$
t	=	*time between sampling,* $(t_2 - t_1)$	*days*
$COD^{sol,tot}$	=	*COD supernatant, total sample*	
$COD_{i,e}$	=	*COD influent, effluent*	

RESULTS

Effect on reactor performance

In the first series of the 200 ml UASB reactor experiments, phosphate deficiency of the influent resulted in a decrease of the maximum methane production capacity of granular sludge (table 3). Feeding a P-free substrate (<0.0 mgP.l^{-1}) resulted in a 35% decrease of methanogenic activity relative to substrate containing 10 mgP.l^{-1} after 63 days of exposure in these conditions.

Table 3 The effect of a P-limited VFA substrate degraded for 60 days on the methanogenic activity. 200 ml reactors, series 1.

Phosphate concentration mgP.l^{-1}	0	1	5	10
Methanogenic activity gCOD.(gVSS.d)$^{-1}$	0.81	1.11	1.13	1.24

In the second series of the 200 ml reactor experiments a serious decrease in efficiency was found after omitting phosphate from the influent. This resulted in effluent COD concentrations up to 2 gCOD.l^{-1} (figure 1). A continuous phosphate dosage of 39 mgPO_4-P.l^{-1} following this period immediately resulted in an increased degradation rate. On the other hand a short peak dosage of 39 mgPO_4-P.l^{-1} only resulted in a temporary recovery (figure 1c). Feeding the system at a constant influent concentration of 1 mgPO_4-P.l^{-1} resulted in an efficiency increase up to 80% (figure 1d). An influent concentration of 39 mgPO_4-P.l^{-1} for a prolonged period following the P-deficient period resulted in an almost complete recovery of the efficiency. This efficiency was maintained when subsequently a phosphate concentration of 4 mgPO_4-P.l^{-1} was supplied (figure 1b).

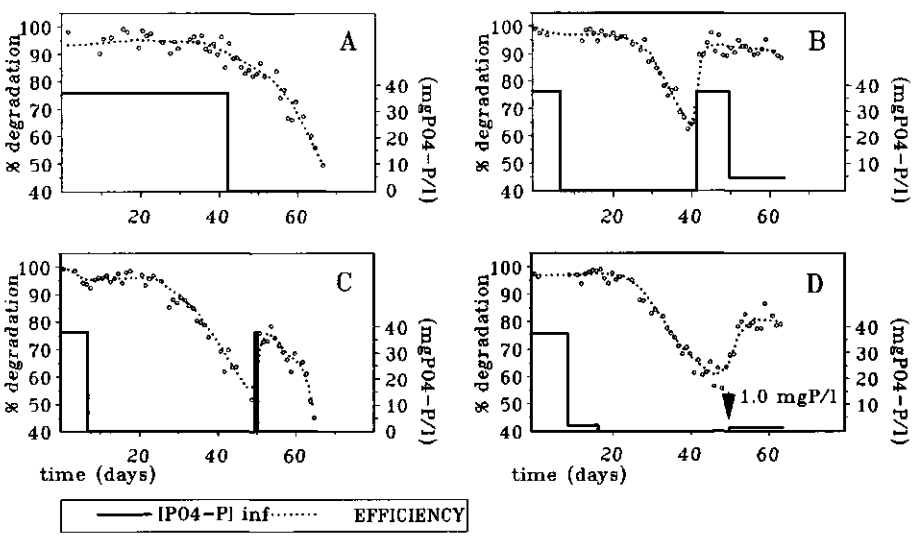


Figure 1 The effect of P-dosage on the treatment efficiency of a 200 ml UASB reactors fed with a P-deficient VFA substrate. For experimental conditions see table 1, series 2.

The treatment efficiency ($COD_{influent}$ - $COD_{effluent}$) / $COD_{influent}$ (%)) of the 3.2 litre UASB reactors fed with gelatin and a total P-dosage of 1.1 mgP.l^{-1} remained unaffected during a period of 60 days (reactor 2) (table 4). However batch tests reveal that the methanogenic activity, and consequently the maximum sludge load of the reactor, decreased to less than 50% of its original value (table 5). A complete recovery of the methanogenic activity occurs after addition of phosphate (table 5).

Table 4 Reactor performance of the 3.2 litre reactors treating gelatin substrate. Average data of 68 and 95 days (started 20 days after reactor start-up) for reactor 1 and reactor 2 and 3 respectively.

		Reactor 1	Reactor 2	Reactor 3
Efficiency (%)	$COD_{CH4}.COD_{input}^{-1}.100$	60	85	90
Maximum sludge load	$gCOD.(gVSS.d)^{-1}$	0.5	0.65[a]	>0.9[a]
granule Yield[b] (%)	$gVSS.gCOD^{-1}.100$	-0.7	3.4	4.1
particle wash-out[b] (%)	$gVSS.gCOD^{-1}.100$	2.8	2.9	3.9
suspended growth[b] (%)	$gVSS.gCOD^{-1}.100$	3.1	6.7	6.5

[a] Loading rate beyond day 115. For reactor 3 the reactor design limits the loading rate.

[b] Calculated according the formula described before.

Sludge characterization

When yeast extract was omitted from the nutrient solution in the beginning (influent 0.6 $mgP.l^{-1}$), the treatment efficiency decreased to 50% (table 4). The sludge loading rate however did not exceed the methanogenic activity while the activity of the sludge was comparable with sludge in reactor 2 (table 4). When phosphate was added to the solution an 100% increase of specific activity was observed in methanogenic activity tests using the sludge from reactor 1. The maximum specific activity however was lower than found for the other reactors (table 5).

Table 5 Granule characteristics for the 3.2 litre reactors treating gelatin substrate. Average data at day 88 and 115 for reactor 1 and reactor 2 and 3 respectively.

		Reactor 1	Reactor 2	Reactor 3
Activity[a]	without P	0.67	0.77	1.56
	with 40 $mgP.l^{-1}$	1.35	1.65	1.58
Density	$kg.m^{-3}$	1034	1029	1029
Median diameter[c]	mm	0.93	1.15	1.33
Mechanical strength	% of start	50	93	95

[a] Methanogenic activity as $gCOD.(gVSS.d)^{-1}$. Batch tests with 40 $mg.l^{-1}$ KH_2PO_4 corresponded to the maximum methanogenic activity tests. The maximum methanogenic activity of the seed sludge was 1.04 $gCOD.(gVSS.d)^{-1}$

[b] The diameter of the seed sludge was 0.89 mm

Phosphorus content of the sludge

The phosphorus content for the sludges present in both 3.2 litre reactors 1 and 2 apparently reached a stable minimum value of approx. 6.5 mgP.gVSS^{-1}, while the phosphorus content of the control sludge remained stable at 10.5 mgP.gVSS^{-1} (figure 2).

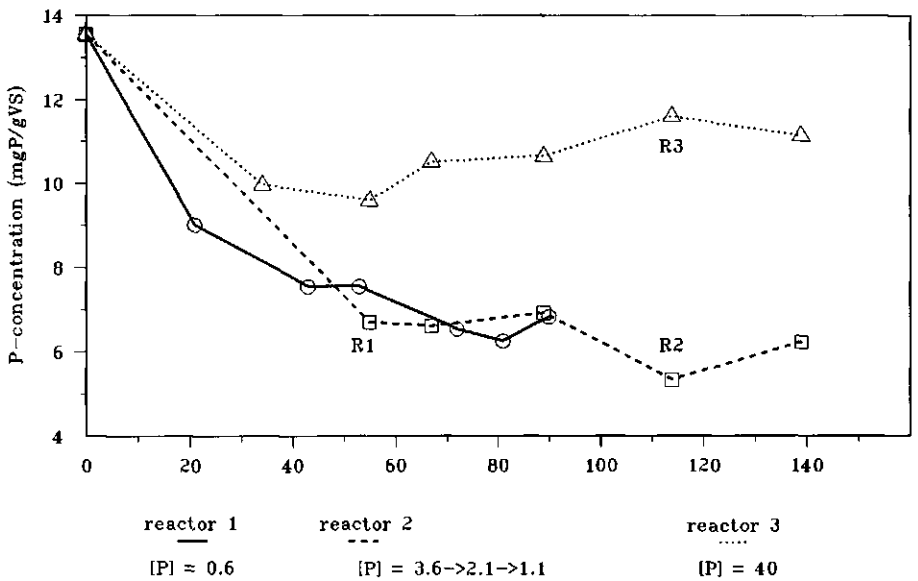


Figure 2 Change in P-content of the sludges in time for the reactors treating P-deficient gelatin containing substrate.

After 88 days of continuous operation an influent P-concentration of 55 mg.l^{-1} was fed to the P-deficient reactor 1 during a period of two hydraulic retention times. From the effluent concentration measurements a total phosphate uptake of 41 mg was calculated, which corresponds to a P-concentration in the sludge of 10.5 mgP.gVSS^{-1}. However, ten days after this P-dosage, a concentration of only 8 mgP.gVSS^{-1} was determined in the sludge samples. The reactor efficiency after phosphate addition increased to 85%, almost to the efficiency of the control reactor. A decrease to 65% was observed however within 10 days after the peak dosage, as also is indicated by the increasing effluent values (figure 3).

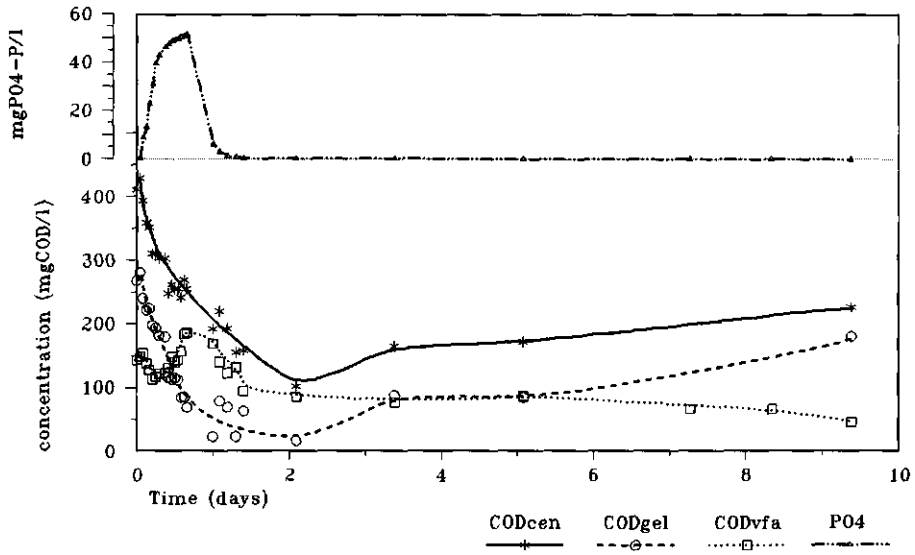


Figure 3 The effect of a P-dosage at day 88 on the effluent composition of the 3.2 litre UASB reactor 1 treating P-limited gelatin substrate. COD_{cen} = COD of the supernatant, COD_{gel} = COD_{cen} - COD_{vfa}.

Phosphate uptake

To assess the phosphate uptake, continuously shaked batch experiments (1 litre serum flasks) were applied using granular sludge from the 3.2 litre gelatin fed reactors 2 and 4 (table 2, P content approx. 6.1 $mgP.gVSS^{-1}$). The recovery of the methanogenic activity of the sludge was examined for different initial phosphate concentrations in the liquid. figure 4 illustrates the average results of the various series carried out.

The maximum recovery of the methanogenic activity was achieved at an initial phosphate concentration of 6 $mgPO_4\text{-}P.l^{-1}$. Assuming total uptake of the phosphate available by the sludge, a maximum phosphorus-sludge concentration of 10.5 $mgP.gVSS^{-1}$ should be achieved. This (calculated) concentration corresponds with the equilibrium phosphorus sludge-concentration observed in the gelatin fed control reactor number 3 (table 2, figure 2).

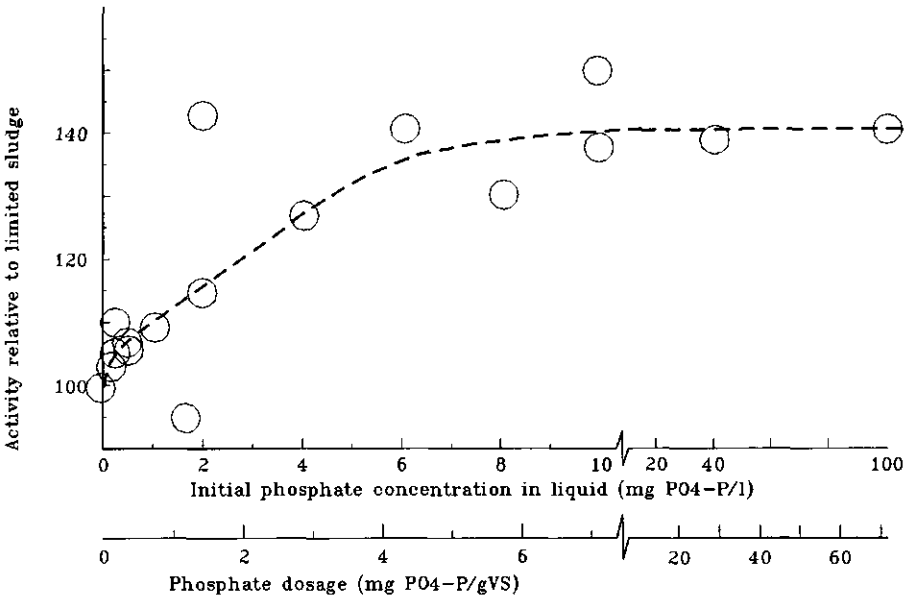


Figure 4 The recovery of the methanogenic activity of a P-limited sludge from 3.2 litre reactor 2 as a result of phosphate addition. The 100% level (no P added) corresponds to an activity of 0.66 gCOD.(gVSS.d)$^{-1}$. The initial phosphorus content of the sludge was 6.1 mgP.gVSS^{-1}.

Phosphate uptake velocity

The phosphate uptake velocity was measured in 1.0 litre serumflasks and in 2.5 litre batch reactors (3 gVSS.l^{-1}) which allowed a more frequent liquid sampling. Sludge from a reactor fed with a gelatin and sufficient phosphate containing influent (sludge loading rate 0.9 gCOD.(gVSS.d)$^{-1}$, reactor data not presented) was used as a control to examine the influence of the initial phosphorus content of the sludge. The initial phosphate-liquid concentration applied in the serum flask experiments was 10 mgP.l^{-1} and decreased during the experiments to approx. 6 mgP.l^{-1}. The phosphate concentration in the stirred batch reactor experiments varied from 20 mgP.l^{-1} to approx. 13.5 mgP.l^{-1}. The relation between phosphate-uptake and methanogenic activity of the sludge was investigated by monitoring both the VFA concentration and the phosphate concentration in the liquid (figure 5).

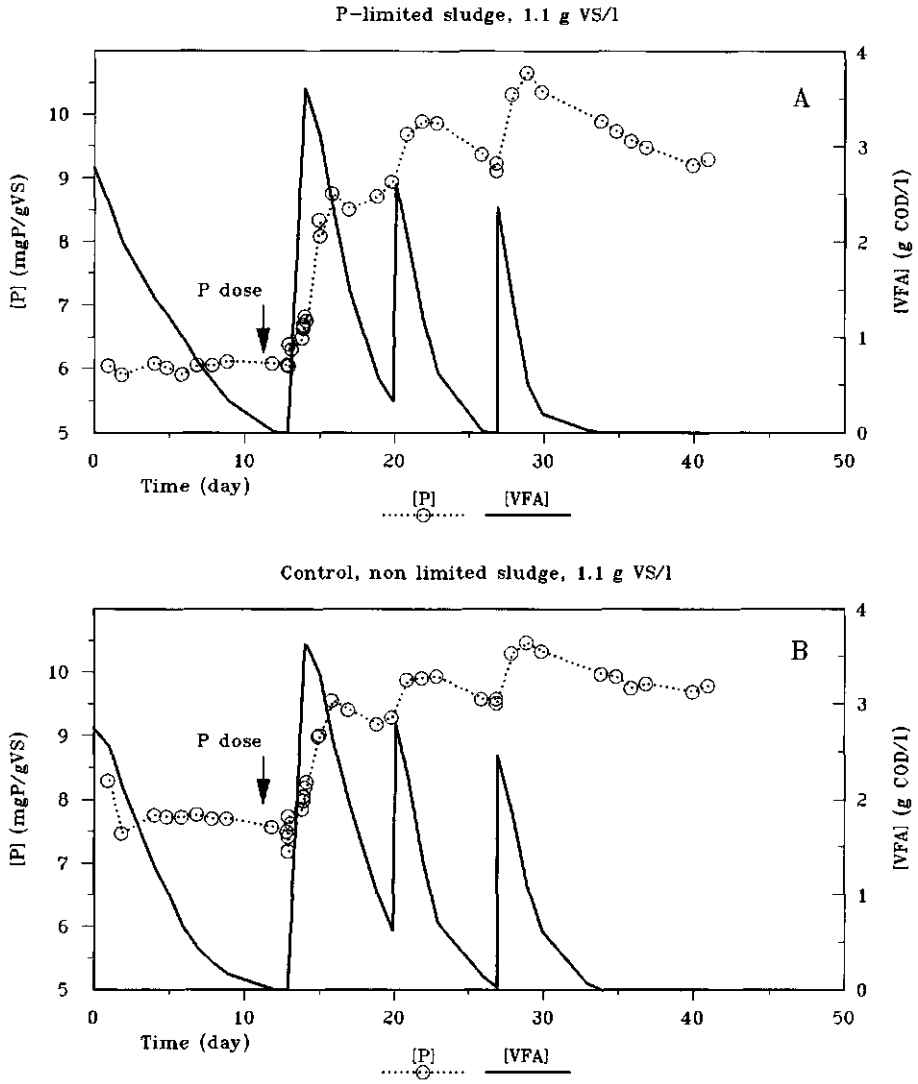


Figure 5 The phosphorus concentration of the sludge calculated from the liquid phosphate concentration, related to the VFA utilization.

For the P-limited sludge as well as for the control sludge, a equilibrium concentration of approx. 10 mgP.gVSS^{-1} was found, fluctuating due to the phosphorus-uptake and -release resulting from substrate utilization (figure 5). A rapid phosphate uptake (0.2 mgP.(gVSS.hr)$^{-1}$) was observed during the first 3 hours after the addition

of phosphate and substrate to phosphorus limited sludge. In all other situations a phosphorus uptake rate of 0.025 mgP.(gVSS.hr)$^{-1}$ (std 0.011) was found (average values over all batch experiments).

During the periods in which no substrate was available a phosphorus release was observed. The release rate was 0.0036 mgP.(gVSS.hr)$^{-1}$ (std 0.0012) again with an exception for the first phosphate uptake of phosphorus-limited sludge, after which no phosphorus release was observed. From data obtained in batch experiments applying various VSS concentrations, a relation between phosphorus uptake and substrate degradation has been derived. For the first feeding with phosphorus-limited sludge the value found for this factor amounted to 1.05 mgP.gVFA$_{degr}^{-1}$ (std 0.18), for all other experiments with a final phosphorus sludge content below 10 mgP.l^{-1} the factor amounted 0.56 mgP.gVFA$_{degr}^{-1}$ (std 0.17).

DISCUSSION.

Large differences in the phosphorus content generally were found for granular sludge samples from different full-scale reactors (6.5 up to 37 mgP.gVSS^{-1}, data not published). Although the P-concentrations of methanogens are different (Scherer et al. 1983) and the chemical composition of bacteria can be strongly influenced by environmental conditions (Harder and Dijkhuizen 1983), high phosphorus contents will indicate the presence of inorganic precipitates in the sludge (Fukuzaki et al. 1991, Grotenhuis et al. 1991[b]).

Besides the effect of P-deficiency on the anaerobic treatment process also the timescale in which effects become manifest will be of great importance for practice. A delayed effect of a phosphorus deficiency was observed in the UASB experiments treating VFA (figure 1). A similar delay also was reported for UASB reactors treating petrochemical effluent (Britz et al. 1988). When the sludge was fed under laboratory conditions for a prolonged time, the delay shortened (figure 1). This indicates that phosphorus-precipitates, which may present in the seed sludge, serve as an internal phosphorus source.

Both the continuous reactor experiments as well as the batch tests demonstrate the reversibility of the methanogenic activity decline resulting from a P-deficiency. Similar results were reported by Lettinga et al. (1980), who observed an immediate efficiency increase (from 50% up to 95%) upon phosphate dosage after 270 days treatment of a nutrient deficient sugar waste. The effect of a peak dose however appeared to be rather temporary (figures 1c, 3). This probably is related to the release of phosphorus, as was observed in batch experiments after omitting the phosphate supply (figure 5). This

release reduces the applicability of employing a peak dosage as P-dosage strategy in practice.

When comparing the data of the 3.2 litre UASB reactors (table 4) with the maximum methanogenic activity values under P-limiting conditions (table 5) it can be concluded that phosphorus limitation does not significantly reduce the treatment efficiency when the applied sludge loading rates remains below the methanogenic activity of the sludge under P-limiting conditions. Consequently, when moderate loading rates are applied, the performance of phosphorus limited reactors are satisfactory.

The absence of yeast extract resulted in an importantly stronger decrease of treatment efficiency in UASB reactors fed with phosphorus deficient substrate (table 4).. Although other limitations than merely phosphorus may have prevailed, the results clearly indicate the significance of phosphorus shortage also in this situation. All nutrients of greater importance must be present in sufficient amount before a less essential nutrient will demonstrate stimulation (Speece 1983) (table 5).

Phosphorus limitation does not affect the sludge quality seriously, except probably for the granular strength. The differences in granule density, -diameter and -strength presumably are related to differences in the granule yield (effecting the sludge retention time) rather than to any direct effect of phosphorus limitation (table 5). Regarding the reversible effect of phosphorus limitation, apparently no decay of bacteria took place during the P-limited period. Conforming with the findings of Speece (1983) and Archer (1985), the nett granule yield was related to the phosphorus concentration in the influent (table 3).

The observed equilibrium value of the phosphorus-content in P-limited sludge (approx. 6 $mgP.gVSS^{-1}$) is possibly related to the minimum phosphorus content of bacterial matter (figure 2). A dosage of phosphate, both in methanogenic activity assays and continuous experiments results in a P-content of 10 $mgP.gVSS^{-1}$, comparable to the P-content in the control (reactor 3, table 2) and the values reported in literature. Speece and McCarty (1964) gave 10.5 $mgP.g^{-1}$ as the phosphorus content of anaerobic sludge, Scherer et al. (1983) gave 9.5 $mg.g^{-1}$ wt/wt as the phosphorus content of an acetate utilising *Sarcina*.

Since any phosphate uptake was not found in the absence of a VFA substrate, the phosphate uptake undoubtedly is related to the activation of the sludge due to substrate addition. The increase of methanogenic activity when lower phosphorus concentrations are added indicated the capability of the granular sludge organisms to handle concentrations of ≥ 0.1 $mgPO_4\text{-}P.l^{-1}$.

Even at higher sludge-phosphorus contents, phosphate-uptake occurres during substrate utilization. This may indicate an supplementary phosphorus requirement at maximum methanogenic activity. The maximal phosphorus contents mentioned before were achieved during biological active conditions of the sludge. However, during substrate limited conditions a decrease of the phosphorus content will take place due to phosphorus release. Such a sequential phosphorus uptake and release was also found by Archer (1985).

CONCLUSIONS

The negative influence of phosphorus limitation on the methanogenic activity of the sludge are reversible.

Phosphorus limitation will lead to a minimal phosphorus content of 6 $mgP.gVSS^{-1}$. The phosphorus content immediately will increase when phosphate and substrate are available.
Because phosphate uptake only takes place when organic substrate is available, in practice phosphate dosage merely is profitable during biologically active periods.

Phosphate uptake stimulate the biological activity even at very low concentrations of 0.1 $mgPO_4\text{-}P.l^{-1}$. It also was found that phosphorus precipitates, as well as the organic phosphorus present in gelatin and yeast extract are available for the methanogenic population.

The presence of excess phosphorus is not advantageous for the anaerobic treatment efficiency. Effluent concentrations exceeding 1 $mgP.l^{-1}$) are not advantageous and should be avoided.

Phosphorus limitation of a sludge can be checked easily in batch tests by determining the course of the phosphate concentration in the liquid and/or by determining the methane production rate after the addition of phosphorus.

13 SUMMARY / GENERAL DISCUSSION

13 SUMMARY / GENERAL DISCUSSION

Introduction

Although granular sludge is not essential for wastewater treatment using UASB reactors, most such reactors in the field are designed in such a way that granular sludge is required for proper operation. Accurate knowledge of the mechanisms of granular growth, granule disintegration, and biomass wash-out are essential to the proper operation of these UASB reactors.

Granule formation from flocculant seed material is a time-consuming process: it takes several months before in newly started, full-scale reactors, the design loading rates can be applied (de Zeeuw 1984; Hulshoff Pol 1989). Today however, almost all full-scale reactors are started up using granular seed sludge instead (Lettinga and Hulshoff Pol 1991). As granulation is no longer a rate-limiting step, the start-up period for full-scale UASB reactors now takes only a few days.

In using granular sludge as seed sludge in a new reactor, the environmental conditions may change considerably for the granules. Also the type of wastewater fed can be very different from that to which the granules are accustomed. As a result, the composition of the granule population, the spatial organization inside the granules, the precipitates present, and physical parameters such as granule size, granular strength, and granule density will generally not correspond to the actual situation. Consequently, some of these parameters may alter considerably during the adaptation of the sludge to the new situation.

During such adaptation periods, sludge granules can be more or less unstable. Possibly many problems occurring in full-scale UASB reactors can be considered as delayed start-up problems. in most UASB reactors, low sludge quality may remain an important factor for a relatively long period, due to the low granular sludge yields.

Granular sludge

In the introduction of this thesis granular sludge is defined as a specific biomass consisting of relatively large, separate entities which can be regarded as well balanced micro ecosystems. Most important with regard to the functioning in UASB reactors are the high specific (methanogenic) activity and high sedimentation velocity.

The quality of granular sludge should be related to the extent to which it allows UASB reactors to operate properly. A clear definition of the quality of granular sludge cannot be given, because each situation may require different sludge characteristics.

Both the sludge characteristics themselves and the sludge characteristics needed for proper operation are closely related to the process conditions applied and the composition of the wastewater treated. The relations involved in the granulation process are presented in figure 1 of the introduction.

The present research focused on gaining more insight into the factors determining the growth and quality of anaerobic granular sludge. The results obtained can help overcome some of the major problems experienced in wastewater treatment plants in the field with regard to the character of granular sludge. This chapter summarizes and discusses the main results. At the end the results are presented in a very condensed form, as a trouble shoot list.

Granule porosity

The main mechanism for the transport of soluble substrates, intermediates, and end products through granules is based on diffusion. Convection processes may enhance the transport through larger pores. The transport through granules is determined by the diffusion velocity and the total pore volume available for transport. The transport of molecules of different sizes will be controlled by porosity and pore size distribution. For assessing the importance of the factors involved, a new method based on size exclusion chromatography was developed which measures the total available pore volume and pore size distribution in granular sludge (chapter 3).

The results obtained using this method show that for most granular sludge types, the total available pore volume varies from 40% to 80%. For some types of granular sludge, however, values below 10% were found, while for others they were found to exceed 95% (chapter 3 and 4). Among molecules with molecular weights up to 1000 $g.mol^{-1}$, no great differences in available pore volume were observed. Consequently, segregation of bacteria populations over granule radii is not caused by limitation of the transport of substrates with molecular weights below 1000 $g.mol^{-1}$ (chapter 3).

For some granule types, the size exclusion method showed a very low porosity, even when optically the porosity seemed to be very high. Size exclusion chromatography applied to granules developed in settled sewage wastewater (van der Last 1991) revealed a porosity of as low as 7%. Such a low porosity contrasts sharply with the microscopic observation of large holes inside the granules. Apparently, non-penetrable barriers reduce the available pore volume. Photographs of cross sectioned granules show dark layers which, possibly, are those barriers impenetrable to substrate (chapter 4).

The porosity of various fractions (diameters mm 0.5-1.0, 1.0-2.0 and >2.0) of sludge samples taken from different full-scale UASB reactors was measured, and found to be inversely related to granule size. In other words, small granules have a high

porosity, while the porosity of large granules is lower. A possible explanation is that bacteria populations in large (and supposedly older) granules are more compact due to continuous bacterial growth inside the granules. Another explanation could be that pores are clogged, possibly by material released through cell lysis.

Transport capacity through the granule

Both granule growth and granule quality depend largely upon the degree to which substrate, intermediates, and end products can be transported through the granule, i.e. the transport capacity. Substrate transport limitation causes a low specific activity, and may causes granule disintegration (chapter 6). Limitation of gas transport through the granule cause gas bubbles to be enveloped, resulting in sludge flotation and granule wash-out (chapter 8).

The mechanism mostly responsible for the transport of soluble substrates and intermediates through the granule is diffusion (Beeftink 1987; Dolfing 1985; Goodwin et al. 1991). In our research into several granular sludge types, the diffusion constant for acetate was estimated using a simple test. The values found ($0.5 - 1.0 \times 10^{-9}$ $m^2.s^{-1}$) are in the same range as those used by Henze and Harremoës (1983), and are almost equal to the diffusion constant for acetate in water (chapter 4). Possibly because our test included stirring, the values we obtained are 4 to 10 times higher than those found by Nilsson and Karlsson (1989) and Kitsos et al. (1992), who used gentler methods.

The velocity at which substrate is transported affects (together with the activity and the amount of bacteria present) the depth to which substrate is able to penetrate the granule. The effect of substrate transport limitation was examined in tests using sieved fractions of samples of granular sludge taken from full-scale reactors (diameters mm 0.5-1.0, 1.0-2.0 and >2.0). For both intact and crushed granules, the Monod K_s-value and the maximum methanogenic activity were measured. For all investigated sludge types, the methanogenic activity was found to be inversely related to granule size (chapter 4). Furthermore, it was found that crushing affected the methanogenic activity in sludge fractions of samples to only a slight degree. For two of the investigated sludge types, the K_s values turned out to be positively affected by crushing, which is additional evidence of the importance of substrate diffusion for these types of sludge. An explanation for the fact that for a third type of sludge no effect of crushing on the K_s value was observed (although the granule size was found to be inversely related to methanogenic activity), could be that in this type of sludge, the methanogenic activity is restricted to specific granule layers. After bacteria in the cores of large granules have starved to death, those on the outside will still be viable, while the cores consist of inactive organic material. In such a case, crushing has no effect on either activity or K_s. The same applies to methanogenic granule cores having layers of non-methanogenic organic matter, for example acidogenic bacteria, on the outside (figure 1).

Substrate concentration, and loading rate

The results presented in chapter 6 show a positive correlation in a UASB reactor between average granule size and the sludge loading rate applied. According to observations made by Grotenhuis et al. (1991a), granule size and influent concentration are also positively correlated. Both sludge loading rate and influent concentration are positively correlated with the prevailing substrate concentration in a reactor, and thus with the substrate penetration depth. Presuming that viable biomass contributes considerably to granule stability, the maximum granule size of granular sludge will be related to the depth to which substrate can penetrate into granules. Since the mixing characteristics of the experimental reactors used were not sufficiently clear, no distinction could be made between the effects of influent concentration and those of sludge loading rate on granule size.

Stable granular sludge consists of up to 95% organic material, which mainly originates from (viable) bacteria. Probably, bacteria are crucial to the stability of granules, so that a decline in viable bacterial biomass results in a lower mechanical strength of sludge granules. Such a decline may be due to starvation caused by substrate limitation of bacteria inside the granule. The latter could occur as a result of changes in process conditions such as loading rate (chapter 6), influent concentration, substrate composition, process temperature, or due to the total absence of substrate during storage.

According a study of Beeftink (1987) concerning the formation and the performance of acidogenic aggregates in gas-lift reactors, substrate limitation causes the formation of hollow cores, and thus leads to mechanically vulnerable aggregates. Methanogenic granules are particularly sensitive to the internal forces related to biogas production, which could lead to their destruction. Moreover, hollow granules are easily washed out as a result of flotation induced by trapped gas.

Substrate limitation will occur in the cores of granules if their radii exceed the substrate penetration depth (figure 1). Granules with inactive cores will develop if their growth is insufficiently compensated by granule shear, disintegration, wash-out, or the removal of excess sludge. In other words, substrate limitation will occur in the cores of granules if the granules become too large (chapter 4 and 6).

The formation of large, hollow granules in reactors operated at low temperatures and low influent concentrations seems to contrast with the positive correlation found between maximum granule size and substrate penetration depth, but in fact it illustrates the great importance of biological activity to granule size. Since under such conditions (low temperature, low [COD]) in the reactor the internal forces on the granule (as a result of gas production) and the external forces (as a result of the superficial gas

velocity) are weak, due to a relatively high methane solubility and a relatively low gas production rate, fragile granules can remain intact in the reactor.

Since granular sludge can be stored unfed for long periods of time without serious deterioration (Hulshoff Pol 1989, Wu et al. 1985), the deterioration observed in our study (chapter 6) was probably due to a decline of the acidogenic population in the granules as a result of feeding the sludge with non-acidified substrate. We found that the decay rates of acidogenic bacteria (approx. 0.15 d^{-1}) are 15 to 100 times higher than the rate in which the methanogens loose their activity (approx. 0.005 d^{-1}) (chapter 8). Consequently, granular sludge containing high amounts of acidogenic bacteria is much more susceptible to changing conditions or unfed storage than granular sludge in which methanogens dominate.

When UASB reactors are started up using granular seed sludge, it is strongly recommended to use a seed sludge which contains a small as possible fraction of acidogenic bacteria. Important in this respect is the phenomenon observed by De Zeeuw (1984): he found that the death rate (loss of activity) of *Methanothrix* is higher than its decay rate. Possibly, methanogens stabilize the granule structure by forming chains, even after their cells have died, resulting in the high resistance of methanogenic granules to long, unfed storage

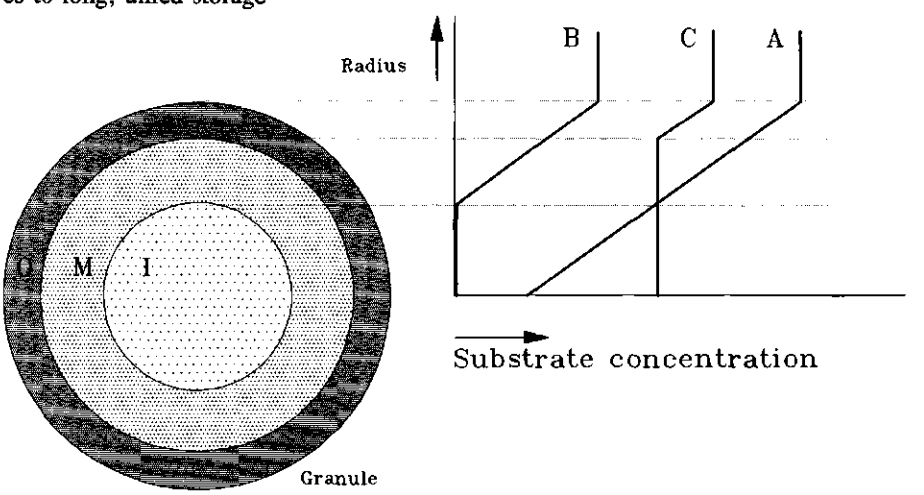


Figure 1 Diagram showing the relationship between bulk substrate concentration and granule diameter. A: The bulk substrate concentration and the substrate gradient in the granule and the granule radius are in balance. Substrate is available for bacteria throughout the granule. B: The granule radius exceeds the substrate penetration depth. Substrate is available in the surface area (O,M) only. Bacteria in the granule core (I) may die due starvation. C: The substrate is degraded only in the most exterior layer of the granule (O). The granule core (I, M) consists of inactive organic matter, possible due conditions as shown in (B).

Wastewater composition

Obviously, granular sludge characteristics depend heavily on the composition of wastewater. Whenever the substrate composition changes, the microbial population in granular sludge has to adapt to the new situation. Over a long period of time, this may result in significant changes in granular sludge characteristics. Our investigations in this respect focused on the effect of the suspended solids content of the wastewater (chapter 7), the degree of wastewater pre-acidification (chapter 8), the sulphate content (chapter 9 - 11), and at last the effect of the phosphorus content of wastewater on the functioning of granular sludge.

Suspended acidogenic bacteria

Anaerobic wastewater treatment in full-scale UASB reactors can be seriously impeded by the presence of suspended material in the influent (Hulshoff Pol 1989, Lettinga et al. 1985). One of the major sources of suspended material in influent is wastewater pre-acidification. Usually, pre-acidification results in the formation of a substantial amount of suspended acidogenic bacteria in the influent of methanogenic reactors. Our results show that such bacteria may cause serious sludge flotation, which in this case is caused by the agglomeration of individual granules into large aggregates, and by the gas trapped in these aggregates. Results of experiments conducted with ultrasonically disintegrated suspended bacteria suggest that flotation is not correlated to the suspended bacterial matter itself, but to a degraded product resulting from the lysis of acidogenic bacteria.

With regard to the process stability of the methanogenic reactor it is highly recommended to remove suspended matter, especially acidogenic bacteria, from the influent before entering the UASB reactor.

Non-acidified and partly acidified wastewater

Granular sludge may include all bacterial species necessary for the degradation of the organic pollutants present in the wastewater to which it is exposed. In reactors treating non-acidified wastewater, the granular sludge population largely consists of acidogenic bacteria. Obviously, the extent to which the wastewater is pre-acidified, strongly affects the composition of the granule population. Laboratory-scale reactor experiments were performed using non-acidified solutions of sucrose and gelatin as substrates, in order to assess to what extent the functioning of the granular sludge in a methanogenic UASB reactor system is affected by non-acidified wastewaters (chapter 8).

The results of the experiments we performed with lab-scale, one-phase UASB reactors in which the reactors were fed with non-acidified gelatin, show that sludge

loading rates of up to 1.2 gCOD.(gVSS.d)$^{-1}$ cause no problems. Moreover, we found that feeding non-acidified gelatin results in the formation of an excellent type of granular sludge. However, sucrose-containing media were treated satisfactorily only at moderate sludge loading rates, i.e. up to approx. 0.5 gCOD.(gVSS.d)$^{-1}$. Both experimental results as model calculations reveal that at higher loading rates an abundant growth of acidogenic bacteria will displace the methanogenic population present.

Furthermore, the results of our experiments show that sucrose-containing substrate may cause serious problems with regard to sludge retention. Such problems are mainly due to the occurrence of abundant growth of filamentous, probably acidogenic, bacteria. These partially attached to granules and partially in suspension growing bacteria lower the granule sedimentation and increase the sludge volume index and thus the performance of sucrose-fed reactors. These findings agree with the experiences of Anderson et al. (1991); Hulshoff Pol et al. (1983b) and Mendez-Rapin et al. (1986).

Granule flotation is also caused because the biogas produced in some granules becomes trapped between the original granule surface and a layer of acidogenic bacteria on top of that surface, probably because gas transport is hindered in the acidogenic layer.

The experiments showed that most of the acidogenic bacteria in a reactor can be removed by gently stirring the sludge bed; in our case, it resulted in a considerable improvement in reactor performance (chapter 8). Due to the small scale of the reactors used, the applied upward liquid velocities and the applied superficial gas velocities were low compared to those in full-scale reactors. It is possible that in full-scale reactors less flotation will occur than was observed during the experiments. Consequently, intermittent intensified mixing will reduce the negative effects of the abundant growth of acidogenic bacteria. Treatment of non-acidified and only partly acidified wastewaters in one-step UASB reactor systems is feasible. To prevent problems related to abundant growth of acidogenic bacteria, in some cases only moderate loading rates can be applied however.

Substrate competition between methane-producing bacteria (MPB) and sulphate-reducing bacteria (SRB)

Substrate competition between MPB and SRB, as occurs in sulphate-containing wastewaters, has far-reaching consequences for the quality of granular sludge.

In accordance with Hulshoff Pol (1989), who states that granule formation is mostly induced by the removal of dispersed growing bacteria (the selection pressure), the results of our investigations show that the formation of mixed sulphidogenic/methanogenic granular sludge is promoted by high upward velocities and

short hydraulic retention times. The relatively high substrate conversion by methanogens found in the reactor with the highest selection pressure, suggests that granule formation is mainly related to the growth of methanogens. This indicates the important role played by methanogens in granule formation (chapter 9).

Although a situation in which there are more SRB than MPB has thermodynamic and kinetic advantages, most literature on high-rate anaerobic treatment systems shows that acetate-utilising SRB are less competitive in such systems than MPB. According to Isa et al. (1986 a,b), this could be due to an inferior attachment capacity of SRB. When we conducted sulphidogenic UASB experiments in which methanogenesis was inhibited, we indeed noticed hardly any granule formation after 160 days of operation, while in similarly operated mixed sulphidogenic/methanogenic reactors, granulation was observed within 100 days, whereas in a purely methanogenic reactor it started already on day 50 (chapter 10).

On the other hand, we also found that sulphate-reducing bacteria are fully capable of participating in the formation of granules in mixed methanogenic/sulphidogenic systems, and could even keep granules intact after the methanogens present in those granules were killed by chloroform (chapter 9 and 10).

Moreover, the results we obtained in fluidized-bed experiments using pumice as inert carrier material clearly show that sulphate-reducing bacteria are fully capable of growing on inert carrier material and forming biofilms on it, without the presence of methanogens (chapter 11).

It was concluded that the low level of granule formation occurring in sulphidogenic UASB systems could be due to the low selection pressure in such systems instead of the poor attachment capacity of the SRB. Because of the generally very low biogas production, the wash-out of dispersed biomass in sulphidogenic reactors is significantly lower than in methanogenic reactors. If no extensive effluent recirculation is applied, granule formation may not proceed due to insufficient selection pressure.

The evolution of methanogenic and sulphidogenic activity in systems fed with wastewater containing high SO_4 / COD ratios, and seeded with methanogenic granules, indicates that in the long term SRB will dominate methanogens in such situtions (chapter 10, 11).

Nutrient limitation

Sufficient supply of nutrients is essential for optimum wastewater treatment (Hulshoff Pol et al. 1983, Speece 1987). Our research shows that phosphate deficiency limits the degradation capacity of granular sludge, but does not directly affect the granule quality. Furthermore, it shows that the negative effect of P-limitation on methanogenic activity is entirely reversible (chapter 12). Batch tests showed that no

rapid phosphate uptake by P-limited sludge takes place, unless both a COD source and phosphate are available.

The phosphorus content of the biological portion of the granular sludge in our laboratory-scale reactors varied from between approx. 6 mgP.gVSS^{-1} for sludge fed with P-deficient substrate, and 10.5 mgP.gVSS^{-1} for sludge supplied with sufficient phosphorus. These values correspond with data published by Speece and McCarty (1964) and Scherer et al. (1983). In granular sludge from full-scale reactors, we found phosphorus content values of up to 45 mgP.gVSS^{-1} (chapter 12), which indicates the presence of phosphate precipitates.

In sludge samples from full-scale reactors, phosphorus content values for granular sludge exceeding 10.5 mgP.gVSS^{-1} were found, which indicates overdosage of phosphorus. On the other hand, phosphorus content values below approx. 8 mgP.gVSS^{-1} indicate phosphorus limitation.

Since the uptake of phosphate by phosphorus-limited sludge occurs very rapidly, phosphorus limitation of a sludge can be checked easily in batch tests by determining the course of the phosphate concentration in the liquid after the addition of phosphorus. Phosphorus limitation can also be checked by determining the methane production rate, as the methanogenic activity of phosphorus-limited sludge rapidly changes when phosphate is added. Possibly similar tests are feasible checking limitation of other nutrients and trace elements.

TROUBLE SHOOT LIST

There are several factors affecting the increase in net biomass and the performance of granular sludge in a reactor. Due to the low sludge yield in methanogenic systems, stable UASB performance might be severely restricted by insufficient sludge production, if not by biomass wash-out. Wastewater treatment is also impaired by insufficient biological activity. Table 1 summarizes the practical implications of the research described in this thesis. It should be noted, however, that we found it difficult to discern early signs and consequences of process failure.

Table 1 Schematic overview of the problems observed during our study with regard to the functioning of granular sludge in UASB reactors.

	Problem		Cause		Solution
1	Insufficient sludge growth	1.1a	Trace-element- or nutrient limitation.	1.2a	Raising the nutrient- and/or trace-element concentration in the UASB influent
		1.1b	Too high a degree of influent pre-acidification	1.2b	Reducing the degree of pre-acidification.
		1.1c	Too low a sludge loading rate	1.2c	Increasing the loading rate (removing sludge)
		1.1d	Granular sludge wash-out (see 4, 5)		
		1.1e	Wash-out flocculant sludge, granule disintegration (see 6)		
2	Insufficient methanogenic capacity, (reactor overloaded).	2.1a	Not enough sludge in the reactor	2.2a	Reducing the loading rate. Raising the amount of sludge. Using external seed sludge. Promoting sludge growth (see 1), reducing wash-out (see 3-6)
		2.1b	Insufficient methanogenic activity (see 3).	2.2b	Decreasing sludge loading rate, increasing (methanogenic) activity of the sludge (see 3.2)
3	Insufficient methanogenic activity	3.1a	Nutrient- or trace-element deficiency (see 1.1a)		
		3.1b	An abundant growth of acidogenic bacteria	3.2b	Increasing the degree of wastewater pre-acidification. Reducing the loading rate.
		3.1c	Accumulation of organic suspended material in the sludge bed	3.2c	Ensuring that the influent does not contain suspended material.
		3.1d	A too low process temperature	3.2d	Increasing temperature
		3.1e	Toxic compounds in the wastewater fed or activity inhibiting conditions (see 6.1d).		
4	Granule wash-out.	4.1a	Gas trapped in hollow granules. formation of too big granules due to insufficient forces: low temperature, low loading rate, low influent concentration (see also 6.1a,b)		Increasing forces on granules, reducing the granule size.

		4.1b	Gas entrapment due the the formation of a layered structure, covering the granules with (acidogenic) biomass.	4.2b	Applying more stable process conditions, increasing the degree of wastewater pre-acidification.
5	Sludge wash-out, formation of bulking sludge and fluffy granules	5.1a	The conglomeration of individual sludge granules, related to suspended acidogenic bacteria in the influent.	5.2a	Withdrawing suspended matter from the influent. Diminishing the degree of pre-acidification.
		5.1b	Extensive growth of suspended or more or less at the granule surface attached acidogenic bacteria.	5.2b	Increasing the degree of pre-acidification. Intensifying the mixing applied.
		5.1c	Formation of very fluffy granules, strong growth of attached acidogenic bacteria.	5.2c	Increasing the degree of pre-acidification, Decreasing the sludge loading rate.
6	Granule disintegration.	6.1a	"Delayed" start-up problems, see 6.1 b-d.	6.2a	Applying other start-up strategy (faster increase of sludge loading rate), choosing another type of seed sludge.
		6.1b	Sudden variations in loading rate and/or influent concentration.	6.2b	Applying a more stable process conditions.
		6.1c	Sudden increase in the degree of pre-acidification. Starvation of acidogenic bacteria.	6.2c	Applying a more constant pre-acidification; (at start-up: choosing another type of seed sludge).
		6.1d	(Periodically) exposure to toxic compounds harmful conditions.	6.2d	Removing or detoxifying the toxic compound. Keeping longer .aption periods. Using a larger hydraulic buffer.
		6.1e	Too strong mechanical forces	6.2e	Preventing too strong mechanical forces, decreasing sludge loading rate.
		6.1f	Formation flocculant sludge due to an insufficient selection pressure.	6.2f	No problem if the process is stable. Otherwise increasing the selection pressure (effluent recirculation).

14 SAMENVATTING / SLOT DISCUSSIE

14 SAMENVATTING / SLOT DISCUSSIE

Inleiding

Hoewel korrelslib in principe niet essentieel is voor het functioneren van een UASB reactor worden de meeste UASB reactoren zodanig ontworpen dat de aanwezigheid van korrelslib noodzakelijk is voor een goed verlopende zuivering. Voor een optimale afvalwaterzuivering met behulp van deze UASB reactoren is kennis van de factoren die bepalend zijn voor de groei, de desintegratie en de retentie van anaëroob korrelslib essentieel.

De vorming van korrelslib vanuit een vlokkig entmateriaal is een tijdrovend proces. Hierdoor kan het vele maanden duren voordat de ontwerp belasting in een nieuw opgestarte UASB reactor ook werkelijk haalbaar is (de Zeeuw 1984; Hulshoff Pol 1989). Om dit probleem te ondervangen worden vrijwel alle UASB reactoren vandaag de dag bij de opstart geënt met een grote hoeveelheid uit andere reactoren afkomstig korrelslib (Lettinga en Hulshoff Pol 1991). Hierdoor wordt de duur van de opstartperiode niet langer bepaald door de snelheid van het korrelvormingsproces, en kan de opstartperiode tot een aantal dagen gereduceerd worden.

Tijdens de opstart wordt het entslib blootgesteld aan condities die sterk kunnen afwijken van de condities in de reactor(en) waaruit het entslib afkomstig is. Als gevolg hiervan kunnen de slibeigenschappen na de opstart, of na andere ingrijpende conditieveranderingen, sterk wijzigen. De populatiesamenstelling, de ruimtelijke opbouw, de chemische samenstelling (precipitaten) en ook de fysische korreleigenschappen zoals de sterkte, de diameter en de dichtheid van het als entmateriaal gebruikte korrelslib zijn niet in evenwicht met de nieuwe condities. In de periode dat het entslib zich aanpast aan de nieuwe situatie zijn de slibkorrels in meer of mindere mate instabiel.

Mogelijk kunnen een aantal van de problemen die zich in UASB reactoren voordoen beschouwd worden als "uitgestelde" opstart problemen. Door de soms zeer lage korrelslibaanwas kan het lang duren voor het slib een nieuwe evenwichtssituatie bereikt.

Het in dit proefschrift beschreven onderzoek heeft zich gericht op een verbetering van het inzicht in de factoren die bepalend zijn voor de groei en kwaliteit van anaëroob korrelslib. Aan de hand van de resultaten zullen een aantal van de in de praktijk voorkomende problemen beperkt of verhinderd kunnen worden. Dit hoofdstuk geeft een samenvatting van de resultaten. Aan het slot worden de resultaten verder

gecomprimeerd in de vorm van een "trouble shoot" lijst met betrekking tot het functioneren van UASB reactoren in de praktijk.

Korrelslib

Vanaf het midden van de jaren zeventig is onderzoek verricht naar de vorming van stabiele bacterie aggregaten vanuit een vlokkige biomassa, zoals die plaatsvindt bij de behandeling van afvalwater in upflow reactoren. Hoewel een gedeelte van dit onderzoek gericht was op de aggregaatvorming door verzurende bacteriën (Beeftink 1987; Mulder 1990; Zoetemeyer 1982; Zoutberg 1990) en op de vorming van denitrificerende slibkorrels (van der Hoek 1988), wordt het begrip korrelslib, of, in het engels "granular sludge", vrijwel uitsluitend gebruikt voor de (methanogene) bacterie aggregaten die in UASB reactoren worden gevormd.

Definitie korrelslib

Anaëroob korrelslib kan gezien worden als een zuiveringsslib (biomassa) met een aantal specifieke eigenschappen die het zeer geschikt maken voor het functioneren in UASB- en andere opwaarts doorstroomde anaërobe reactoren. Hulshoff Pol (1989) noemt in dit kader met name de uitstekende sedimentatie eigenschappen en de hoge methanogene activiteit van de slibkorrels. Microbiologisch gezien kunnen slibkorrels beschreven worden als uitgebalanceerde miniatuur eco-systemen; slibkorrels bevatten alle noodzakelijke bacteriën die noodzakelijk zijn voor een totale afbraak van de in het afvalwater aanwezige organische stof.

In tegenstelling tot aggregaten met een toevallige structuur, zoals vlokken, kunnen anaërobe slibkorrels gezien worden als mechanisch stabiele, individuele eenheden. Morfologisch worden slibkorrels gekenmerkt als relatief grote ($d > 0.5$ mm) deeltjes met een min of meer regelmatige vorm en een duidelijk gedefinieerd oppervlak. Samen met een relatief hoog soortelijke gewicht leidt deze morfologie tot de uitstekende sedimentatie eigenschappen van korrelslib.

Inert dragermateriaal is niet essentieel bij de vorming van korrelslib. Ook de stabiliteit van een slibkorrel is niet afhankelijk van de aanwezigheid van inert materiaal. Doordat de vorming van korrelslib gebaseerd is op autoimmobilisatie bestaat korrelslib voor een groot gedeelte uit (actief) biologisch materiaal. In tabel 1 worden de belangrijkste kenmerken van korrelslib op een rijtje gezet.

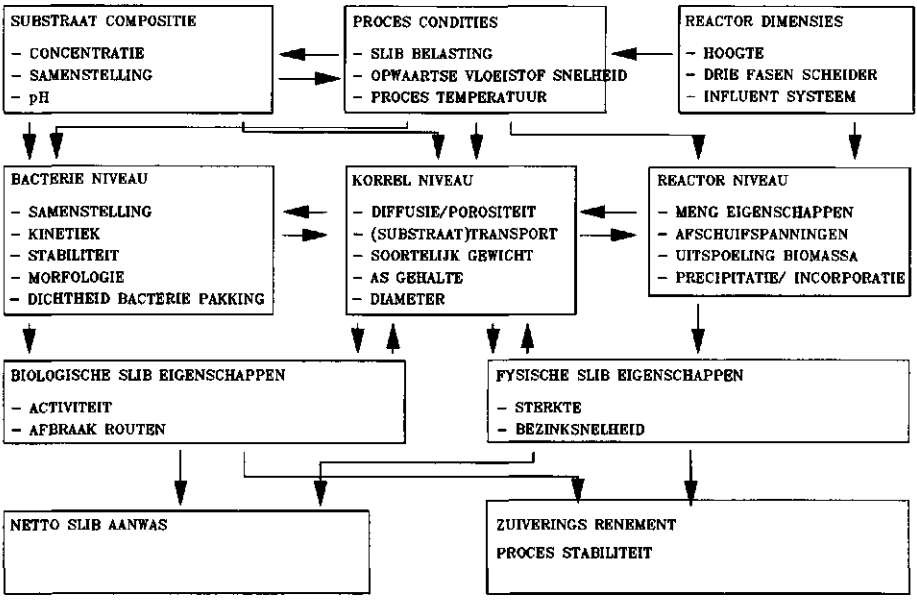
Tabel 1 De belangrijkste eigenschappen m.b.t. het functioneren van korrelslib in UASB reactoren.

Eigenschap	Beschrijving
Biologische activiteit	Het belangrijkste doel van biologische afvalwaterzuivering is de conversie en verwijdering van de organische afval componenten. In UASB systemen zijn de methaan producerende en / of de sulfaat reducerende capaciteit van het slib de meest kenmerkende eigenschappen.
Sedimentatie snelheid	In UASB reactoren wordt de slibretentie met name bepaald door de sedimentatie eigenschappen van het slib. De sedimentatiesnelheid van het slib is hiermee één van de belangrijkste factoren m.b.t. een stabiele procesvoering.
Mechanische sterkte	In de meeste UASB reactoren is de slibretentie gebaseerd op de aanwezigheid van korrelslib. Om een te groot verlies aan biomassa te voorkomen moeten de aanwezige slibkorrels voldoende weerstand bieden tegen interne en externe krachten om desintegratie en afschuring te beperken.
Ontwikkeling	Het ontstaan van korrelslib is gebaseerd op een continu proces van autoimmobilisatie van bacteriën. Slechts een geringe fractie van het korrelmateriaal bestaat uit inert dragermateriaal.

Korrelslib kwaliteit

Zoals reeds eerder genoemd is korrelslib niet essentieel voor de behandeling van afvalwater in een UASB reactor. In veel UASB reactoren wordt het functioneren echter wel degelijk bepaald door de kwaliteit van het aanwezige korrelslib. Het ligt voor de hand de korrelslib kwaliteit te relateren aan de mate waarin het korrelslib beschikt over de voor het functioneren van de UASB reactor benodigde eigenschappen. Een eenduidige definitie van korrelslib kwaliteit is echter niet te geven; de voor een optimale zuivering noodzakelijke slibeigenschappen verschillen per situatie.

Behalve de eisen die voor een optimaal functioneren aan korrelslib gesteld worden zijn ook de slibeigenschappen zelf afhankelijk van de toegepaste proces condities en de afvalwater samenstelling. In figuur 1 worden de hierbij betrokken relaties schematisch weergegeven. De figuur dient om de enorme complexiteit van de vele microbiologische en fysische factoren die bij het korrelvormingsproces betrokken zijn aan te geven, en pretendeert niet een compleet beeld van de verschillende oorzaak - gevolg relaties te geven.



Figuur 1 Schema van de complexe relaties tussen proces condities, afvalwatersamenstelling en korrel kwaliteit.

Korrel porositeit

Diffusie is het belangrijkste transport mechanisme voor opgelost substraat, tussenprodukten en eindprodukten in de korrel. Bij aanwezigheid van grotere poriën gaat ook convectie een rol spelen. Het transport door de korrel is dus afhankelijk van de diffusiesnelheid en het beschikbare porievolume. De porie diameter verdeling van de korrel lijkt bepalend voor de transport mogelijkheden van moleculen van verschillende diameter. Om een beter inzicht te krijgen in de betekenis van deze factoren is een, op size exclusion chromatografie gebaseerde, methode ontwikkeld waarmee het totaal beschikbare porievolume en de diameterverdeling van de in korrelslib aanwezige poriën bepaald kunnen worden (hoofdstuk 3).

De met deze methode verkregen resultaten geven aan dat het beschikbare porievolume voor de meeste slibsoorten varieert tussen de 40% en 80%. Sommige slibsoorten hebben echter een beschikbaar porievolume lager dan 10%, terwijl dit voor andere hoger is dan 95%. Het maximale porievolume is beschikbaar voor moleculen tot een molecuulgewicht van $\pm$ 1000 g/mol. Hieruit kan geconcludeerd worden dat eventuele segregatie van de in de korrel aanwezige populatie niet gebaseerd is op transport limitatie van stoffen met een lager molecuulgewicht (hoofdstuk 3).

Opvallend is dat de gevonden porositeit niet altijd correspondeert met optische waarnemingen aan het slib. Bij experimenten met bezonken rioolwater worden zeer grote en in belangrijke mate holle korrels gevormd (van der Last 1991). Het beschikbaar porievolume blijkt echter van slechts 7% te zijn. Kennelijk zijn er in deze korrels voor substraat ondoordringbare lagen aanwezig die grote delen van de korrel afsluiten. Doorsneden van deze korrels geven inderdaad een aanwijzing voor het bestaan van dit soort lagen (hoofdstuk 4).

Uit experimenten met naar diameter gefractioneerde korrelslib monsters (diameter (mm) 0.5-1.0, 1.0-2.0 en >2.0) blijkt dat het beschikbare porievolume afhankelijk is van de diameter van de korrels: kleine korrels hebben een duidelijk hogere porositeit dan de grotere korrels uit het zelfde slibmonster (hoofdstuk 4). Het lagere beschikbare porievolume van grotere korrels kan mogelijk verklaard worden te stellen dat de grotere slibkorrels uit een monster gemiddeld ouder zullen zijn. Door een continue groei van bacteriën in de korrels ontstaat een steeds dichtere pakking van het korrelmateriaal en neemt de porositeit af.

Een andere verklaring voor de met toenemende korrelgrootte afnemende porositeit kan zijn dat in de grotere korrels de beschikbare poriën verstopt raken, mogelijk door grote moleculen die vrijkomen bij het lyseren van de aanwezige bacteriën.

Substraat transport capaciteit

Zowel korrelgroei als de korrelkwaliteit zijn in belangrijke mate gerelateerd aan de transportcapaciteit van substraat en produkten door de korrel. Substraat transport limitatie leidt tot een lage slibactiviteit en kan leiden tot korreldesintegratie (hoofdstuk 6). Limitatie van gastransport kan leiden tot het ophopen van biogas in de korrel, en zo de uitspoeling van slib veroorzaken (hoofdstuk 8).

Zoals reeds genoemd is het transport van opgelost substraat en intermediaren door de korrel voornamelijk gebaseerd op diffusie (Beeftink 1987, Dolfing 1985, Goodwin et al. 1991). Middels een eenvoudige test werd de diffusie constante voor acetaat voor verschillende korrelslib soorten geschat op 0.5 tot 1.0 x 10^{-9} $m^2.s^{-1}$ (hoofdstuk 4). De verkregen waarden liggen dicht bij de diffusie constante van acetaat in water en komen overeen met de waarden die door Henze en Harremoës (1983) genoemd worden. Waarschijnlijk doordat de slibmonsters in de test krachtig werd geroerd zijn deze waarden vier tot tien maal groter dan de waarden welke door Nilsson en Karlsson (1989) en door Kitsos et al. (1992) gevonden werden met rustiger methoden.

De substraat transport snelheid bepaald (in combinatie met de activiteit en de pakking van de aanwezige bacteriën) de penetratiediepte van het substraat in de korrel. De invloed van substraat limitatie in het centrum van korrelslib werd onderzocht aan

de hand van verschillende diameter fracties van slib uit praktijk installaties (hoofdstuk 4). Zowel voor de intacte korrels als voor gemalen slibmonsters werd de maximale methanogene activiteit en de Monod-K_s waarde gemeten. De maximale methanogene activiteit blijkt af te nemen met een grotere korreldiameter. De maximale methanogene activiteit van de verschillende fracties wordt echter nauwelijks beïnvloed door het malen. Waarschijnlijk bevatten de grotere korrels een hoger gehalte inactief organisch materiaal.

In overeenstemming met de veronderstelde rol van substraat transport limitatie blijkt het malen van de slibkorrels bij twee van de onderzochte slibsoorten te leiden tot een lagere K_s waarde. Het ontbreken van een effect van het malen bij een derde type slib kan verklaard worden door te stellen dat de methanogene activiteit in dit slib beperkt is tot een specifieke laag; mogelijk zijn alleen in de buitenste regionen van de korrel actieve bacteriën aanwezig en zijn de bacteriën in de korrelcentra door substraatgebrek afgestorven. In dergelijke gevallen, en ook wanneer een actieve methanogene kern wordt omgeven door een mantel van niet methanogeen organisch materiaal (b.v. verzurende bacteriën) zal het malen van het slib niet leiden tot een lagere K_s waarde voor het substraat voor de methanogene populatie (figuur 2).

Substraat concentratie en slibbelasting

Uit de resultaten van het in hoofdstuk 6 beschreven onderzoek blijkt dat gemiddelde de korreldiameter toeneemt bij de toepassing van hogere slibbelastingen. Een vergelijkbare relatie is door Grotenhuis et al. (1991a) gevonden tussen de gemiddelde korreldiameter en de influent concentratie. Het is aannemelijk dat beide relaties gebaseerd zijn op een toename van de substraatconcentratie in de reactor, en dus op een toename van de substraat penetratie diepte in de korrel. De mengeigenschappen van de reactoren zijn onvoldoende bekend om onderscheid te kunnen maken tussen de invloeden van de slibbelasting en de substraat concentratie.

Stabiel korrelslib kan voor meer dan 95% uit organisch materiaal bestaan, voor het overgrote deel (afkomstig van) levende bacteriën. Het is waarschijnlijk dat bacteriën een essentiële rol spelen bij de korrelslib stabiliteit. Afsterving van de bacteriën, b.v. door substraat gebrek, kan dan leiden tot een ernstige verzwakking van de korrels. Dit kan onder andere optreden bij een plotselinge wijziging van de procescondities zoals de slibbelasting, de influent concentratie, de substraatsamenstelling, de proces temperatuur en bij een totaal gebrek aan substraat, b.v. tijdens de opslag van korrelslib.

Indien actief biologisch materiaal in belangrijke mate bijdraagt aan de korrelstabiliteit zal de gemiddelde korreldiameter gerelateerd zijn aan de penetratie diepte van het substraat. Beeftink (1987) heeft aangetoond dat substraat transport limitatie bij

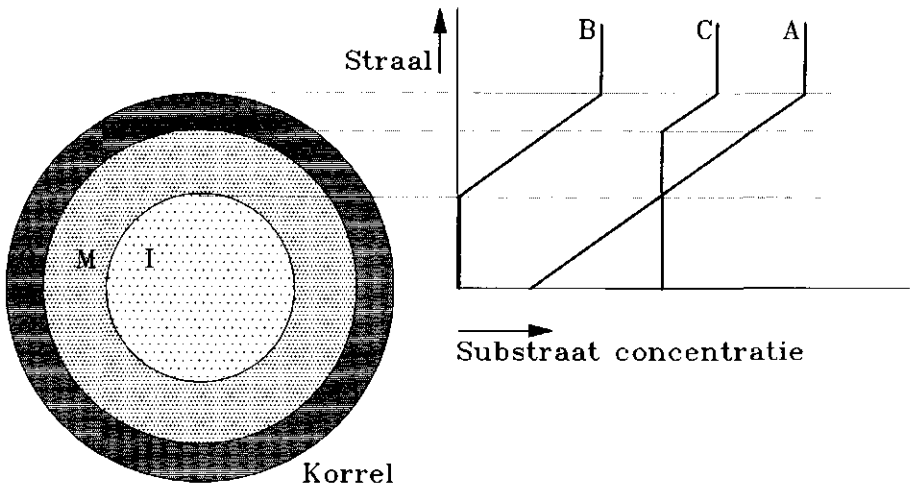
verzurende aggregaten in gaslift reactoren leidt tot holle, en als gevolg hiervan mechanisch zwakke, aggregaten. Bij methanogeen korrelslib zullen met name de met interne gasproduktie gepaard gaande krachten in dergelijke gevallen leiden tot korrelslib desintegratie. Bovendien zullen holle korrels zeer snel uitspoelen als gevolg van ophoping van biogas.

Substraat limitatie in het korrelcentrum treedt op indien de straal van de slibkorrel groter is dan de substraat penetratie diepte (figuur 2). Dergelijke korrels ontstaan indien de groei van nieuwe bacteriën onvoldoende wordt gecompenseerd door afschuring, door korreldesintegratie, door uitspoeling of door het afvoeren van spuislib. Met andere woorden, substraat limitatie treedt op wanneer de korrels te groot zijn voor de gegeven omstandigheden (hoofdstuk 4 en 6).

De vorming van zeer grote, holle korrels in reactoren gevoed met huishoudelijk afvalwater met een lage influent concentratie en een lage influent temperatuur (van der Last 1992) lijkt in tegenspraak met de hiervoor beschreven relaties. In feite illustreren deze korrels echter de cruciale en complexe rol die de biologische activiteit speelt bij de bepaling van de korreldiameter. Door de relatief hoge oplosbaarheid van methaan en de lage gasproduktie onder deze condities zijn de interne en externe krachten op het korrelslib beperkt, waardoor ook zwakke en holle korrels zich in de reactor kunnen handhaven.

Een van de kenmerkende eigenschappen van methanogeen korrelslib is dat het gedurende lagere tijd zonder voeding kan worden opgeslagen zonder dat noemenswaardige schade optreedt (Hulshoff Pol 1989, Wu et al. 1985). De in hoofdstuk 6 waargenomen desintegratie van korrelslib moet in verband hiermee waarschijnlijk worden gerelateerd aan de afsterving van de in de slibkorrels aanwezige verzurende populatie. Uit in hoofdstuk 8 beschreven experimenten blijkt dat de decay rate (de snelheid waarmee de bacteriën uit elkaar vallen) van verzurende bacteriën ($\pm$ 0.15 d^{-1}) tussen de 15 en 100 maal hoger ligt dan de afsterfsnelheid van de methanogene populatie in korrelslib ($\pm$ 0.005 d^{-1}). Als gevolg hiervan zullen slibkorrels die voor een groot gedeelte uit verzurend bacteriemateriaal bestaan beduidend kwetsbaarder zijn voor conditie veranderingen dan slibkorrels die voornamelijk uit methanogene bacteriën bestaat.

Bij de opstart van reactoren moet bij voorkeur gebruik gemaakt worden van entslib met een zo laag mogelijk gehalte aan verzurende bacteriën. Een interessant aspect hierbij is dat de afsterfsnelheid van *Methanothrix* (de afname van de activiteit) hoger is dan de snelheid waarmee deze bacteriën uiteen vallen de Zeeuw (1984). Mogelijk worden methanogene slibkorrels ook na het afsterven van een aanzienlijk deel van de actieve populatie gestabiliseerd door de aanwezige methanothrix ketens.



Figuur 2 Schematische weergave van de relatie tussen de substraatconcentratie in de bulkvloeistof in de reactor en korreldiameter. A: De substraatconcentratie in de bulk en de substraatgradiënt in de korrel verhouden zich zodanig dat het substraat tot in het korrelcentrum kan doordringen, de hele korrel is actief B: De substraatconcentratie in de bulk is te laag ten opzichte van de substraatgradiënt in de bulk. Het substraat dringt alleen in de buitenste lagen van de korrel door. De bacteriën in het korrelcentrum (I) zijn niet actief en sterven na verloop van tijd door gebrek aan substraat. C: Het substraat wordt alleen in de buitenste laag van de korrel afgebroken. Het centrum (M en I) bestaat, mogelijk als gevolg van een situatie als in B, uit inactief materiaal.

De samenstelling van het afvalwater

De korrelslib kwaliteit wordt sterk beïnvloed door de samenstelling van het afvalwater. Direct na een verandering in substraatsamenstelling moet de aanwezige populatie adapteren aan de nieuwe situatie. Op de langere termijn zullen alle korreleigenschappen zich (moeten) aanpassen aan de nieuwe situatie, en veranderd de populatie. In het in dit proefschrift beschreven onderzoek zijn een aantal aspecten van de relatie tussen de samenstelling van het afvalwater en het functioneren van korrelslib onderzocht: de invloed van gesuspendeerd materiaal in het influent (hoofdstuk 7), de invloed van onverzuurd materiaal in het influent (hoofdstuk 8) en de invloed van in het influent aanwezig sulfaat (hoofdstuk 9 - 11). Ten slotte is invloed van fosfaatgebrek op het functioneren van het slib onderzocht (hoofdstuk 12).

Gesuspendeerd verzuringsslib UASB influent

De aanwezigheid van gesuspendeerd materiaal in het influent wordt gezien als een belangrijke oorzaak van problemen bij de behandeling van afvalwater met UASB

reactoren (Hulshoff Pol 1989; Lettinga et al. 1985). Een van de meest frequent in UASB influent voorkomende typen gesuspendeerd materiaal bestaat uit gesuspendeerde verzuringsbacteriën. Bij de (voor) verzuring van afvalwater kunnen relatief grote hoeveelheden verzuringsbacteriën gevormd worden. De gesuspendeerd in het influent aanwezige bacteriën kunnen worden meegevoerd naar de UASB reactor. Uit de in hoofdstuk 7 gepresenteerde resultaten blijkt dat dit tot een zeer ernstige slibflotatie kan leiden; na verloop van tijd blijken de in de reactor aanwezige korrels samen te klonteren tot grotere aggregaten waartussen zich biogas ophoopt. Experimenten met ultrasoon gedesintegreerde verzuringsbacteriën geven aan dat dit niet direct aan het gesuspendeerde karakter van het materiaal te wijten is. Mogelijk wordt het verkleven van de individuele korrels veroorzaakt door een afbraakprodukt van het gesuspendeerd materiaal, of door groei van specifiek op dit materiaal groeiende bacteriën.

In verband met een stabiele procesvoering van methanogene UASB reactoren wordt het sterk aanbevolen het in het influent aanwezige gesuspendeerd materiaal te verwijderen vóór het de reactor wordt ingevoerd.

De invloed van de mate van voorverzuring van het afvalwater

Korrelslib bevat over het algemeen alle bacterie species die noodzakelijk zijn voor de totale omzetting van de in het afvalwater aanwezige organische verontreiniging. In reactoren die een gedeeltelijk onverzuurd afvalwater behandelen zullen zich slibkorrels ontwikkelen die voor een belangrijk gedeelte uit verzurende bacteriën bestaan. De populatiesamenstelling (en dus de korrelkwaliteit) wordt sterk bepaald door de mate van voorverzuring van het afvalwater. In hoofdstuk 8 worden een aantal experimenten beschreven waarin werd onderzocht inhoeverre een methanogeen UASB systeem wordt beïnvloed door niet verzuurd afvalwater.

Onverzuurde gelatine blijkt tot een slibbelasting van 1.2 gCOD.(gVSS.d)$^{-1}$ zonder problemen behandeld te kunnen worden in een één-fase UASB reactor. In deze reactoren wordt bovendien korrelslib van een zeer goede kwaliteit gevormd.

De behandeling van sucrose houdend afvalwater blijkt daarentegen slechts mogelijk bij een slibbelasting lager dan 0.5 gCOD.(gVSS.d)$^{-1}$. Zowel de resultaten van de uitgevoerde experimenten als van modelberekeningen geven aan dat bij hogere slibbelastingen de methanogene populatie verdrongen wordt door een snelle aanwas van verzurende bacteriën. Bovendien geven de experimenten aan dat onverzuurde sucrose in het reactor influent aanleiding kan geven tot problemen m.b.t. de slibretentie. Een sterke groei van los aan het korreloppervlak gebonden of gesuspendeerd levende draadvormende verzurende bacteriën veroorzaakt een daling van de sedimentatiesnelheid van het slib en een stijging van de slib volume index. Vergelijkbare problemen zijn waargenomen door Anderson et al. (1991); Hulshoff Pol et al (1983b) en Mendez-Rapin et al. (1986). Daarnaast treedt uitspoeling van individuele korrels op door-

dat zich biogas opgehoopt tussen de oorspronkelijke methanogene korrel en een hier omheen groeiende laag van verzurende bacteriën.

In praktijk reactoren zijn de opwaartse vloeistofsnelheid en de gassnelheid vele malen hoger dan in de gebruikte laboratorium reactoren. Bij de laboratorium experimenten kon een belangrijk gedeelte van de in het slibbed aanwezige verzurende bacteriën relatief makkelijk verwijderd worden door het slibbed te roeren. Hierdoor werd een belangrijke verbetering van het zuiveringsproces bereikt. In verband hiermee geven de experimenten mogelijk een te somber beeld van de problemen. Gesteld wordt dat eventuele problemen bij de behandeling van onverzuurd afvalwater als gevolg van gesuspendeerde groei van bacteriën door een (periodieke) intensieve menging van het slibbed kunnen worden verminderd. De behandeling van onverzuurd afvalwater in een eentraps UASB reactor systeem is goed mogelijk. Wel moet in een aantal situaties rekening gehouden worden met een relatief lage maximale slibbelasting.

Substraat competitie tussen methaan producerende bacteriën (MPB) en sulfaat reducerende bacteriën (SRB)

De competitie om substraat tussen sulfaat reducerende bacteriën (SRB) en methaan producerende bacteriën (MPB) bij de behandeling van sulfaat houdend afvalwater kan grote consequenties hebben voor de kwaliteit van het korrelslib.

Hulshoff Pol (1989) noemt de specifieke selectie voor goed bezinkbare deeltjes als één van de belangrijkste condities die de vorming van methanogeen korrelslib stimuleren. De resultaten van de in hoofdstuk 9 beschreven experimenten geven aan dat dit ook bij de behandeling van sulfaat-houdend afvalwater geldt: korrelvorming wordt gestimuleerd door een korte vloeistof verblijftijd en een hoge opwaartse vloeistofsnelheid in de reactoren. Het feit dat een snellere korrelvorming in deze experimenten gekoppeld is aan een hoger percentage methaan producerende bacteriën wijst op een belangrijke rol van de methanogene populatie bij de vorming van korrelslib in UASB reactoren.

Ondanks het feit dat SRB zowel m.b.t. de thermodynamische als kinetisch eigenschappen ten opzichte van MPB in het voordeel zijn, zijn er vele aanwijzingen dat MPB zich, ook bij aanwezigheid van sulfaat in het afvalwater, prima in high rate anaërobe reactoren kunnen handhaven. Als mogelijke verklaring worden door Isa et al. (1986a,b) de slechtere hechtingseigenschappen van de SRB genoemd. Uit de in hoofdstuk 10 beschreven experimenten blijkt dat de methanogene populatie inderdaad een belangrijke rol speelt bij de vorming van korrelslib. In een puur sulfaat reducerend systeem werd ook na 160 dagen niet of nauwelijks korrelslib vorming waargenomen, terwijl korrelvorming in een vergelijkbaar gemengd sulfaatreducerend /

methaan producerend systeem binnen 100 dagen duidelijk waarneembaar is. In afwezigheid van sulfaat wordt reeds na 50 dagen korrelvorming waargenomen.

In tegenstelling tot de verklaring van Isa et al. (1986a,b) voor de relatieve dominantie van MPB in high rate reactoren blijkt uit de in hoofdstuk 9 en 10 beschreven experimenten dat sulfaat reducerende bacteriën goed in staat zijn om zich om in gemengde sulfidogeen / methanogeen korrelslib te handhaven. Ook zijn de SRB in staat de korrelstructuur te handhaven wanneer de methanogene populatie selectief wordt afgedood.

In hoofdstuk 11 wordt bovendien aangetoond dat de sulfaatreducerende bacteriën onder bepaalde condities over uitstekende hechtingseigenschappen blijken te beschikken, en ook zonder de aanwezigheid van een methanogene populatie in staat zijn om zich te handhaven in high rate anaërobe reactoren. Op puimsteen als dragermateriaal worden in een fluidized bed reactor stabiele, puur sulfidogene aggregaten gevormd.

Mogelijk moet de slechte korrelvorming in sulfidogene UASB reactoren niet verklaard worden door een gebrek aan hechtingscapaciteit van de SRB, maar is dit veeleer terug te leiden tot een te lage selectiedruk in sulfidogene systemen. Door de zeer geringe biogasproduktie in deze systemen is de uitspoeling van vlokkig materiaal (en dus de noodzaak tot aggregatie) veel geringer dan in vergelijkbare methanogene reactoren; waarschijnlijk is een zeer hoge opwaartse vloeistofsnelheid essentieel om een voldoende selectie richting immobilisatie te verkrijgen.

Het verloop van de methaan producerende- en sulfaat reducerende activiteit bij gebruik van substraat met een hoge sulfaat/COD verhouding geeft aan dat de sulfaat reducerende populatie in deze situaties op de lange termijn de methanogene populatie zal gaan overheersen.

Nutriënt gebrek

Een optimale biologische zuivering van afvalwater kan slechts plaatsvinden wanneer de voor de aanwezige bacteriën noodzakelijke nutriënten en spore elementen in voldoende mate beschikbaar zijn (Hulshoff Pol et al. 1983a; Speece 1987).

Uit de in hoofdstuk 12 beschreven resultaten blijkt dat fosfaatgebrek een duidelijk negatieve invloed heeft op het zuiveringsrendement van UASB reactoren. De korrelkwaliteit lijkt echter op korte termijn niet ernstig te worden beïnvloed. De negatieve invloed van fosfaatgebrek op de methanogene activiteit van korrelslib is volledig reversibel. In aanwezigheid van organisch substraat blijkt fosfaat zeer snel door fosfor gelimiteerd korrelslib te worden opgenomen.

Het fosfor gehalte in het korrelslib van de laboratorium reactoren gevoed met gelatine varieert tussen de 6 $mgP.gVSS^{-1}$ voor slib uit reactoren gevoed met een vrij-

wel fosfaatvrij influent, tot 10.5 mgP.gVSS^{-1} voor slib uit reactoren met een voldoende aanbod van fosfaat. Deze gehaltes komen overeen met de door Speece en McCarty (1964) en Scherer et al. (1983) gegeven fosfor gehaltes voor methanogeen slib en methanogene bacteriën. De (veel) hogere fosfaat gehaltes die soms in slib van praktijkreactoren worden gevonden (tot 45 mgP.gVSS^{-1}), worden waarschijnlijk veroorzaakt door de aanwezigheid van fosfaat precipitaten.

Fosfaatgehaltes in het slib hoger dan 10.5mg P.gVSS^{-1} zijn een indicatie dat een eventuele fosfaat dosering teruggebracht kan worden. Fosfaatgehaltes lager dan $\pm$ 8 mgP.gVSS^{-1} wijzen daarentegen juist op fosfaat limitatie van het slib.

Door de snelle opname van fosfaat door fosfaat gelimiteerd korrelslib en door de snelle respons van de methaanproduktiesnelheid wanneer fosfaat wordt toegevoegd is fosfaatgebrek in anaërobe reactoren makkelijk aantoonbaar: een sterke toename van de methanogene activiteit na fosfaat dosering in een (in eerste instantie fosfaatvrije) activiteitstest, en/of een snelle afname van het fosfaatgehalte in de vloeistof zijn duidelijke aanwijzingen voor fosfaatgebrek van het korrelslib. Mogelijk zijn vergelijkbare testen bruikbaar om limitatie van andere nutriënten en spore elementen aan te tonen.

TROUBLE SHOOTING

Verschillende factoren zijn van invloed op de slibaanwas, de slibkwaliteit en het functioneren van korrelslib in de reactor. Veel problemen met UASB reactoren in de praktijk zijn gerelateerd aan een te lage slibgroei of een te sterke uitspoeling van slib. Ook een te lage activiteit van het in de reactor aanwezige slib kan de zuivering van afvalwater negatief beïnvloeden. In tabel 2 wordt een zeer compacte samenvatting van de voor de praktijk relevante aspecten van het uitgevoerde onderzoek gegeven. Hierbij moet worden opgemerkt dat het bij veel problemen in de praktijk moeilijk is om onderscheid te maken tussen oorzaken, gevolgen en bijkomende zaken.

Tabel 2 Schematisch overzicht van de in dit onderzoek waargenomen problemen m.b.t. het functioneren van UASB reactoren.

	Probleem		Oorzaak		Remedie
1	Onvoldoende slib groei	1.1a	Gebrek aan nutriënten en / of spore elementen.	1.2a	Verhogen of aanpassen van de nutriënt en / spore element dosering.
		1.1b	Een te hoge mate van voorverzuring.	1.2b	Verlaging van de mate van voorverzuring.
		1.1c	Een te lage slibbelasting.	1.2c	Verhoging COD invoer, verwijderen slib.
		1.1d	Uitspoeling korrelslib (zie 4, 5).		
		1.1e	Uitspoeling vlokkig slib (zie 6).		
2	Overbelasting, onvoldoende methanogene capaciteit.	2.1a	Onvoldoende slib aanwezig.	2.2a	Verlagen slibbelasting. Opvoeren van de slibhoeveelheid: enten met korrelslib, slibgroei stimuleren (zie 1), slibuitspoeling beperken (zie 3-6).
		2.1b	Onvoldoende methanogene activiteit (zie 3).	2.2b	Verlagen slibbelasting, verhogen (methanogene) activiteit slib ((zie 3.2)
3	Onvoldoende methanogene activiteit.	3.1a	Gebrek aan nutriënten en/of spore elementen (zie 1.1a).		
		3.1b	Een te sterke groei van verzurende bacteriën.	3.2b	Opvoeren van de mate van voorverzuring. Verlagen van de slibbelasting.
		3.1c	Accumulatie van inert organisch materiaal in het slib.	3.2c	Wegnemen organisch materiaal uit influent.
		3.1d	Te lage proces temperatuur.	3.2d	Opvoeren temperatuur.
		3.1e	blootstelling slib aan toxische componenten / condities (zie 6.1d).		
4	Uitspoeling korrels.	4.1a	Gas ophoping in holle korrels. Te grote korrels door te lage krachten: lage temperatuur, lage belasting, lage influent concentratie (zie ook 6.1a,b).		verhogen krachten op korrels, grote korrels verwijderen / breken.
		4.1b	Gas ophoping in gelaagde korrelstructuur. Groei van verzuringsslib op korreloppervlak.	4.2b	Toepassen meer constante proces condities, Verhoging mate van voorverzuring, verlaging slibbelasting.

5	Uitspoeling slib, vorming van volumineus slib.	5.1a	Samenklonteren individuele slibkorrels tot grote aggregaten, gerelateerd aan inspoeling gesuspendeerd verzurings slib.	5.2a	Verwijderen verzuringsslib uit influent reactor, verlaging mate van voorverzuring.
		5.1b	Sterke groei van gesuspendeerde of zeer los gepakte verzurende bacteriën in reactor.	5.2b	Verhogen van mate van voorverzuring, verhogen van menging van slibbed.
		5.1c	Vorming van zeer slappe korrels, sterke ingroei van verzurende bacteriën.	5.2c	Verhogen van de mate van voorverzuring, verlagen slibbelasting.
6	Uitspoeling vlokkig slib, korrel desintegratie.	6.1a	"Uitgestelde" opstart problemen samenhangend met 6.1 b-d.	6.2a	Andere opstart strategie (sneller opvoeren slibbelasting), gebruik ander entslib.
		6.1b	Plotselinge variaties is slib belasting en/of influent concentratie.	6.2b	Toepassen meer stabiele proces condities in UASB.
		6.1c	Plotselinge verhoging van de mate van voorverzuring, afsterving verzurende organismen.	6.2c	Toepassen meer stabiele proces condities (bij opstart gebruik ander entslib).
		6.1d	(periodieke) blootstelling aan toxische componenten en/of lethale condities.	6.2d	Wegnemen of neutraliseren van toxische component, aanpassen procescondities, langere adaptatie periode, grotere hydraulische buffer.
		6.1e	Te grote mechanische krachten	6.2e	Verminderen mechanische krachten (aanpassing invoer systeem, effluent recirculatie), verlaging belasting.
		6.1f	Vorming vlokkig slib door te lage selectiedruk.	6.2f	Bij stabiele procesvoering in principe geen probleem, anders selectiedruk verhogen door effluent recirculatie.

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CURRICULUM VITAE

Pieter Arne Alphenaar werd op 29 oktober 1959 geboren in Wormerveer. In 1978 behaalde hij het Atheneum B diploma aan de scholengemeenschap "Bertrand Russell" te Krommenie en start hij met studeren aan de Landbouwhogeschool te Wageningen. In 1987 werd de studie Milieuhygiëne, specialisatie waterzuivering, voltooid. Van oktober 1987 tot juli 1992 was hij als tijdelijk wetenschappelijk medewerker verbonden aan de vakgroep Milieutechnologie van de Landbouwuniversiteit en werkte hij aan het in dit proefschrift beschreven onderzoek.
Samen met Kees Theunis werd Macro Consult Wageningen opgericht, een bedrijf dat zich bezig houdt met dienstverlening op het gebied van microscopisch onderzoek.

Sinds 1992 werkt hij als onderzoeker bij de Research and Development afdeling van Tauw Milieu, een ingenieursbureau in Deventer, aan oplossingen voor de problemen rond verontreinigde bodems.

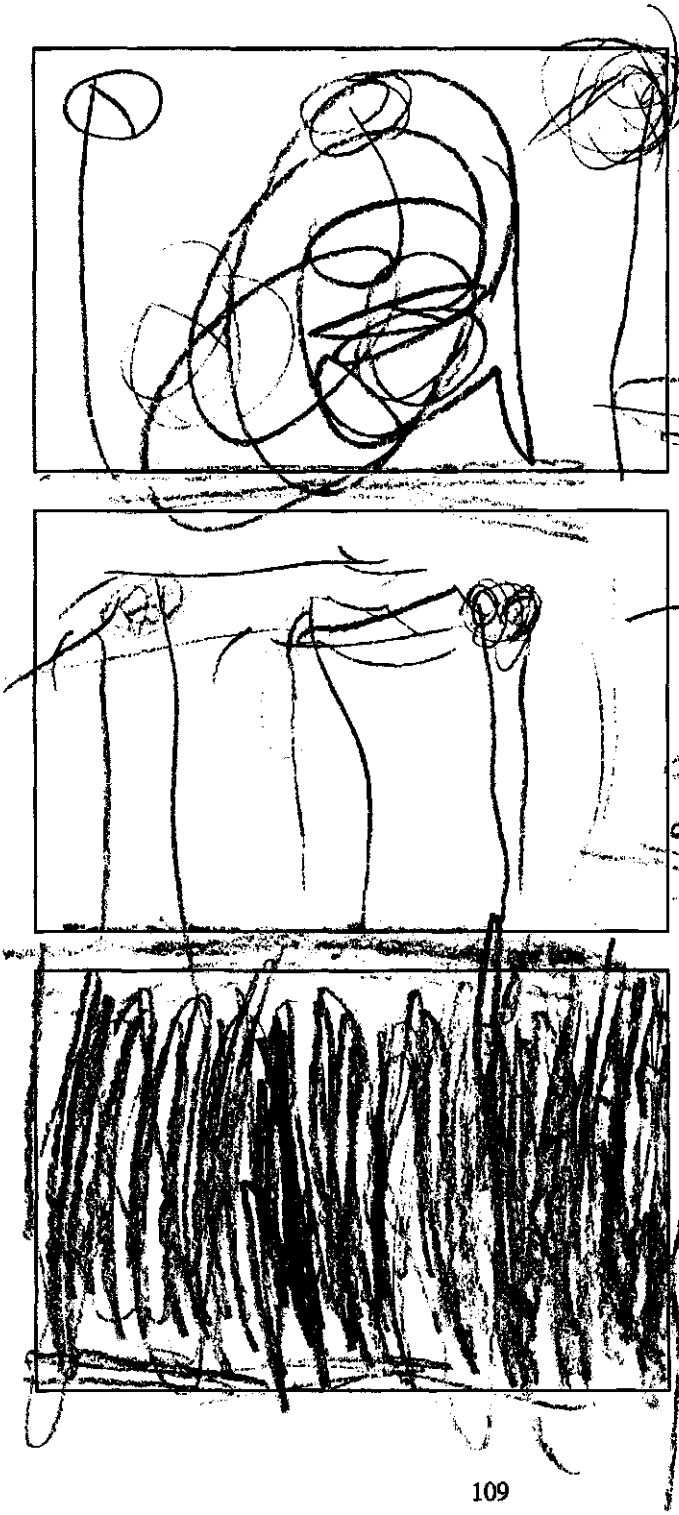

Figure 6

a: Example of the external layer developed when granular sludge was fed with 30% non-acidified sucrose during 30 days. Sludge loading rate: 0.5 gCOD.(gVSS.d)$^{-1}$, HRT 5.5 hr. Bar indicates 1 mm.

b: Granular sludge fed with 25% non acidified sucrose during 55 days. Sludge loading rate 1.2 gCOD.(gVSS.d)$^{-1}$, HRT 9.4 hr. Bar indicates 1 mm.

c: Sludge washed out from a papermill wastewater treating UASB reactor. Gas entrapment in methanogenic granules covered with newly formed (acidogenic) biomass. Bar indicates 1 mm.