Sulfate reducing processes at extreme salinity and temperature:
extending its application window

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Marcus Vinícius Guerini Vallero

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des namiddags te vier uur in de Aula

Cover: Two bioreactor concepts (UASB and SAMBaR) utilized in the investigations described in this thesis. The inserts show the respective view of the macro (sludge type) and micro (scanning electron and light microscopy) of the biomass present in each reactor concept.

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meu irmão Marcelo

e a Marina

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ABSTRACT

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The characteristics of various sulfate-rich wastewaters, such as temperature, pH and salinity, are determined by the (industrial) process from which they originate, and can be far from the physiological optima of the sulfur cycle microorganisms. The main goal of the research described in this thesis was to investigate and develop high rate sulfate reducing wastewater treatment processes for the treatment of inorganic sulfate-rich wastewaters under extreme conditions, i.e. high temperature and high salinity. In this thesis, several simple organic bulk chemicals were tested as electron donor, viz. lower alcohols (methanol and ethanol) and volatile fatty acids (formate, acetate and propionate).

With respect to the start-up of upflow anaerobic sludge bed (UASB) reactors at high salinity or high temperature, the results obtained in this investigation indicate that the appearance of a targeted metabolic property (sulfate reduction at high salinity or at high temperature) is independent of the strategy for biomass acclimation (direct exposure vs. stepwise exposure). The stepwise adaptation of thermophilic sulfidogenic methanol degrading biomass to a high osmolarity environment, both at 55°C or at 70°C, likely does not occur in UASB reactors, as probably no methanol halotolerant thermophilic sulfate reducing bacteria (SRB) were present in the thermophilic inoculum sludge used in the investigations described in this thesis. Exposing the sludge directly to a very high salinity (50 g $NaCl.L^{-1}$) stimulated the growth of a mesophilic (30°C) propionate- and ethanol-utilizing halotolerant SRB population, which supported high rate sulfate reduction (up to 3.6 g $SO_4^{2-}.L^{-1}.day^{-1}$) in a UASB reactor. The start-up of thermophilic (55 to 65°C) and extreme thermophilic (70°C or higher) anaerobic bioreactors inoculated with mesophilic sludges at the targeted temperature proceeded fast and stable, as it provoked the rapid selection of (extreme) thermophiles. Therefore, the key for the successful treatment of high salinity or hot wastewaters is to invest enough time for the growth of the targeted microorganism in the biomass.

The results of this investigation show that the competition between SRB, methane producing archaea and acetogenic bacteria for substrate is highly dependent of the type of substrate and operational conditions imposed to the bioreactor. This thesis describes a situation where the production of acetate and methane was completely suppressed in methanol-fed sulfate reducing UASB reactors operated at 70°C. As a result, for the first time a fully sulfate reducing granular sludge has been cultivated in a methanol-fed thermophilic sulfate reducing reactor (with sulfate reduction rates as high as 14.4 g $SO_4^{2-}.L^{-1}.day^{-1}$), provided that an operational temperature of 70°C is kept. The production of methane can be easily suppressed in thermophilic methanol fed reactors, either by running the reactor at temperatures equal or higher than 65°C or by exposing 55°C operated reactors to a short (2 days) temperature (65 – 70°C) shock. Methanogenesis can also be easily suppressed in mesophilic propionate- and ethanol-fed reactors, provided high salinity conditions prevail (e.g. above 50 $mS.cm^{-1}$). It seems, however, that the production of acetate, with the exception of methanol-fed reactors operated at 70°C, is unavoidable both in thermophilic and mesophilic reactors.

This thesis also describes the use of specialized microorganisms, the halophilic SRB *Desulfobacter halotolerans*, in bioreactors for the treatment of saline sulfate-rich wastewaters. Very high specific sulfate reduction rates (up to 6.6 g $SO_4^{2-}.gVSS^{-1}.day^{-1}$) can be obtained in completely mixed tank reactors where the biomass grows in suspension and can be efficiently retained by membranes which are submerged in the reactor system. This investigation showed that anaerobic membrane bioreactors can be operated over extended periods of time at a fixed flux, if this flux is substantially below the nominal critical flux determined experimentally (18-21 $L.m^{-2}.h^{-1}$). Chemical cleaning of the membranes will be required only at about 106 days, as long a low constant flux is imposed (4.7 $L.m^{-2}.h^{-1}$) and intermittent backflush (e.g. 1 minute each 10 minutes) is adopted as operational strategy.

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Chapter 1

Introduction

INTRODUCTION

The presence of high sulfate concentrations in specific wastewaters restricts the application of the anaerobic treatment technology, due to the production of the toxic, corrosive and odorous hydrogen sulfide (H_2S). The H_2S formation results from the proliferation of sulfate reducing bacteria (SRB) in anaerobic bioreactors, where they compete with methane producing archaea (MPA) and homoacetogenic bacteria (AB) for common substrates such as hydrogen, acetate and methanol (Lens *et al.*, 1998). For achieving an effective methanogenesis, a complete suppression of sulfate reduction and a complete conversion of the organic substrate into methane is the most desired option. In the last two decades, however, progress has been made with novel biotechnological processes that utilize sulfate reduction conversions up to a point that these processes are now successfully introduced into the market. These processes include the production of biogenic sulfide for heavy metal removal from heavy metal laden wastewaters and acid mine drainage as well as biological desulfurization of natural gas, flue gases, LPG, oil and rubber.

The characteristics of various of these wastewaters, such as temperature, pH and salinity, are determined by the (industrial) process from which they originate, and can be far from the physiological optima of the sulfur cycle microorganisms (Lens and Kuenen, 2001). In addition, constraints in water supply as well as restrictive environmental regulations have been stimulating industrial processes to re-examine their water management strategies. As a result, reuse/recycling of process water is becoming a valuable tool to reduce both fresh water intake and effluent disposal (Levine and Asano, 2002). One of the major problems of recycling process water by deliberately closing the water loops is the toxicity that can be exerted due to build up of high salt concentrations in the circuit water. In addition to high salinity, certain streams from chemical factories and in the biodesulfurization of flue gases are hot wastewaters that must be treated before their reuse in the process. The easiest way to guarantee the activity of SRB present in SRB-based bioreactors when they need to be applied under these extreme conditions would be the modification of the characteristics of these parameters towards the physiological optima of the microorganisms, e.g. by dilution (for saline streams), pH correction (for acid or alkaline streams) and cooling (for very hot streams) of the sulfate-rich wastewater prior to entering in the bioreactor. As this strategy is limited in case of water shortage or very high salinity (for saline streams) or in cases which require the direct reuse of the treated hot water into the process (for hot streams), treatment systems that are tolerant to high salt concentrations and/or hot temperatures must be developed in order to efficiently treat these types of (waste)waters to a quality allowing their final discharge or enabling their reuse in a given (industrial) process. For the biological desulfurization of inorganic wastewaters, two factors are of paramount importance to determine the economic feasibility of a selected electron donor (van Houten, 1996): (1) the cost of the electron donor

needed to support the sulfate reduction process and (2) the need for minimization of formation of undesired side-products, such as methane and acetate.

The main goal of the research described in this thesis was to investigate and develop high rate sulfate reducing wastewater treatment processes for the treatment of inorganic sulfate-rich wastewaters under extreme conditions, ie. **high temperature and high salinity**. In this thesis, several simple organic bulk chemicals were tested as electron donor, viz. lower alcohols (methanol and ethanol) and volatile fatty acids (formate, acetate and propionate).

STRUCTURE OF THIS THESIS

Chapter 2 presents a review of the current knowledge of sulfur-cycle based bioprocesses, covering both microbiological and technological aspects of the treatment of sulfur rich waste streams involving the formation of metal sulfides and elemental sulfur. **Chapter 3** presents the investigation on the effect of increasing sulfate concentrations (COD/SO_4^{2-} of 10, 5 and 0.5) on the anaerobic treatment of methanol containing wastewater in an upflow anaerobic sludge bed (UASB) reactor operated at a temperature of 55°C. The investigation focused on the effect of the presence of sulfate on conversion rates, metabolic shifts and possible process disturbances. **Chapter 4** presents the investigation on the feasibility and constraints of thermophilic (55 to 65°C) and extreme thermophilic (55 to 80°C) sulfate reducing processes (COD/SO_4^{2-} = 0.5) in methanol- or formate-fed UASB reactors inoculated with mesophilic granular sludge. It is also presented the use of temperature as a tool to steer the competition to sulfide as well as the negative effect of high NaCl concentrations towards methanol degrading thermophilic (70°C) granular sludges. **Chapter 5** highlights the negative effects of NaCl (25 $g.L^{-1}$) on thermophilic (55ºC) methanol conversion in the presence of excess of sulfate (COD/SO_4^{2-} = 0.5) in UASB reactors inoculated with granular sludge previously not adapted to NaCl.

In view of the observed process constraints due to the presence of high NaCl concentration in thermophilic sulfate reducing reactors (presented in **Chapters 4 and 5**), further research was focused on the development of strategies to overcome the salt stress to bacterial cells present in sulfate reducing bioreactors. **Chapter 6** presents the investigation on the assessment of the stepwise addition of NaCl on the acclimation of a sludge of a methanol-fed sulfate reducing thermophilic (55°C) UASB reactor operating at increasing NaCl concentrations (0.5 to 12.5 g $NaCl.L^{-1}$). **Chapter 7** presents results of batch investigations on the use of different antagonistic salts and osmoprotectants, viz. glutamate, betaine, ectoine, choline, a mixture of compatible solutes and K^+ and Mg^{2+}, to alleviate the acute NaCl toxicity on sulfate reducing granular sludges developed in methanol degrading thermophilic (55ºC) UASB reactors. **Chapter 8** presents the investigation on the feasibility and constraints of

mesophilic (30°C) sulfate reducing processes (COD/SO_4^{2-} = 0.5) at very high salinities (up to 70 g $NaCl.L^{-1}$ and conductivity of 90 $mS.cm^{-1}$) in UASB reactors, this time fed with acetate, propionate or ethanol and inoculated with unadapted mesophilic granular sludge. **Chapter 8** also presents the attempts of entrapping (and the subsequent colonization) of the acetate oxidizing halotolerant SRB *Desulfobacter halotolerans* in the sludge, which would result in the introduction of a previously absent metabolic capacity (oxidation of acetate via sulfate reduction at high salinity). **Chapter 9** discusses the feasibility of sulfate reduction in salt rich wastewaters (50 g $NaCl.L^{-1}$ and 1 g $MgCl_2.6H_2O.L^{-1}$; conductivity 60-70 $mS.cm^{-1}$) in a submerged anaerobic membrane bioreactor (SAMBaR) fed with acetate or ethanol and inoculated solely with the halotolerant sulfate reducing bacterium *Desulfobacter halotolerans*. Finally, **Chapter 10** summarizes the main findings achieved by the investigations described in this thesis. Overall aspects for the treatment of sulfate-rich wastewaters at high salinity and high temperature are discussed in **Chapter 10.**

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Chapter 2

Biotechnological treatment of sulfur-containing wastewaters

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INTRODUCTION

In recent years, increased anthropogenic activity has contributed to local imbalances in the natural sulfur cycle, leading to several serious environmental problems: acid rain (sulfuric acid production out of SO_x emissions); odor nuisance from polluted rivers, landfills or treatment systems; corrosion of steel and concrete; heavy metal and sulfuric acid release from oxygen-exposed mineral ores and soils (which are transported off-site in the so-called acid mine drainage). Industrial wastewaters containing sulfur compounds also contribute to the sulfur imbalances (Colleran *et al.,* 1995; Lens *et al.*, 1998a). Examples of sulfate-rich wastewaters are those produced by industries that use of sulfuric acid (e.g. food and fermentation industry) or sulfate-rich feed stocks (e.g. sea-food processing industry). Reduced sulfur compounds can also be present in wastewaters, such as sulfide (tanneries, Kraft process for wood pulping), sulfite (sulfite pulping), thiosulfate (fixing of photographs) or dithionite (pulp bleaching). An extensive review of sulfur-rich wastewaters is presented elsewhere (Lens *et al.*, 1998a). Recently, considerable attention is also given to the deleterious presence of organosulfur compounds in petroleum and other fossil fuels. In addition to the inorganic sulfur species such as elemental sulfur, sulfate, sulfite, thiosulfate and sulfide, more than 200 sulfur-containing organic compounds have been identified in crude oil. These include sulfides, thiols, thiophenes, substituted benzo- and dibenzothiophenes, and many considerably more complex molecules (Londry and Suflita, 1998).

As for other pollutants, environmental technology processes were primarily focused on the elimination or removal of the sulfur compounds that cause damage to the environment. In the last two decades, however, sulfur cycle conversions are also adopted as well-usable microbial conversion techniques in a range of environmental biotechnological processes to abate pollution by sulfurous compounds, heavy metals, xenobiotics and nitrogen compounds with clear opportunities for resources recovery. This chapter covers both microbiological and technological aspects of the treatment of sulfur rich waste streams involving the formation of metal sulfides and elemental sulfur. Furthermore, new possible applications of biological sulfur transformations are also presented, with special emphasis on the removal of organosulfur compounds from refineries wastes streams.

THE SULFUR CYCLE IN ENVIRONMENTAL TECHNOLOGY

The biological sulfur cycle

Biogeochemical cycles represent the motion and the conversion of matter by biochemical activities in the ecosystems. The sulfur cycle is a natural environmental process in which sequential transformation reactions convert sulfur atoms in a variety of oxidation states (Table 1). Physically sulfur can be present in gaseous, liquid or solid states while

chemically sulfur can be present in organic and inorganic compounds. Despite that some sulfur-transformations occur at considerable rates chemically, the global sulfur cycle is strongly influenced by microorganisms in both the oxidative and reductive sides of the sulfur cycle (Fig. 1). Obviously, different groups of microorganisms (mostly bacteria) are able to use sulfur compounds in a specific redox state. Sulfur- and sulfide-oxidizing bacteria produce sulfate, and sulfate reducing bacteria (SRB) use sulfate as electron acceptor in anaerobic respiration and produce hydrogen sulfide. The formation and degradation of a vast array of organic sulfur compounds are not solely microbial processes, and numerous other organisms participate in them. Higher plants, algae, fungi and most prokaryotes use sulfate as a sulfur source for biosynthesis.

Table 1. Oxidation state of key sulfur compounds (After: Steudel, 2000).

Compound	Oxidation state
Organic Sulfur (R-SH)	- 2
Sulfide (H_2S, HS^-, S^{2-})	- 2
Disulfane (H_2S_2) , disulfide (S_2^{2-}), polysulfides (-S(S_n)S-)	- 1
Elemental sulfur (S^0), organic polysulfanes (R-S_n-R)	0
Dichlorodisulfane (Cl-S-S-Cl)	+ 1
Sulfur dichloride (SCl_2), sulfoxylate (SO_2^{-2})	+ 2
Thiosulfate ($S_20_3^{2-}$)	+ 2 (average per S)
Tetrathionate ($S_4O_6^{2-}$)	+ 2.5 (average per S)
Dithionite ($S_2O_4^{2-}$)	+ 3
Sulfur dioxide (SO_2), sulfite (SO_3^{2-})	+ 4
Dithionate ($S_2O_6^{-2}$), sulfonates (RSO_3^-)	+ 5
Sulfur trioxide (SO_3), sulfate (SO_4^{2-}), peroxosulfate (SO_5^{2-})	+ 6

Three types of solid sulfur storage compounds are found in nature (Fig. 1). The bulk of the sulfur of the earth is present in sediments and rocks in the form of sulfate minerals (primarily as gypsum, $CaSO_4^{2-}$), sulfide minerals (primarily as pyrite, Fe_2S) and sulfur deposits (S^o), which have been formed in various different geological periods. In fact, environmental technology also uses the same insoluble solid intermediates that accumulate in nature ($CaSO_4$, metal-sulfides and S^o) for the abatement of sulfur pollution, as the solid phase can be separated from the liquid phase. It is important to note that all gaseous sulfur compounds (e.g. H_2S and volatile organic sulfur compounds such as dimethyl sulfide and mercaptans) are toxic, corrosive and odorous. Thus, the formation of a gaseous end product is not an option in the sulfur cycle, as opposed to the carbon and nitrogen cycles where the production of, respectively, CO_2/CH_4 and N_2 is the common method applied for their removal.

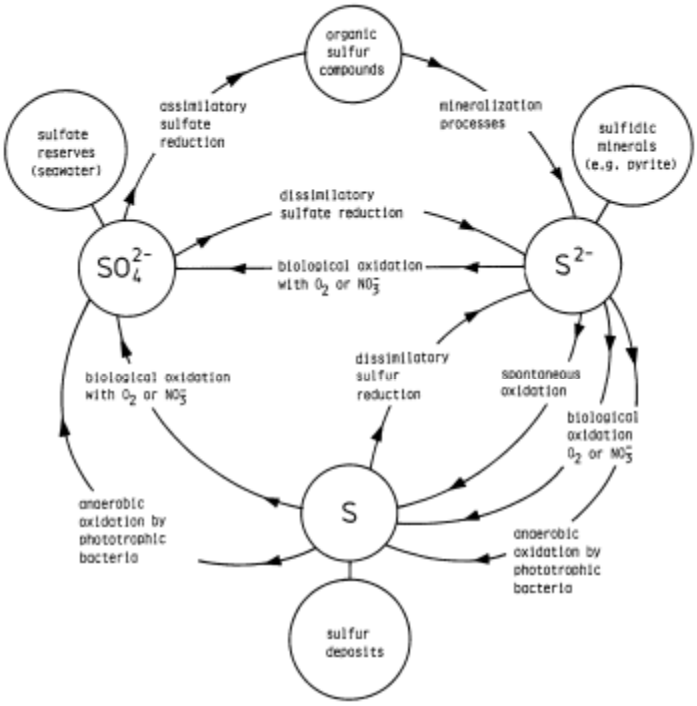

Figure 1. The sulfur cycle (After: Robertson and Kuenen, 1992).

Treatment of sulfate-rich wastewaters in anaerobic bioreactors

Being chemically inert, non-volatile and non-toxic, sulfate itself does not constitute a direct threat to the microbiota in anaerobic reactors. The negative effect of sulfate is associated with the active role of sulfate reduction in anaerobic digestion process, as sulfate will be used as terminal electron acceptor resulting in the production of hydrogen sulfide. Considering the negative effects of sulfide formation in anaerobic bioreactors with carbon removal as the prime target (Table 2), the process conditions should favor the development of methanogens and avoid the growth and metabolism of the SRB as much as possible.

Table 2. Effects of sulfide formation in anaerobic reactors (After: Lens *et al.*, 2000).

DISADVANTAGES	Advantages
- Reduced COD removal efficiency due to the presence of H_2S in the effluent - Corrosion - Accumulation of inert material in the sludge (e.g. metal sulfides) - Diminished availability of trace metals for bacteria (e.g. CoS) - Less methane formation - Poor biogas quality + need for H_2S-removal from the biogas - Malodor - Potential toxicity - Deterioration of aerobic post treatment system (activated sludge bulking, excessive growth of phototrophs)	- Removal of oxidized sulfur compounds (sulfate, sulfite and thiosulfate) from: - organic wastestreams - inorganic wastestreams - Heavy metal removal - Precipitated metal sulfides (e.g. FeS) form good precursors for granulation - Degradation of xenobiotics - Production of alkalinity

Biological sulfate reduction – microbiological aspects

Dissimilatory sulfate reduction is a process in which sulfate is used as electron acceptor. It differs from the process of assimilating sulfur compounds in the cell material (Widdel, 1988). Many bacteria and some archaea can carry out dissimilatory sulfate reduction. They are a metabolically versatile group of microorganisms, belonging to many different families and genera. On the basis of their oxidative capacity, the SRB can be subdivided into two groups: genera that oxidize organic compounds completely to CO_2 and those which carry out incomplete oxidation, usually to acetate as end product (Widdel, 1988). Complete oxidizers include *Desulfobacter, Desulfobacterium, Desulfonema, Desulfosarcina, Archaeoglobus* and *Desulforhabdus* (Widdel and Hansen, 1991; Oude Elferink *et al*., 1995). Incomplete oxidizers include *Desulfomicrobium, Desulfobulbus, Desulfobotulus, Thermodeulfobacterium* and the majority of the traditional sulfate reducing genera *Desulfovibrio* and *Desulfotomaculum* (Widdel, 1988). The most usual electron donors include organic acids, fatty acids, alcohols and hydrogen. However, the range of electron donors is broad (Widdel, 1988; Hansen, 1993). An overview of the range of energy substrates that are known to be metabolized by sulfate reducers is given in Table 3.

Table 3. Energy substrates for SRB (Data from: Hansen, 1993).

Compound Class	Individual Compound Used
Aliphatic monocarboxylic acid	Formate, acetate, propionate, butyrate, isobutyrate, 2 methylbutyrate, 3 methylbutyrate, 3 methylvalerate, fatty acids up to C_{20}, pyruvate, lactate
Dicarboxylic acids	Succinate, fumarate, malate, oxalate, maleinate, glutamate, pimelate
Alcohols	Methanol, ethanol, propanol-1 and 2,butanol-1 and 2.isobutanol, pentanol-1, ethylene glycol, 1-2 propanediol, 1-3 propanediol, glycerol
Amino acids	Glycine, serine, alanine, cysteine, cystine, threonine, valine, leucine, isoleucine, asparate, glutamate, phenylalanine
Sugars	Fructose, glucose, manose, xylose, rhamnose
Aromatic compounds	More than 35 aromatic compounds including benzoate, phenol, indol, resorcinol, catechol, p-cresol, quinoline, nicotine acid, phenylacetate, vanillin, syringaldehyde, trimethoxybenzoate, etc.
Miscellaneous	Very varied group including betaine, choline, furfural, acetone, cyclohexanone, alkanes, etc.
Inorganic compounds	H_2/CO_2

The SRB compete with methane producing archaea (MPA) and homoacetogenic bacteria (AB) for common intermediates of the anaerobic mineralization process such as

H_2/CO_2, formate, acetate and methanol (Oude-Elferink *et al.*, 1994; Lens *et al.*, 1998a). Heterotrophic as well as autotrophic SRB are found in nature. Heterotrophic SRB uses organic compounds as substrates (Eq. 1). Autotrophic SRB utilize CO_2 as carbon source while the electrons are obtained from the oxidation of H_2 (Eq. 2). In wastewater treatment systems, H_2 can be directly supplemented from the environment or can be generated as an intermediate product from the breakdown of various electron donors present in wastewaters such as propionate or glucose (Lens and Kuenen, 2001).

$$\text{organic matter} + SO_4^{2-} \rightarrow HS^- + H_2O + 3\ HCO_3^- \quad (1)$$

$$H_2 + 2\ SO_4^{2-} \rightarrow H_2S + HS^- + 5\ H_2O + 3\ OH^- \quad (2)$$

Despite the vast array of possible electron donors, H_2S is always the end product and incomplete sulfate reduction to S^0 has thus far not been reported. Only two cases are reported in literature where minor amounts of sulfite or thiosulfate were observed as the end products (Fitz and Cypionka, 1990). This implies that the formation of S^0 in a single anaerobic reactor can not be accomplished. Moreover, elemental sulfur production under anaerobic conditions would impose problems, as S^0 can be used for anaerobic respiration (e.g. by SRB) or react with sulfide to form the toxic polysulfides.

Reduction of sulfite and thiosulfate is also very common among many SRB (Widdel, 1988; Hao *et al.*, 1996). This metabolic capability of SRB is employed in the treatment of sulfite-rich scrubbing waters. Certain types of SRB are also capable of a unique form of energy metabolism called disproportionation, using sulfur compounds of intermediate oxidation states. For example, *Desulfovibrio sulfodismutans* can disproportionate thiosulfate and sulfite (Bak and Pfennig, 1987):

$$S_2O_3^{2-} + H_20 \rightarrow SO_4^{2-} + HS^- + H^+ \quad (3)$$

$$4\ SO_3^{2-} + H^+ \rightarrow 3\ SO_4^{2-} + HS^- \quad (4)$$

$$4\ S^0 + 4\ H_2O \rightarrow 3\ H_2S + SO_4^{2-} + 2\ H^+ \quad (5)$$

In addition to using sulfate as an electron acceptor, many SRB can utilize nitrate (NO_3^-) as an electron acceptor, reducing NO_3^- to ammonia (NH_3). They can also use certain organic compounds for energy generation by fermentative pathways in the complete absence of sulfate (Widdel, 1988; Madigan *et al.*, 1997). The most common fermentable compound is pyruvate, which is converted via a phosphoroclastic reaction to acetate, CO_2 and H_2 (Madigan *et al.*, 1997). Recent findings also indicates that sulfate reducers may also be acetogens. For instance, *Syntrophobacter fumaroxidans*, isolated from a culture enriched from anaerobic granular sludge, oxidizes propionate syntrophically in co-culture with the hydrogen- and formate-utilizing *Methanospirillum hungateii*, and is able to oxidize propionate and other

organic compounds in pure culture with sulfate or fumarate as the electron acceptor (Harmsen *et al.*, 1998). This flexibility in metabolic pathways allows them to survive in anaerobic reactors under conditions of sulfate deprivation (O'Flaherty and Colleran, 1999a,b).

Competition between methanogens and sulfate reducers in bioreactors

Competition between MPA and SRB in high-rate anaerobic reactors is dictated by many factors such as growth kinetics, thermodynamics, their immobilization properties (Isa *et al.*, 1986a,b), substrate limitations inside the biomass aggregates (Liu and Fang, 1998), environmental conditions such as the undissociated sulfide concentration (O'Flaherty and Colleran, 2000), medium composition (Lens *et al.*, 1998a), temperature (Visser *et al.*, 1993a; Weijma *et al.*, 2001) and pH (O'Flaherty and Colleran, 2000). Moreover, the competition can also be dictated by the bacterial composition of the seed sludge (Omil *et al.*, 1998; O'Flaherty *et al.*, 1999a,b). The outcome of this competition is of utmost importance, as it determines to what extent sulfide and methane, the end-products of the anaerobic mineralization process, will be produced. Notwithstanding the various factors influencing the outcome of the competition between SRB and MPA in high-rate anaerobic reactors (Table 4), kinetic properties of SRB, MPA and AB can be used as a simple tool to predict the outcome of the competition of common substrates and intermediates of the oxidized organic matter (Lovley *et al.*, 1982; Kristjansson *et al.*, 1982; Robinson and Tiedje, 1984; Lupton and Zeikus, 1984). Both from a thermodynamic (Table 5) and a kinetic point of view, SRB are expected to outcompete the methanogenic consortia during growth on these substrates, viz. hydrogen, acetate and methanol. Previous results have shown that, when sulfate is supplied in excess, propionate and butyrate are degraded faster by SRB than by the syntrophic consortia (Colleran *et al.*, 1995; O'Flaherty *et al.*, 1999a,b).

Competition for hydrogen: It is well reported that hydrogen is completely consumed by SRB in anaerobic reactors where sulfate is added in excess (Colleran *et al.*, 1995; Lens *et al.*, 1998a). As a matter of fact, the activity of hydrogenotrophic methanogens is completely suppressed within a few weeks when sulfate is added in reactors with immobilized biomass (Visser *et al.*, 1993a). By addition of sulfate the hydrogen partial pressure becomes so low that thermodynamically hydrogenotrophic methanogenesis is no longer possible (Oude Elferink *et al.*, 1994; Lovley *et al.*, 1982). This concept has been successfully applied to favor sulfate reduction in H_2 utilizing anaerobic reactors. Thus, when supplying hydrogen to a sludge at H_2 limiting concentrations (or sulfate in excess), H_2 consuming SRB will consume H_2 to a concentration that is too low to allow H_2 consumption by MPA. Therefore, under these operating conditions MPA are outcompeted and a fully sulfidogenic sludge develops.

Competition for acetate: In contrast to the dominance of SRB in the consumption of hydrogen, the competition for acetate in anaerobic digesters is less clear. Complete

conversion of acetate by MPA, even at an excess of sulfate, has been reported (Isa *et al*., 1986a,b; Qatibi *et al.*, 1990; Visser *et al.*, 1993a,b; Ueki *et al.*, 1988, Ueki *et al.*, 1989; Yoda *et al.*, 1987; O'Flaherty *et al.*, 1999a,b). In other studies, however, a predominance of acetate-degrading sulfate reducers was found (Gupta *et al.*, 1994a,b; Rinzema and Lettinga, 1988; Visser, 1995; Omil *et al.*, 1996).

Table 4. Factors determining the outcome of the competition between SRB and MB in high-rate anaerobic reactors (Data from: Lens *et al.*, 2000).

Measure	Reference
A. *Inoculum composition*	
- Type of seed sludge	McCartney and Oleszkiewicz, 1991
- Bacterial composition	Harada *et al.*, 1994; Omil *et al.*, 1998
- Attachment properties of bacteria	Isa *et al.*, 1986a,b
- Experimental run time	Harada *et al.*, 1994; Omil *et al.*, 1998
- Inoculation with new bacterial species	Omil *et al.*, 1997, O'Flaherty *et al.*, 1999a,b
B. *Influent composition*	
- Type of COD	Polprasert and Haas, 1995
- Acetate concentration	Yoda *et al.*, 1987
- Sulfate concentration	Overmeire *et al.*, 1994
- Sulfide concentration	Omil *et al.*, 1996; Weijma *et al.*, 2001
- Ca^{+2} and Mg^{+2} concentration	de Smul *et al.*, 1999
C. *Operational Conditions*	
- Two stage anaerobic reactor digestion	Visser *et al.*, 1996; de Smul *et al.*, 1999
- Multi step process	Visser *et al.*, 1992; Lens *et al.*, 1998b

Work of Kristjansson *et al*. (1982) has indicated that the predominance of *Desulfobacter postgatei* in marine sediments could be explained by its higher affinity for acetate than *Methanosarcina barkeri*. The K_m values were 0.2 and 3.0 mM, respectively. However, in bioreactors *Methanosarcina* sp. are only present in high numbers when the reactors are operated at a high acetate concentration (Grotenhuis, 1992). Generally, *Methanosaeta* sp. are the most important acetoclastic methanogens in anaerobic bioreactors (Grotenhuis, 1992; MacLeod *et al.*, 1990; Morvai *et al.*, 1992; Nishio *et al.*, 1993). *Methanosaeta* sp. have a higher affinity for acetate than *Methanosarcina* sp.; their K_s is about 0.4 mM (Jetten et al, 1992). The kinetic properties of two acetate-degrading sulfate reducers, *Desulforhabdus amnigenus* and *Desulfobacca acetoxidans* (Oude Elferink *et al.*, 1995; Oude Elferink *et al.*, 1999), are only slightly better than those of *Methanosaeta* (Table 6).

Table 5. Stoichiometry of the anaerobic degradation of propionate, acetate and molecular hydrogen by SRB and MPA (Data from: Thauer *et al.*, 1977) (ΔG^0 at 37ºC in kJ/reaction).

Reactions	**ΔG^0**
Propionate	
$CH_3CH_2COOH^- + 3\ H_2O \rightarrow CH_3COO^- + HCO_3^- + H^+ + 3\ H_2$	+ 76.1
$CH_3CH_2COOH^- + 0.75\ SO_4^{2-} \rightarrow CH_3COO^- + HCO_3^- + 0.75\ HS^- + 0.25\ H^+$	- 37.7
$CH_3CH_2COOH^- + 1.75\ SO_4^{2-} \rightarrow 3\ HCO_3^- + 1.75\ HS^- + 0.5\ H^+ + 0.25\ OH^-$	- 88.9
Acetate	
$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$	- 31.0
$CH_3COO^- + SO_4^{2-} \rightarrow HS^- + 2\ HCO_3^-$	- 47.6
Methanol	
$4\ CH_3OH \rightarrow 3\ CH_4 + HCO_3^- + H^+ + H_2O$	- 313.0
$4\ CH_3OH + 3\ SO_4^{2-} \rightarrow 4\ HCO_3^- + 3\ HS^- + 4\ H_20 + H^+$	- 362.0
$4\ CH_3OH + 2\ HCO_3^- \rightarrow 3\ CH_3COO^- + H^+ + 4\ H_20$	- 220.0
Hydrogen	
$4\ H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3\ H_2O$	- 136.0
$4\ H_2 + SO_4^{2-} + H^+ \rightarrow HS^- + 4\ H_20$	- 151.9

Table 6. Kinetic properties of acetotrophic MPA and SRB (Data from: Hulshoff Pol *et al.*, 2001).

Acetotrophic microorganism	**μ_{max} (day^{-1})**	**V_{max} ($\mu mol.min^{-1}.g\ protein^{-1}$)**	**K_M (mM)**	**Threshold (μM)**
Acetotrophic methanogens				
Methanosarcina sp.	0.5-0.7	-	3.0	200-1200
Methanosaeta sp.	0.1-0.3	32-170	0.4-0.7	7-70
Acetotrophic sulfate-reducers				
Desulforhabdus amnigenus	0.1-0.2	21-35	0.2-1	<15
Desulfobacca acetoxidans	0.3-0.4	29-57	0.1-1	<15

Putting all kinetic information together it seems that the growth of acetate-degrading SRB is only slightly higher than that of MPA. Therefore, it can be expected that the initial relative cell number affects the outcome of the competition (Omil *et al*., 1998). This is in particular the case for methanogenic sludge from bioreactors where a major part of the microbial biomass may consist of *Methanosaeta*. When methanogenic bioreactors are fed with sulfate, the initial population of acetate-degrading sulfate reducers in a predominantly methanogenic seed sludge has to compete with huge numbers of acetoclastic *Methanosaeta* species. It is worth to remind that the sludge retention time in high-rate anaerobic reactors,

such as the Upflow Anaerobic Sludge Bed (UASB) reactor can be as high as 0.5-1 year (Hulshoff Pol, 1989). Visser (1995) simulated the competition between sulfate-reducing bacteria and methanogens for acetate using a maximum specific growth rate of 0.055 and 0.07 day^{-1} for the methanogenic and sulfate-reducing bacterium, a K_s value of 0.08 and 0.4 mM, respectively, and different initial ratios of bacteria. Starting with a ratio of methanogens/sulfate reducers of 10^4, it will take already one year before the numbers of acetate-degrading sulfate-reducing bacteria and acetate-degrading methanogens are equal. Nevertheless, long-term UASB reactor experiments of Visser (1995) showed that sulfate reducers are able to outcompete the methanogens.

Competition for methanol: Temperature is an important factor in the competition between SRB and MPA for methanol degradation. Despite the fact that thermodynamically the SRB would outcompete the MPA in the presence of sulfate (Table 5), this is not apparent under mesophilic conditions (Weijma, 2000) with just a small fraction of the methanol being converted via sulfate reduction (Glombitza, 2001). In contrast, the presence of sulfate in excess (COD/SO_4^{2-} lower than 0.67) greatly affects the methanol conversion rate and degradation pathway under thermophilic (55-65ºC) conditions, with sulfate reduction accounting for over 80 % of the consumed methanol-COD (Weijma *et al.,* 2000).

Sulfide toxicity

The inhibitory effect of sulfide is presumed to be caused by unionized hydrogen sulfide as only neutral molecules can permeate well through the cell membrane (Schlegel, 1981). H_2S may interfere with the assimilatory metabolism of sulfur, while it possibly also may affect the intracellular pH (Oude Elferink *et al.*, 1995). Hydrogen sulfide dissociates in water according to the following equations (Garrels and Christ, 1965):

$$H_2S \leftrightarrow H^+ + HS^- \qquad (K_a = \pm 1.0 \times 10^{-7})$$

$$HS^- \leftrightarrow H^+ + S^{2-} \qquad (K_a = \pm 1.0 \times 10^{-14})$$

Above pH 8.0-9.0, almost all dissolved sulfide is present in its ionized form. At low pH values toxicity increases, as unionized sulfide is the predominant species. Also, as the pKa-value of this acid-base equilibrium is about 7, small pH-variations in the pH range of 6 to 8 (typical of methanogenic reactors) significantly affect the H_2S concentration in the biogas. Unfortunately, much of the published literature on sulfide toxicity does not take pH and adaptation of the biomass into consideration, which makes general conclusions about toxicity levels rather difficult. Due to the fact that sulfide readily reacts with most metals to form insoluble metal sulfides, the toxicity of sulfide is also related to metal concentrations present in both the influent and the sludge.

In an extensive review about sulfur problems in anaerobic digestion, O'Flaherty and Colleran (2000) underscored that, although one would expect a direct correlation between the unionized H_2S concentration and the extent of the inhibition, this is not always true and, in fact, other parameters like total sulfide concentration can correlate better with the observed inhibition. This indicates that both total sulfide and unionized H_2S may exert an inhibitory effect on the microorganisms. Therefore, one may have two inhibition thresholds, one for undissociated H_2S and another for total sulfide (O'Flaherty and Colleran, 2000). The levels of undissociated H_2S required for 50 % inhibition of the different bacterial groups (the so-called IC_{50} value) were found to be much lower than the total sulfide IC_{50} value (O'Flaherty, *et al.*, 1998), indicating that the undissociated H_2S was clearly the more toxic form of sulfide. It was found, however, that propionate degrading SRB had a much lower threshold for total sulfide than the other bacteria studied (O'Flaherty *et al.*, 1998).

Studies under both mesophilic and thermophilic conditions showed that granular sludge is less inhibited by H_2S than suspended sludges at low and neutral pH, whereas the inhibition is very similar at high pH values (Visser *et al.*, 1996). In suspended sludges, inhibition is determined by the H_2S concentration both at low and high pH values (McCartney and Oleszkiewicz, 1993) and 50 % inhibition was found at unionized H_2S concentrations ranging from 50 to 130 $mg.l^{-1}$. In sludge granules a 50 % inhibition was found at unionized H_2S concentrations of 250 and 90 $mg.l^{-1}$ at pH values of 6.4-7.2 and 7.8-8.0, respectively (Koster *et al.*, 1986). The inhibition of the MPA is significantly higher than the inhibition of the SRB at pH values above 7.8. At a lower pH range ($pH < 7.0$) there is not a distinct difference in the degree of inhibition (Koster *et al.*, 1986). Methanogens are more sensitive than acidogens and acetogens to H_2S inhibition both in suspended (Oleszkiewicz *et al.*, 1989) and granular (Shin *et al.*, 1995) sludges, with the exception of syntrophic propionate degrading bacteria. In a sulfate-reducing fixed bed reactor treating a mixture of acetate and sulfate, process failure occurred even at H_2S concentrations of about 50 $mg.l^{-1}$ (Stucki *et al.*, 1993). This suggests a rather high susceptibility of acetotrophic sulfate-reducing bacteria (ASRB). In the pH range of 7.5 to 9, sulfide inhibition of ASRB is determined by the total sulfide concentration rather than the H_2S concentration, both in flocculent (Oleszkiewicz *et al.*, 1989) and granular sludge (Koster *et al.*, 1986; Visser *et al.*, 1996). Besides the pH, also the COD/SO_4^{2-} influences the susceptibility of sludge to sulfide toxicity, because of the development of different bacterial associations (McCartney and Oleszkiewicz, 1991). In practice, anaerobic treatment always proceeds successfully for wastewater with a COD/SO_4^{2-} exceeding 10. For such wastewaters, the H_2S concentration in the anaerobic reactor will never exceed the presumed critical value of 150 $mg.l^{-1}$ due to the stripping effect of the biogas production (Rinzema and Lettinga, 1988). At COD/SO_4^{2-} lower than 10, process failures of anaerobic reactors have been reported, while in other cases the process proceeds successfully when precautions are taken to prevent sulfide toxicity (Hulshoff Pol *et al.*, 1998).

Reactor technology

For effective methanogenesis, a complete suppression of sulfate reduction and a complete conversion of the organic substrate into methane is the most desired option. As a result, attempts have been made to selectively suppress sulfate reduction by using specific inhibitors, e.g. sulfate analogues (Yadav and Archer, 1989), transition elements (Clancy *et al.*, 1992) or antibiotics (Tanimoto *et al.*, 1989). However, so far, no effective selective inhibitor of SRB has been found, implying that sulfate reduction can not be prevented in practice.

In principle, all common methanogenic bioreactor designs can be applied for the treatment of sulfate-containing wastewaters, provided that proper measures are taken: a) to prevent the occurrence of high H_2S concentrations in the liquid or in the gas phase (Table 7); b) to take into account the precipitation of inorganic sulfides (leading to less active biomass); c) to consider the low mass transfer efficiencies due to the lower biogas generation (Lens *et al.*, 2000).

To prevent sulfide inhibition, different process configurations can be proposed to integrate sulfate reduction, methanogenesis and sulfide removal in order to achieve the removal of both organic matter and sulfurous compounds (Lens *et al.*, 2000). In some cases, the sulfide concentration in the reactor can be reduced by diluting the influent, for example, with a sulfate-free process water. In case the plant is equipped with a sulfide removal unit, sulfide free effluent can be recirculated (Fig. 2D). A rational method is to split the sulfide production from the methane production in two-stage anaerobic digestion (Fig. 2B). In the first step, acidification and sulfate reduction would occur and sulfide is removed between the two anaerobic bioreactors (Lens *et al.*, 1998b). This configuration aims at the total sulfate reduction in the first phase. In practice, however, about 80 to 95 % of the sulfate is converted to sulfide in the acidification phase (Rinzema and Lettinga, 1988; Reis *et al.*, 1995).

Sulfide produced in the process can be removed in a single reactor by the formation of metal-sulfide precipitates (Fig. 2C). Iron is the most common heavy metal used for sulfide precipitation (McFarland and Jewell, 1989; Dezham *et al.*, 1988; Särner, 1986; Gupta *et al.*, 1994a). Evidently, this method has the major disadvantage associated with the costs of iron addition, potential clogging of the inlet pipes, and the accumulation of precipitated FeS in the reactor. As an example, the precipitation of metal sulfides in biogranules (accumulated on the bacterial surface) caused a drastic inhibition of the biological activity of the sludge in the treatment of a benzoate-rich wastewater containing up to 7.5 $g.L^{-1}$ of sulfate (Liu and Fang, 1998). Alternatively to the precipitation, sulfide can be stripped from anaerobic reactors (Fig. 2E). Yamaguchi *et al*. (1999) used a sulfide stripping device to purge the sulfide-rich effluent stream with the biogas evolved in the bioreactor at a rate of 5 – 20 $L.L_{column}^{-1}.min^{-1}$. The sulfide adsorption column was packed with ferrous oxide pellets. Desulfurized liquid was returned to the UASB reactor through the feed recycle at a ratio 2:1 (recycle:influent). Such strategy alleviated

the sulfide inhibitory effects observed before the installation of the sulfide-stripping device, with consequent improvement of the process performance. Stripping methods, however, require a careful regulation of the pH to minimize CO_2 release from the wastewater, which can vary between 2 % (pH 8) and 30 % (pH 7), thus disturbing the alkalinity equilibrium and eventually leading to reactor instability (van Groenestijn *et al.*, 1995).

Table 7. Measures to reduce the sulfide concentration, thus allowing the integration of methanogenesis and sulfate reduction (After: Lens *et al.*, 2000).

Measure	Reference
A. Dilution of influent	
- Non sulfate containing process water	Rinzema and Lettinga, 1988
- Recycle of effluent after a sulfide removal step by:	
Sulfide stripping	Jensen and Webb, 1995; Yamaguchi *et al.*, 1999
Sulfide precipitation	Särner, 1990
Biological sulfide oxidation to elemental sulfur by:	
Thiobacillus sp., oxygen	Buisman *et al*., 1990
Thiobacillus denitrificans, nitrate	Gommers *et al*., 1988
Chlorobium limicola, sunlight	Kim *et al*., 1993
Chemical oxidation to elemental sulfur	
Ferric sulfate/silicate supported reactor	De Smul and Verstraete, 1999
B. Decrease of the unionized sulfide concentration	
- Elevation of the reactor pH	Rinzema and Lettinga, 1988
- Elevation of the reactor temperature	Rintala *et al*., 1991
- Precipitation of sulfide, e.g. with iron salts	McFarland and Jewell, 1989
- Stripping of the reactor liquid using:	
High mixing degree inside the reactor	
Recirculation of biogas after scrubbing	Särner, 1990
Other stripping gas (e.g. N_2)	Lens *et al.*, 2003
C. Separation of sulfide production and methanogenesis	
- Two stage anaerobic reactor digestion	Rinzema and Lettinga,1988
- Multi step process	Sipma *et al*., 1999
D. Selective inhibition of SRB	
- Sulfate analogues (e.g. MoO_4^{2-})	Yadav and Archer, 1989; Ranade *et al.*, 1999
- Transition element (e.g. Co, Ni or Zn)	Clancy *et al*., 1992
- Antibiotics	Tanimoto *et al*., 1989
- Chloroform	Weijma *et al.*, 2002

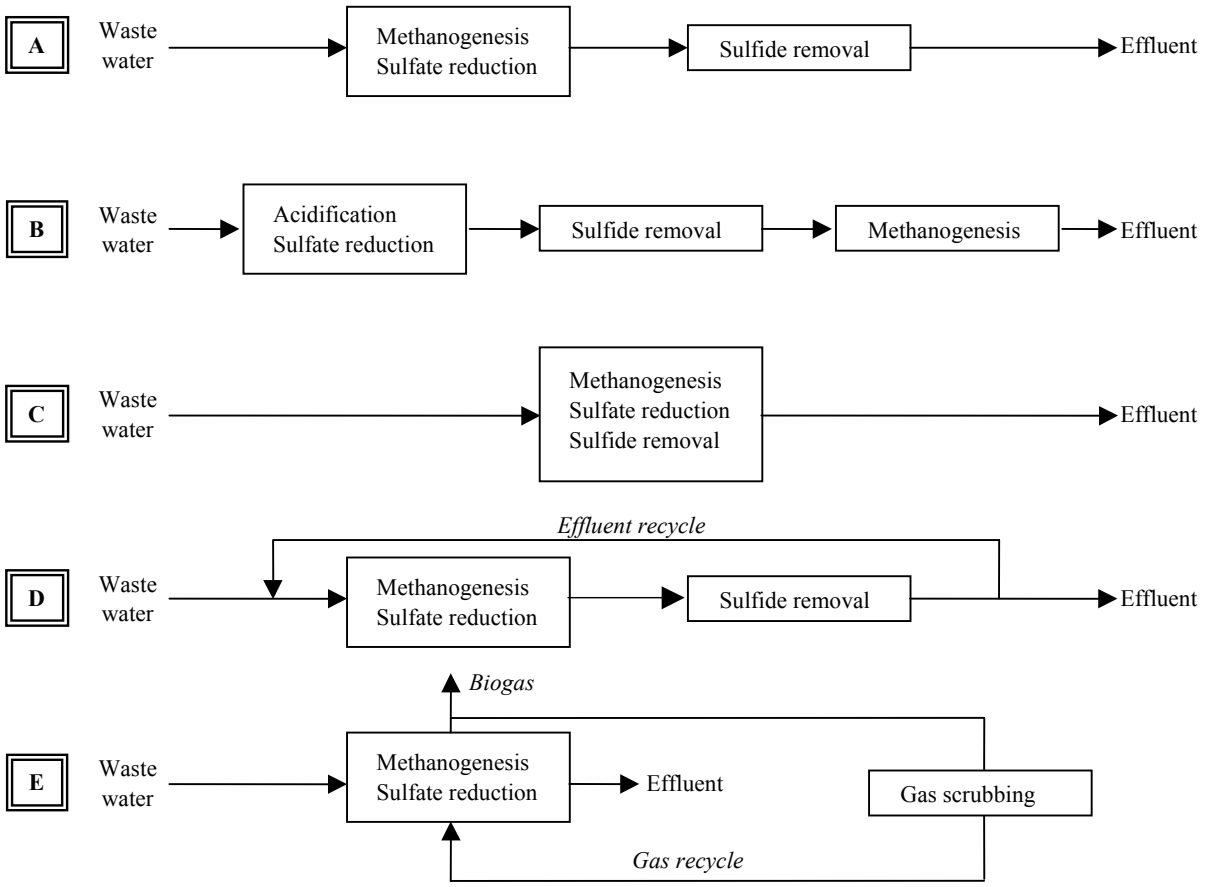

Figure 2. Process configurations integrating methanogenesis with sulfate reduction (Figure from: Lens *et al.*, 2000).

Treatment of sulfur-rich wastewaters in sulfur-cycle-based bioreactors

Biological reduction of sulfate to sulfide, and subsequent biological conversion of sulfide to elemental sulfur, were successfully developed as a cost-effective method for the removal of sulfur from waste streams (Visser, 1995; Janssen, 1996; van Houten, 1996; Weijma, 2000; Lens *et al.,* 2000; Hulshoff Pol *et al.*, 2001). Since sulfur is a colloidal solid, it can be separated as valuable raw material for sulfuric acid production or used for soil amendment (Janssen *et al.*, 2000). Moreover, the biologically produced sulfur can be used as a substrate for the bioleaching of metal-polluted soils and sediments. In the bioleaching process, indigenous microorganisms such as *Thiobacillus ferroxidans* oxidize elemental sulfur to release sulfuric acid, that is used to leach the metals (Tichy *et al.*, 1998).

In contrast to the general case of anaerobic wastewater treatment where carbon removal is the prime target, sulfate reducing reactors are designed to have maximal sulfate reduction, coupled to a, if possible, complete suppression of methanogenesis. Based on thermodynamics (Table 5) it is expected that the organic substrates and their reducing equivalents are channeled to the SRB in bioreactors operating under excess of sulfate ($COD/SO_4^{2-} < 0.67$). Moreover, the sensitivity of other trophic groups to sulfide (see section

2.2.3) might stimulate the prevalence of the SRB in bioreactors aimed at sulfate reduction. However, as outlined in section 2.2.2, a multitude of factors determine the outcome of the competition in modern high-rate anaerobic reactors, selective conditions can be imposed that allow the proliferation of the SRB.

Criterions for electron donor selection: For inorganic sulfate containing wastewaters with no or insufficient electron donor and carbon source for a complete sulfate reduction, addition of an appropriate electron donor is required. The choice of the electron donor is determined by two factors (van Houten, 1996):

- The cost of the electron donor per unit of sulfate converted to sulfide.
- The electron donor should result in little, if any, remaining pollution, which always should be easily removable.

Based on the last criterion, the use of relatively pure, fully degradable bulk chemicals offer two significant advantages. First of all, post treatment is not required provided that the electron donor is completely degraded. Secondly, the well-defined composition of such chemicals makes the understanding, description and prediction of the bioprocess easier (van Houten, 1996). Therefore, simple organic compounds (ethanol, methanol, acetate) or H_2/CO_2 are preferred over complex wastes (e.g. molasses). The successful application of pure chemicals like acetate, ethanol and lactate for sulfate reduction has been demonstrated in lab-scale bioreactors (Table 8).

Hydrogen: Hydrogen is an attractive electron donor in sulfidogenic bioreactors under mesophilic conditions. Sulfate loading rates as high as 30 g $SO_4^{2-}.L^{-1}.day^{-1}$ were achieved in a gas lift reactor fed with a mixture of H_2 and CO_2 (80:20%) operating at 30ºC within 10 days of operation (van Houten *et al*., 1994). This high sulfate elimination rate was achieved only when the free H_2S concentration is kept below 450 $mg.l^{-1}$. The gas lift reactor was used because it provides good mass transfer rates. Pumice was used as carrier material to immobilize the SRB (van Houten *et al*., 1994). These experiments revealed that the hydrogenotrophic sulfate-reducing bacteria (HSRB) were not autotrophic and needed acetate as carbon source. Acetate is formed by homoacetogens. Due to the low affinity of the homoacetogens for H_2, it is possible that under conditions of H_2-limitation insufficient amounts of acetate become available for the HSRB, which may result in a predominance of the hydrogenotrophic methanogenic archaea (HMA). H_2-gas is too expensive to be used in its pure form, but synthesis gas (a mixture of H_2, CO and CO_2) is an attractive and economic alternative (van Houten *et al.*, 1994). It appeared that CO is not used as electron donor by SRB, and it exerts a toxic effect on SRB and thus limits the sulfate loading rate to 10 g $SO_4^{2-}.l^{-1}.day^{-1}$ at CO concentrations in the gas phase between 5% and 20% (van Houten *et al.*, 1996). With CO, layered biomass particles developed. Homoacetogenic *Acetobacterium* sp. were mainly located in the periphery, whereas *Desulfovibrio* sp. were located inside the aggregate (van Houten *et al.*, 1994).

Table 8. Sulfate and sulfite reduction rates and competition between sulfate reducing bacteria and methane producing archaea found in biological removal of sulfate and sulfite with different electron donors (After Weijma, 2000).

Electron donor	T (ºC)	Bioreactor type	SO_4^{2-} removal ($g.L^{-1}.day^{-1}$)	SO_3^{2-} removal ($g.L^{-1}.day^{-1}$)	COD to HS^-/CH_4 (%/%)	Reference
molasses	**31**	packed bed	6.5	na[a]	nr[b]	Maree and Strydom, 1987
molasses	**30**	UASB[c]	4.3	nr	90/10	Annachhatre and Suktrakoolvait, 2001
m.s.d.[d]	30	packed bed	na	46.0	100/0	Selvaraj *et al.*, 1997
lactate	R.T.[e]	plug-flow	0.41	na	nr	Hammack *et al.*, 1994
acetate	35	packed bed	65.0	na	100/0	Stucki *et al.*, 1993
acetate	33	EGSB[f]	9.4	na	nr	Dries *et al.*, 1998
acetate	32	UASB	14	na	nr	Muthumbi *et al.*, 2001
acetate[g]	35	EGSB	2.1	na	37.5/62.5	de Smul, 1998
acetate[h]	35	EGSB	2.9	na	65.0/37.5	de Smul, 1998
ethanol	35	UASB	6.0	na	100/0	Kalyuzhnyi *et al.*, 1997
ethanol	R.T.	fluidized bed	6.3	na	100/0	Nagpal *et al.*, 2000
ethanol	35	EGSB	9.6	na	nr	de Smul *et al.*, 1997
formate[i]	35	EGSB	9.5	na	100/0	de Smul *et al.*, 1997
synthesis gas	30	gas-lift	10.0	na	100/0	van Houten *et al.*, 1995
H_2/CO_2	30	packed bed	1.2	na	100/0	du Preez & Maree, 1994

Table 8. continued

H_2/CO_2	30	CSTR[j,k]	0.90	na	100/0	Fedorovich *et al.*, 2000
H_2/CO_2	30	gas-lift	30.0	na	100/0	van Houten *et al.*, 1994
H_2/CO_2	55	gas-lift	7.5	11.1	[l]	van Houten *et al.*, 1995
H_2/CO_2	55	gas-lift	7.5	9.3	50/50	Kaufman *et al.*, 1996
CO	30	packed bed	2.4	na	100/0	du Preez & Maree, 1994
methanol	65	EGSG	15.0	na	100/0	Weijma *et al.*, 2000
methanol	65	EGSB	14.0	18.0	98/2	Weijma *et al.*, 2002
methanol	30	EGSB	nr	na	12/85	Weijma *et al.,* 2003
methanol	R.T.	packed bed	3.2	na	nr	Glombitza, 2001

a) na = no sulfate or sulfite added; b) nr = not reported; c) UASB = upflow anaerobic sludge bed; d) m.s.d. = municipal sewage digest; e) R.T. = room temperature; f) EGSB = expanded granular sludge bed; g) reactor fed with demineralized/Ca^{+2}-rich tap water (90/10%); h) reactor fed with Ca^{+2}-rich tap/demineralized water (90/10%); i) methanogenic activity suppressed by two consecutive doses of 3.85 $g.L^{-1}$ of BES (2 bromo-ethane sulfonate); j) CSTR = completely stirred tank reactor; k) H_2/CO_2 supplied using hydrophobic membranes; l) strong competition between SRB and MPA is reported.

Ethanol: The use of ethanol as electron donor in sulfate-reducing systems is already applied in full-scale plants (Buisman *et al.*, 1996) and sulfate elimination rates as high as 12 g $S.L^{-1}.day^{-1}$ in lab-scale UASB reactors are reported (de Smul, 1998). Apparently, the main drawback of using ethanol as electron donor is the production of acetate, resulting in an effluent with significant residual COD (Nagpal *et al.*, 2000). This could be due to the strong competition between the *Desulfovibrio* (incomplete ethanol oxidizers) and *Desulfobacter* (complete ethanol oxidizers) species in the reactor. Competition between an incomplete oxidizer, *Desulfovibrio* species, and a complete oxidizer, *Desulfobacter* species, has been investigated in a chemostat with limitation by ethanol as the substrate (Laanbroek *et al.*, 1984). The authors observed that *Desulfovibrio* became the dominant strain, although *Desulfobacter* remained present. The unsuccessful incorporation of acetate-utilizing SRB in immobilized-biomass reactors is well reported (Nagpal *et al.*, 2000; O'Flaherty, 1997; Omil *et al.*, 1997) and surely deserves further investigation.

Methanol: The use of methanol as electron donor at mesophilic conditions resulted in methanol degradation mainly by MPA (Weijma, 2000). In contrast, the use of methanol as electron donor under thermophilic conditions results in methanol consumption mainly by SRB in high rate anaerobic reactors (Weijma *et al.*, 2000; Weijma *et al.*, 2001). At a hydraulic retention time (HRT) of 3-4 h, maximum sulfite and sulfate reduction rates of 18 g $SO_3^{2-}.L^{-1}.day^{-1}$ (100% elimination) and 11-14 g $SO_4^{2-}.L^{-1}.day^{-1}$ (about 50 % elimination) were attained in an EGSB reactor, equivalent to a sulfidogenic methanol-conversion rate of 19 g $COD.L^{-1}.day^{-1}$. The sulfate reduction rate was limited by the amount of biomass present in the system (9 to 10 g $VSS.L^{-1}$). The rather poor biomass retention in the reactors was likely due to the flocculent nature of the sludge developed in the reactors, as opposed to the very well settleable granular sludge (20 to 30 g $VSS.L^{-1}$) present in methanogenic EGSB reactors (Rebac *et al.*, 1995). The presence of high numbers of bacteria capable of oxidizing methanol to hydrogen (and presumably CO_2) in the reactor sludge indicates that hydrogen may represent an important intermediate in sulfidogenic methanol degradation at 65ºC (Weijma, 2000).

Biological sulfide oxidation – microbiological aspects

Many bacteria are able to oxidize different reduced sulfur compound, viz. sulfide, elemental sulfur or thiosulfate. Photosynthetic sulfur oxidizing bacteria use sulfur compounds as the electron donor for reductive carbon dioxide fixation during the photolithotrophic growth (Eq. 6), whereas in the non photosynthetic ("colorless") sulfur bacteria, sulfur compounds are oxidized to support chemolithotrophic growth (Madigan *et al.*, 1997).

$$2\ H_2S + CO_2 + h\nu \rightarrow 2\ S^0 + [CH_2O] + H_2O \qquad (6)$$

Colorless sulfur bacteria are frequently found in the gradients at the interface between anoxic, sulfide-containing areas and aerobic areas of waters and sediments where they can effectively compete with the spontaneous chemical oxidation reaction (Robertson and Kuenen, 1992). Practically all morphological forms and types of motility occur among the colorless sulfur bacteria, and representatives growing over most of the pH (pH 1.0-10.5) and temperature (from 4 to 95ºC) ranges can be found (Madigan *et al.*, 1997). Most colorless sulfur bacteria use molecular oxygen as the terminal electron acceptor (Eq. 7), but several species are also able to denitrify (Eq. 8) (Lens and Kuenen, 2001).

$$H_2S + 2\ O_2 \rightarrow SO_4^{2-} + 2\ H^+ \qquad \text{- 798.2 KJ/reaction} \qquad (7)$$

$$5\ H_2S + NO_3 \rightarrow 4\ N_2 + 5\ SO_4^{2-} + 4\ H_2O + 2\ H^+ \qquad \text{- 3571.4 KJ/reaction} \qquad (8)$$

The final product of sulfur oxidation in most cases is sulfate (SO_4^{2-}). Nevertheless, the oxidation of H_2S occurs in stages, and the first oxidation step results in the formation of elemental sulfur (S^0). This oxidation reaction catalyzed, for example, by *Thiobacillus* involves a series of intermediates, including: sulfide, elemental sulfur, thiosulfate, tetrathionate and sulfate (Kelly *et al.*, 1997).

$$SH^- \rightarrow S^0 \rightarrow S_20_3^{2-} \rightarrow S_4O_6^{2-} \rightarrow SO_4^{2-}$$

For environmental biotechnology, it is important that the oxidation stops at elemental sulfur because sulfur, as colloidal solid, can be separated from the wastewater. The physiological type of colorless sulfur bacterium that dominates in biological sulfide removing systems depends mainly on the physico-chemical conditions (pH, temperature) and the composition (i.e. ratio of inorganic/organic compounds) of the wastewater (Robertson and Kuenen, 1992). Many industrial wastestreams and H_2S containing gas-streams contain low amounts of organic material and high concentrations of inorganic reduced sulfur compounds. Therefore, such systems give a selective advantage to obligate chemolithoautotrophic, sulfide oxidizing bacteria (Visser *et al.*, 1997). Indeed, non-sterile laboratory and pilot plant studies, inoculated with samples containing obligately autotrophic species from the genus *Thiobacillus* form stable microbial communities when supplied with high sulfide and low organic influent. Biotechnological sulfide removing methods based on such cultures have been described using *Thiobacillus thioparus*, *Thiobacillus denitrificans* and *Thiobacillus ferrooxidans* (Smet *et al.*, 1998).

Sulfide oxidation - reactor technology

Based on the ability of colorless bacteria to oxidize sulfide partially to elemental sulfur, an aerobic biotechnological sulfide-removing method was developed (Buisman *et al.*, 1990). Usually, colorless sulfur bacteria tend to oxidize sulfide completely to sulfate,

releasing more metabolically useful energy compared to the partial oxidation to S^0 (Kuenen, 1975).

$$2\ HS^- + O_2 \rightarrow 2\ S^0 + 2\ OH^- \qquad \Delta G^o = -\ 169.35\ kJ/mol\ S^- \quad (9)$$

$$2\ HS^- + 4O_2 \rightarrow 2\ SO_4^{2-} + 2H^+ \qquad \Delta G^o = -\ 732.58\ kJ/mol\ S^- \quad (10)$$

For the treatment of spent sulfidic caustics, which have a high pH that has to be neutralized, total oxidation of sulfide to sulfate (Eq. 10) is more interesting, as it decreases the pH and diminishes the acid consumption.

Biological S^0 formation: In order to obtain S^0 as a product, sulfide oxidation must be terminated at the sulfur formation step. This can be accomplished, for example, by applying high sulfide loads or low oxygen concentrations (Stefess et al., 1996). Because the detection limit of currently available oxygen sensors is about 0.1 mg/l, they are not suitable as a measuring device. But these can be successfully replaced by sensors for monitoring the redox potential (Janssen *et al.*, 1998). One drawback frequently mentioned concerning the application of the redox potential is that its value is the result of the contribution of a mixture of dissolved components, which might result in an overall redox potential. However, several authors revealed the existence of a linear relation between the measured redox potential and the logarithm of the hydrogen sulfide concentration in natural environments (Frevert, 1984; Eckert, 1993). In a sulfide-oxidizing bioreactor, the measured redox potential will therefore predominantly be determined by the sulfide concentration (Janssen *et al.*, 1998). Furthermore, Janssen *et al*. (1998) reported the successful application of the redox control in minimizing the sulfate production in a continuous flow air-lift reactor at different sulfide loads.

Properties of biologically produced S^0: The stability and decantability of the elemental sulfur (S^0) is also very important for the biological sulfur removal process. At the physiological conditions that are optimal for growth and activity of both neutrophilic and alkaliphilic sulfide oxidizing bacteria (30-47 ^oC, pH 7-10), the biologically produced S^0 is stable. At higher temperatures and pH, S^0 disproportionates to form sulfite and sulfide (Lomans *et al.*, 2003). Under aerobic conditions, this mixture will react to form thiosulfate. Microbiologically produced S^0 aggregates to good, well-settling particles, in contrast to chemically produced sulfur, as the latter sulfur type is more hydrophobic (Janssen et al., 1996). This results in higher settling rates of biological S^0 particles. When S^0 is reintroduced in the anaerobic bioreactor together with the recycle stream, it will be converted back to sulfide by S^0 reducing bacteria, which are not necessarily SRB. An example is *Wollinella* sp. (Hedderich et al., 1998). Another microbiological S^0 conversion is its disproportionation into HS^- and SO_4^{2-} (Lovley and Phillips, 1994). If environmental conditions favor this reaction, SO_4^{2-} is formed under strictly anaerobic conditions, and thus the performance of a sulfate reducing bioreactor is negatively affected.

Chemical oxidation process: A drawback of biological sulfide-oxidation processes is that meticulous control is essential to prevent the further oxidation of S^0 to sulfate. An alternative to the biological oxidation of sulfide is the chemical oxidation of aqueous sulfide to elemental sulfur by ferric sulfate at low pH, which yields elemental, orthorhombic α-sulfur (de Smul and Verstraete, 1999). The process can be coupled to a membrane assisted extraction (e.g. permeable silicon) of H_2S out of the liquid. After the removal of the sulfur from the ferric solution, the ferric solution can be regenerated by aeration (de Smul and Verstraete, 1999).

Flue-gas desulfurization

Sulfur dioxide (SO_2) represents the main fraction of anthropogenic sulfur emissions worldwide. Combustion of sulfur-containing fossil fuels accounts for approximately 90 % of the anthropogenic emission of SO_2 (Brimblecome *et al.*, 1989). Once SO_2 is oxidized to sulfate it forms sulfuric acid, which is highly soluble in water and is a very strong acid. In this way the release of SO_2 to the environment is a major contributor to acid rain. Furthermore, SO_2 contributes to the formation of acid aerosols, which can cause a haze over large regions (Charlson *et al.*, 1992).

Physical-chemical process: The formation of gypsum ($CaSO_4$) has long been used for the removal of sulfur dioxide from the gases by exposing this gas to a super saturated limestone slurry, in the so-called limestone gypsum process showed in Fig. 3. The calcium in the slurry reacts with the SO_2 to form calcium sulfite or calcium sulfate (gypsum). The calcium sulfite initially formed in the spray tower absorber is oxidized to calcium sulfate by bubbling compressed air through the sulfite slurry. The formed gypsum crystals settle and dewater better than calcium sulfite crystals, but still represent a voluminous waste product that needs to be disposed off. The reuse of produced gypsum, e.g. as building material, is constrained for example by the contamination with heavy metals.

Biological process: Since about a decade, efforts have been made to develop a biotechnological alternative for conventional physical-chemical processes for the removal of sulfur dioxide from flue-gases. This process is called biotechnological flue-gas desulfurization (Bio-FGD) and uses biological sulfur transformations to recover the SO_2 from flue gases into elemental sulfur. The Bio-FGD is composed of four main units, viz: wet-scrubber, anaerobic bioreactor, sulfide oxidation reactor (either biological or chemical) and a physical separator (Fig. 4).

In the first step, the SO_2 is removed in an alkaline based scrubber (e.g. sodium hydroxide solution). Depending the amount of oxygen in the gas (up to 20 % in volume), around 5 to 20 % of the produced sulfite is oxidized to sulfate (Janssen *et al.*, 2000). In the

subsequent step, sulfite and sulfate are reduced under anaerobic conditions to sulfide (see section 2.3.1).

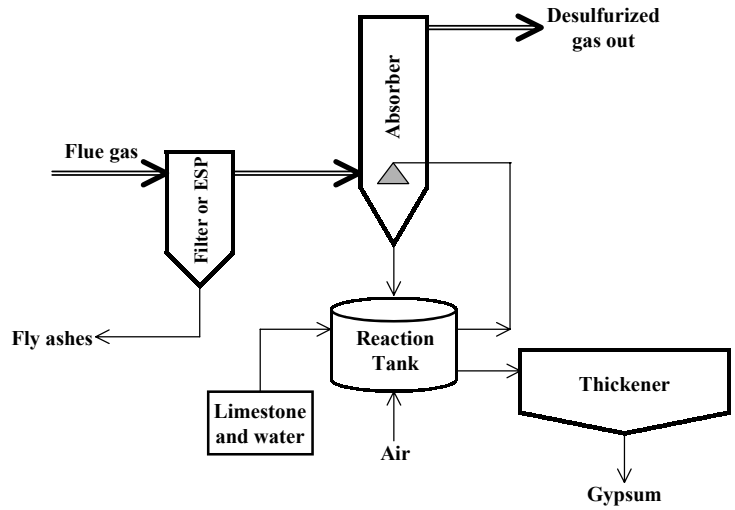

Figure 3. Process diagram of a Flue-Gas Desulfurization (Adapted from: Lagas, 2000).

Addition of an electron donor is necessary (Table 8). In the third step, the produced sulfide can be partially oxidized to elemental sulfur by either autotrophic colorless bacteria or a ferric iron solution (see section 2.3.3). The remaining alkaline solution, with a pH of about 9, can be reused for scrubbing of the SO_2 (Weijma, 2000).

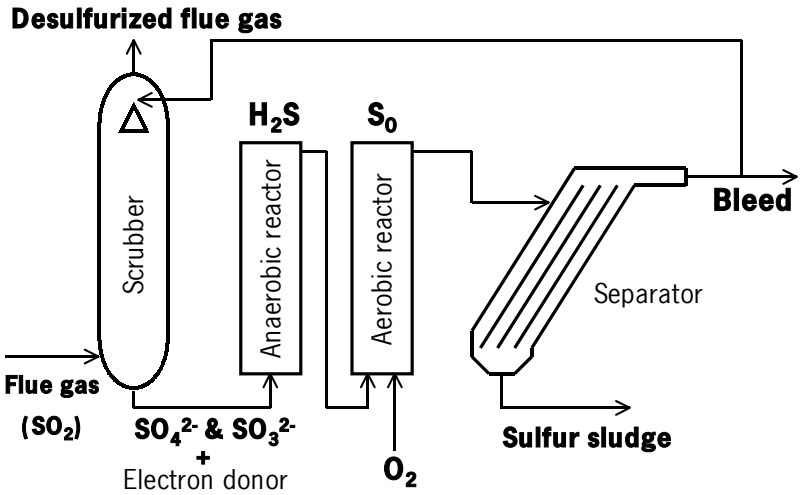

Figure 4. Flow-sheet of the biological flue-gas desulfurization process.

Finally, separation of the solid sulfur particles from the medium enables the recovery of elemental sulfur as a valuable product. This sulfur can either be produced as a sulfur cake (60 % by mass with a purity of 95%) or as liquid sulfur with a higher purity (Janssen *et al.*, 2000). The feasibility of Bio-FGD has been already demonstrated (98 % SO_2 removal) at a pilot scale plant operated from 1992 till 1996. The pilot plant treated 6000 N $m^3.h^{-1}$ (producing 6 Kg sulfur.h^{-1}) flue gas from a coal fired power station at EPZ Geertruidenberg (the Netherlands) and used either ethanol or hydrogen as electron donor.

Removal of heavy metals

In terms of effluent volume, the most significant source of potentially polluting sulfate-rich wastewater is the mining industry, specially the mining of coal and heavy metals. Water draining from the sites of active and derelict mines is frequently enriched with sulfate (100 to > 500 $mg.L^{-1}$) and dissolved iron (and other metals). It may be very acidic (pH < 4), and it is frequently referred as acid mine drainage (AMD). Remediation of AMD has generally been either via the addition of alkaline materials, in combination with aeration to promote ferrous iron oxidation, or by percolating the water through natural or constructed wetlands (Johnson, 2000).

Physical-chemical process: The addition of alkaline chemicals such as limestone, lime, sodium hydroxide, sodium bicarbonate or magnesia results in the raise of the pH followed by the precipitation of metals. These systems, however, generally require the installation of a plant with agitated vessels, precipitators, clarifiers and thickeners with increased cost of reagents, operation, maintenance and disposal of the resulting metal laden sludge (Gazea *et al.*,1996).

Wetland treatment: The remediation of acidic metal-rich wastewaters using natural or constructed wetlands is a passive low-cost approach that found wide application worldwide (Hulshoff Pol *et al.*, 2001). Filtering of suspended material, metal uptake into live roots and leaves, adsorption and exchange by plants, soil and other biological materials, abiotic or microbial-catalyzed metal oxidation and hydrolysis reactions in aerobic zones, and microbial-mediated reduction in anaerobic zones results in the amelioration of the AMD (Gazea *et al.*,1996). A wide range of electron donors such as manure, spent mushroom compost, peat, sawdust and woodchips can fuel the biological process. In some systems vegetation (e.g. cattail) can be a continuous source of reduced carbon (Johnson, 2000). At the former Wheal Jane tin mine a large–scale pilot plant passive system was installed to evaluate the most appropriate configuration for wetland remediation of this and similar drainage waters (Johnson, 2000). The plant was based on a combined treatment in an aerobic constructed wetland and a non-vegetated anaerobic "cell" (a buried wetland to exclude oxygen introduction through plant roots). The aerobic wetland promoted iron and arsenic removal, and the anaerobic cell, using mixtures of hay, sawdust and manure as both electron donor and inoculum, induced precipitation of copper, cadmium and zinc as metal-sulfides. Rock filters were added as a final polishing step for Mn precipitation. The test showed that aerobic-anaerobic constructed wetlands are indeed efficient in treating AMD, especially if anoxic limestone drainage is used as pre-treatment. Zn, Cu and Cd removal was well over 99%. A big drawback is however the large area required.

Biological treatment: More recently, SRB-based bioreactors have been designed to treat AMD and similar wastewaters (Kolmert and Johnson, 2001). The biogenically produced

sulfide is used to remove (and recycle) heavy metals (and to some extent sulfate) from AMD waste streams. Also, as sulfate reduction consumes protons, the process results in a net pH increase of the AMD. Since AMD tends to contain relatively little dissolved organic carbon (< 10 $mg.L^{-1}$), addition of suitable electron donor is necessary (Kolmert and Johnson, 2001). For example, the concept of using biofilm reactors for the treatment of acidic wastewaters is, as shown in a laboratory-scale experiment, a suitable design for the treatment of AMD, as biofilms are robust to environmental changes (Kolmert and Johnson, 2001). Application of membrane assisted extraction (silicone rubber) of H_2S from the liquid phase of a lab-scale SRB bioreactor, used to treat heavy metals rich-wastewater, is also reported (Chuichulcherm *et al.*, 2001). The non-porous membrane prevents the SRB from having direct contact with the toxic metals, extremes of pH, or high salinity in the wastewater. A continuous extractive membrane bioreactor-sulfate-reducing-bacteria (EMB-SRB) system was operated and more than 90 % (w/v) of the Zn^{2+} present in a synthetic wastewater was removed (Chuichulcherm *et al.*, 2001).

A full scale upflow sludge bed reactor to remove sulfate, zinc and cadmium has been applied at Budelco zinc production plant in the Netherlands (Scheeren *et al.*, 1993). The system has been in operation since 1992, treating a flow of 5000 m^3/d. Zn and Cd are removed to an average of 99.7%. Both metal sulfides and elemental sulfur are returned to the smelter. The metals are recovered and the sulfur is converted to sulfuric acid (Scheeren *et al.*, 1993).

Perspectives of biological fossil fuel desulfurization

In addition to the formation of sulfur oxides upon combustion of fossil fuels, leading to acid rain, another important incentive for sulfur removal is that catalytic converters on automobiles are far more effective when the sulfur content in the exhaust is very low, i.e. sulfide acts as a catalyst poison. The consistent global trend toward tighter regulations on petroleum refinery products is driving down sulfur levels. Mandated lower sulfur levels are already in place for diesel fuel in many countries, and strong incentives for decreasing sulfur in gasoline are becoming common.

In refineries, strong alkaline NaOH solutions are used to scrub H_2S and acidic organic sulfur compounds such as thiols from hydrocarbon streams. Once H_2S is absorbed in NaOH, the solution becomes known as a spent sulfidic caustic, that requires disposal. In most locations, environmental regulations prevent the direct disposal of sulfidic caustic to the refinery water outfall without prior treatment. Furthermore the disposal of spent caustic is expensive in many countries and will become more so in the future as regulations controlling its disposal become stricter. Physical-chemical treatment of spent caustic, in general operated at high temperature and pressure, is characterized by high investment and operational costs.

Biological treatment are operated at ambient temperatures and therefore could be an inexpensive alternative.

Degradation of organic sulfur compounds – microbiological aspects

Considerable research has been addressed to the oxidation of methanethiol (MT) and related volatile organic sulfur compounds (VOSC), mainly dimethylsulfide (DMS) and dimethyldisulfide (DMDS). These compounds deserve special attention because they are recognized as having an important role in the biogeochemical cycling of sulfur through the atmosphere (Smith and Kelly, 1988). Bacteria belonging to the genus *Thiobacillus* (Tanji *et al*., 1989; Cho *et al*., 1991; Shinabe *et al*., 1995) have been identified in oxidizing MT and other methyl-sulfides into sulfate. Other bacteria capable of oxidizing MT, DMS and DMDS are *Hyphomicrobium* sp. I55 (Zhang *et al.*, 1991) and *Methylophaga sulfidovorans* (de Zwart *et al*., 1996). The biological oxidation of MT into sulfate proceeds according to:

$$CH_3SH + 3.5\ O_2 \rightarrow CO_2 + H_2SO_4 + H_2O \qquad (11)$$

Biological oxidation of alkylsulfides is still an underdeveloped field of research. Kelly and Smith (1990) mentioned the isolation of an organism that grew on diethylsulfide and was capable of oxidizing DMS, DMDS, diethylsulfide, diethyldisulfide and ethanethiol to sulfate. Butanethiol could be partially oxidized by the same strain. Visscher and Taylor (1993) isolated a marine bacterium capable of oxidizing a large range of alkyl sulfides, amongst others ethanethiol, propanethiol and butanethiol. The strain, which grew both aerobically and anaerobically as denitrifier on alkylsulfides, was designated as a *Thiobacillus* species based on its physiological growth properties and morphology.

Treatment of spent sulfidic caustics

Spent caustics typically have a pH exceeding 12, sulfide concentrations exceeding 2-3 wt%, and a large amount of residual alkalinity. Spent caustics containing exclusively inorganic sulfide can be easily treated in aerobic reactors producing elemental sulfur (see section 2.3.3). Unfortunately, spent caustics may also contain phenols, thiols, amines, and other organic compounds that are soluble or emulsified in the caustic (Rajganesh et al., 1995; Kolhatkar and Sublette, 1996). The most abundant organic sulfur compounds in refinery spent sulfidic caustics are thiols, especially when the waste streams are originating from desulfurization of low boiling hydrocarbon fractions.

The microbial oxidation of methanethiol has been investigated in a bench scale 1.45 L fermenter at 30ºC and pH 7.0 (Subramaniyan *et al.*, 1998). The fermenter was inoculated with several species of *Thiobacilli* including *T. thioparus*, *T. versutus, T. thiooxidans* and *T.*

neopolitanus, together with activated sludge from refinery wastewater treatment plant and an industrial digester. This fermenter was operated with a synthetic MT feed at increasing feed rates for 4 months until a feed rate of 60 mg.h^{1-} was achieved with minimal breakthrough of MT (< 5 mg.l^{-1}). Afterwards H_2S was blended with the MT feed gas. Unfortunately, the culture proved to be intolerant to even low levels of H_2S, indicating that the culture was incapable of oxidizing both organic and inorganic sulfur. After adding of *Thiobacillus denitrificans* strain F to the mixed culture, the fermentor became capable of oxidizing both MT and H_2S. Eventually a combined feed of 60 mg MT/h and 20 mg H_2S/h could be achieved with no breakthrough of H_2S and only little breakthrough of MSH (< 5 mg.l^{-1}) (Subramaniyan *et al.*, 1998).

Up to 5 wt% of NaOH is used to remove H_2S from the hydrocarbon fraction in refineries, resulting in high salinity of the spent caustic (about 1.1 M Na^+). Therefore, microorganisms that can oxidize sulfide at high pH (alkaliphiles) and high salinity (halophiles) are an attractive alternative for dilution and pH neutralizing of the waste. Recently, more than 30 obligatory autotrophic and alkaliphilic sulfur-oxidizing bacteria have been isolated from the most saline soda lakes in Mongolia, Kenya, Egypt and USA, all belonging to the genus *Thioalkalivibrio* (Sorokin *et al*., 2000). Most of these newly described organisms were so called halotolerant types which were able to grow within a broad salinity range of 0.3 to 4.3 M Na^+ and optimal pH of 10-10.1.

Biological desulfurization of coal

Apart from the option of treating flue gas biologically (see section 2.3.4), research programs have been developed in order to remove sulfur biologically prior to combustion. Obviously, as coal is produced at sites differing distinctively in composition, i.e. differences in organic matter, soil composition, presence of metals etc., the composition varies greatly. Even coal found at one site is completely lacking homogeneity (Bos *et al*., 1992).

Sulfur in coal can be of organic origin, i.e. as a part of complex macromolecular structures, as well as inorganic. The most abundant inorganic sulfur compounds are metal sulfides, especially pyrite (Kos *et al.*, 1981). Both the ratio between inorganically and organically bound sulfur and the total sulfur content varies greatly among coals (Bos *et al*., 1992). It is frequently suggested that most organically bound sulfur is present in thiophenic structures (Bos *et al*., 1992).

If coal contains sulfur as sulfidic minerals, physical techniques can be applied, based on differences in the physical characteristics of minerals and coal (Bos *et al*., 1992). As an alternative for these physical processes, biological desulfurization processes, with special emphasis on inorganic sulfur removal, have been developed. The biological process is based on a combination of spontaneous (non-biological) and microbiological oxidation of inorganic

sulfidic minerals present in coal. This combination leads to the dissolution of sulfidic minerals. After separating the coal from the process fluid, a fuel is obtained with a lower sulfur content (Bos *et al*., 1992).

Pyrite, the most common sulfidic mineral in coal, can be oxidized chemically at neutral pH according to:

$$4\ FeS_2 + 15\ O_2 + 2\ H_2O \rightarrow 2\ Fe_2(SO_4)_3 + 2\ H_2SO_4 \qquad (12)$$

The oxidation of pyrite results in the formation of sulfuric acid, and therefore the pH of the liquid phase will drop during the oxidation process. The rate of the chemical pyrite oxidation decreases with decreasing pH. At a pH < 3, the chemical oxidation rate becomes negligible. However, as the pH drops to about 4, acidophilic sulfidic mineral oxidizing bacteria become involved. Typical obligate autotrophs like *Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans* were found to be involved in this process. Also several sulfidic mineral oxidizing facultative autotrophs, e.g. *Sulfolobus* sp., were discovered (Bos *et al*., 1992). The biological oxidation accelerates with decreasing pH until 2 – 1.5, when the maximal oxidation rate is reached. The produced ferric iron can, in turn, serve as an oxidizing agent for other sulfuric minerals:

$$CuFeS_2 + 16\ Fe^{3+} + 8\ H_2O \rightarrow Cu^{2+} + 17\ Fe^{2+} + 2\ SO_4^{2-} + 16\ H^+ \qquad (13)$$

The ferrous iron produced can serve as an oxidizable substrate for the bacteria:

$$4\ Fe^{2+} + O_2 + 4\ H^+ \rightarrow 4\ Fe^{3+} + 2\ H_2O \qquad (14)$$

Uncontrolled microbial oxidation of sulfidic minerals leads to serious environmental problems, as this results in acidic mine effluents (AMD), posing detrimental effects on the quality of the environment in mine regions (see section 2.3.5). However, the same process can be exploited under controlled conditions, for the removal of sulfur from coal or in the leaching of valuable metals e.g. in low-grade copper ores. The microbial removal of inorganic sulfidic minerals from coal is much more selective than physical separation techniques. Physical separation techniques result in substantial losses of carbonaceous material, whereas biodesulfurization does not result in significant changes in the caloric value of the coal (Bos *et al*., 1986). Furthermore, leaching of metals from the coal might be viewed advantageous as this decreases the heavy metal content and fly ash. Another important point of consideration is the formation of jarosite-like precipitates. Jarosites are extremely insoluble, basic ferric sulfates, which might remain in the coal. Jarosite formation can be limited or even completely prevented by operating the biodesulfurization process at mesophilic temperatures with low ionic strength process water (Bos *et al*., 1992).

Microbial oil desulfurization

Removal of organic sulfur is especially of interest in the desulfurization of crude oil. The sulfur compounds in the high boiling fraction, the asphaltenes, are predominantly benzo- and dibenzothiophene (DBT) linkages (Fig. 5). Up to 70% of the sulfur compounds in some Texas crude oils has been reported as dibenzothiophene (Monticello and Finnerty, 1985). The abundance of aromatic thiophene derivatives in nearly all crude oils has led to the use of DBT as a model compound in studies of crude oil desulfurization.

Biodesulfurization, a concept originating 50 years ago, has received renewed attention recently as new "greener" method for desulfurizing crude oils (Monticello, 2000). Bacteria that possess the ability to break down DBT have been isolated (Klein *et al.*, 1988). However, organisms that can selectively remove sulfur from DBT without attacking the carbon skeleton are of most interest. By retaining the carbon structures, the fuel value will not decrease upon desulfurization of the crude oil.

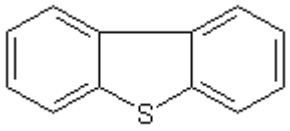

Figure 5. Structure of dibenzothiophene (DBT)

Rhodococcus rhodochrous strain IGTS8 metabolizes dibenzothiophene, a model compound for organic sulfur in fossil fuels, in a sulfur-specific manner by selectively taking out the sulfur moiety, resulting in the formation of 2-hydroxybiphenyl (HBP) as the desulfurized product (Gallagher *et al*., 1993). The conversion of DBT to HBP is catalyzed by a multi-enzyme pathway consisting of two monooxygenases and a desulfinase. The final reaction catalyzed by the desulfinase appears to be the rate limiting step in the pathway. The reason for this selective desulfurization by *Rhodococcus* is that this sulfur acquisition system enables them to obtain sulfur from very stable heterocyclic molecules (Gray *et al*., 1996).

Maghsoudi *et al*. (2001) studied the biodesulfurization of *n*-hexadecane (*n*-C16) containing dibenzothiophene (DBT) and two different diesel oils by *Rhodococcus* sp. P32C1. They used three hydrocarbon–aqueous phase ratios of 25, 50 and 75 vol.% for desulfurization of *n*-C16 containing 1 and 24 mM of DBT as a sulfur source. The maximum specific production rate of 2-hydroxybiphenyl (2-HBP) in the two-phase system equaled 43.5 mmol/(kg dry cell·h).

Based on the optimum conditions determined for desulfurization, diesel oils with different sulfur contents were treated by resting cells. The total sulfur content of 303 ppm in the light diesel oil previously processed through hydrodesulfurization (HDS) was reduced by 48.5 wt.%. Another diesel oil with an initial sulfur content of 1000 ppm was desulfurized by 23.7 wt.% (Maghsoudi *et al*., 2001).

Despite considerable progress in improving the biodesulfurization process, biodesulfurization of crude oil can still be considered to be in its infancy, although several important advances in the elucidation of the mechanisms and the development of a biocatalytic desulfurization process have appeared. Detailed analysis of the rate and extent of desulfurization of real target molecules in a diesel matrix have been described (Monticello, 2000), but are so far still too limited for widespread commercial application. According to Monticello (2000), this can be only achieved if sustained desulfurization rates exceeding 1200 mmol (as substrate Cx-DBTs)/(kg catalyst·h) are needed.

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Chapter 3

Effect of sulfate on methanol degradation in thermophilic (55ºC) methanogenic UASB reactors

A thermophilic (55ºC) lab-scale (0.92 L) methanol-fed upflow anaerobic sludge bed (UASB) reactor (pH 7.0 and hydraulic retention time of 7.5 h) was operated at chemical oxygen demand (COD) to sulfate (SO_4^{2-}) ratios of 10, 5 and 0.5 during 155 days to evaluate the effects of the presence of sulfate on conversion rates, metabolic shifts and possible process disturbances. Methanol was completely removed when operating at an organic loading rate of 20 g $COD.L^{-1}.day^{-1}$ at all COD/SO_4^{2-} ratios tested. At COD/SO_4^{2-} ratios of 10 and 5, methanol was converted both via sulfate reduction (up to 13 % when operating at a COD/SO_4^{2-} of 5) and methanogenesis (85 %). However, when operating at a COD/sulfate ratio of 0.5 (12 g $SO_4^{2-}.L^{-1}$), the sulfate reduction efficiency strongly deteriorated, due to improper immobilization of sulfate reducing bacteria in the sludge bed and the presence of relatively high sodium concentrations (about 6 g $Na^{+}.L^{-1}$) originating from supplying sulfate as its sodium salt. Complete sulfate reduction was achieved when operating at a COD/SO_4^{2-} ratio of 10 (0.6 g $SO_4^{2-}.L^{-1}$) and 5 (1.2 g $SO_4^{2-}.L^{-1}$), corresponding to sulfate removal rates of 2 g $SO_4^{-2}.L^{-1}.day^{-1}$ and 4 g $SO_4^{-2}.L^{-1}.day^{-1}$, respectively. Activity tests showed that methanol was syntrophically converted via H_2/CO_2 by homoacetogenic bacteria, in combination with either sulfate reducing bacteria or methane producing archaea.

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INTRODUCTION

In the last two decades, the anaerobic wastewater treatment process has gained a wide popularity as an established technology for the treatment of a variety of industrial wastewaters, with more than 1160 full-scale plants installed in 65 different countries in 2001 (Frankin, 2001]. The presence of sulfate in some wastewaters restricts the application of the anaerobic treatment technology, due to the production of the toxic, corrosive and odorous hydrogen sulfide (H_2S). The H_2S formation results from the proliferation of sulfate reducing bacteria (SRB) in anaerobic bioreactors, where they compete with methane producing archaea (MPA) and homoacetogenic bacteria (AB) for common substrates such as hydrogen, acetate and methanol (Oude Elferink *et al.*, 1994; Lens *et al.*, 1998). The latter compound deserves special attention, as it has been studied far less than the other direct methanogenic substrates, viz. hydrogen and acetate. Methanol is, nevertheless, a main constituent of several types of sulfur-rich wastewaters. For example, evaporator condensate streams from kraft wood pulp mills contain methanol concentrations ranging from 1.6 to 24.5 $g.L^{-1}$ (Minami *et al.*, 1991; Rintala and Puhakka, 1994). In addition, methanol can be applied as an inexpensive electron donor for the biological treatment of inorganic wastewaters, such as the thermophilic desulfurization of SO_2-containing waste gas scrubbing waters (Weijma *et al.*, 2000).

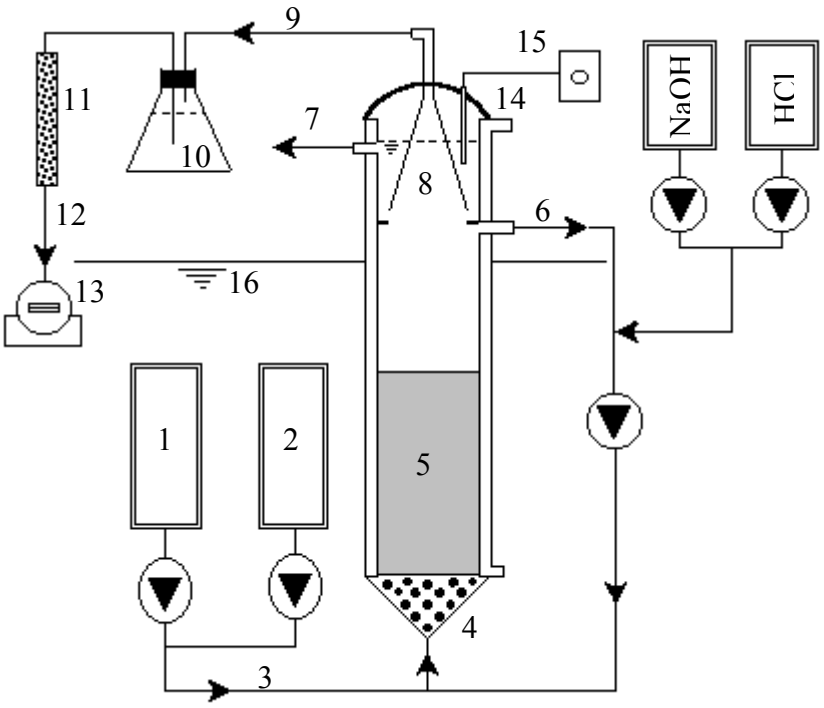
The presence of sulfate, even in excess (COD/SO_4^{2-} lower than 0.67), does not exert considerable effects on the methanol conversion under mesophilic conditions (Weijma *et al.*, 2003), with just a small fraction of the methanol being converted via sulfate reduction (Glombitza, 2001). In contrast, under thermophilic (65ºC) conditions, excess of sulfate (COD/SO_4^{2-} lower than 0.67) greatly affects the methanol conversion rates and degradation pathway, with sulfate reduction accounting for over 80 % of the consumed methanol-COD at 65ºC (Weijma *et al.*, 2000; Weijma *et al.,* 2001). In an expanded granular sludge bed (EGSB) reactor, Weijma *et al.* (2001) obtained a sulfidogenic methanol-conversion rate of 19 g $COD.L^{-1}.day^{-1}$ at a hydraulic retention time (HRT) of 3-4 h, with maximum sulfite and sulfate reduction rates of, respectively, 18 g $SO_3^{2-}.L^{-1}.day^{-1}$ (100% elimination) and 11 g $SO_4^{2-}.L^{-1}.day^{-1}$ (about 50 % elimination). High numbers of bacteria capable of oxidizing methanol to hydrogen (and presumably CO_2) in the EGSB reactor sludge indicated that hydrogen was an important intermediate in sulfidogenic methanol degradation at 65ºC (Weijma, 2000).

So far, little is known about the competition, microbial population dynamics and treatment efficiency of thermophilic methanol-fed reactors seeded with sludge previously not exposed to sulfate. Therefore, a thermophilic (55ºC) methanol-fed methanogenic reactor seeded with such a sludge was operated at decreasing COD/SO_4^{2-} (10, 5 and 0.5) to evaluate metabolic shifts, effects on conversion rates and process disturbances induced by the presence of sulfate in the influent. The effect of increased levels of sulfate on the activity of methanol-degrading microbial groups present in the UASB sludge was also investigated.

MATERIAL AND METHODS

Experimental set-up

To investigate the aims of this work, a 0.92 L UASB reactor with an internal diameter of 6.5 cm and a height of 30 cm (Fig. 1) was operated during 155 days. It was immersed in a glass thermostatic (55ºC) controlled waterbath (Haake, Karlsruhe, Germany). Effluent recycling was applied to obtain a superficial liquid upflow velocity (V_{up}) of 1 $m.h^{-1}$ during the whole experiment. The influent was pumped through the reactor with a peristaltic pump (Watson-Marlow 505S, Falmouth, Cornwall, UK). Basal medium was added to the influent using a vertical axis peristaltic pump (Gilson Minipuls 2, Villiers, France). Both the influent and recirculation flows were combined and submersed in the waterbath before entering the reactor, ensuring an influent temperature of 55ºC. Glass marbles of about 5 mm diameter filled the bottom of the reactor to ensure uniform distribution of the influent in the reactor.



1- Methanol and sulfate
2- Nutrient solution
3- Influent
4- Flux equalizer (marbles)
5- Sludge bed
6- Effluent recirculation
7- Effluent
8- Gas-solid-liquid separator
9- Biogas
10- NaOH scrubber
11- Soda lime pellets
12- Methane gas
13- Wet gasmeter
14- pH electrode
15- pH controller unit
16- Waterbath

Figure 1. Schematic representation of the UASB reactor.

Experimental design

Table 1 summarizes the operational parameters applied to the UASB reactor. The reactor was started with an OLR of 5 g $COD.L^{-1}.day^{-1}$ and a HRT of 10 h. In the next 49 days, both the OLR and HRT were changed as shown in Fig. 2. After day 49, both the OLR and the HRT were kept at around 20 g $COD.L^{-1}.day^{-1}$ and 7.5 h, respectively, till the end of the experiment.

HRT were kept at around 20 g $COD.L^{-1}.day^{-1}$ and 7.5 h, respectively, till the end of the experiment.

Table 1. Summary of the operational parameters applied to the UASB reactor. HRT = hydraulic retention time; OLR = organic loading rate; COD = chemical organic demand; SLR = sulfate loading rate.

	Period I	**Period II**	**Period III**	**Period IV**
Parameter Days	**0 – 75**	**76 – 103**	**104 – 124**	**125 – 155**
Influent flow ($L.day^{-1}$)	2.9 ± 0.4	3.0 ± 0.1	3.0	3.0 ± 0.1
HRT (hour)	7.7 ± 1.5	7.3 ± 0.1	7.3 ± 0.2	7.3 ± 0.2
OLR (g $COD.L^{-1}.day^{-1}$)	18.5 ± 6.5	18.9 ± 1.4	18.5 ± 1.7	17.7 ± 1.8
COD ($g.L^{-1}$)	5.7 ± 1.4	5.8 ± 0.4	5.6 ± 0.5	5.4 ± 0.5
SLR (g $SO_4^{2-}.L^{-1}.day^{-1}$)	2.0 ± 0.8	3.9 ± 0.5	35.3 ± 6.9	3.9 ± 0.6
SO_4^{2-} ($g.L^{-1}$)	0.6 ± 0.2	1.2 ± 0.2	10.8 ± 2.0	1.2 ± 0.2
COD/SO_4^{2-}	9.8 ± 1.7	4.9 ± 0.6	0.5 ± 0.1	4.6 ± 0.7
pH	7.17 ± 0.16	7.29 ± 0.14	7.08 ± 0.14	6.98 ± 0.06
Na^+ ($g.l^{-1}$)*	0.35 ± 0.10	0.69 ± 0.10	6.25 ± 1.18	0.68 ± 0.12

* calculated values

Fig. 2 also gives the evolution of the COD/SO_4^{2-} applied to the UASB reactor. The reactor started at a COD/SO_4^{2-} of 10 (period I), resulting in an influent sulfate concentration of 0.6 $g.L^{-1}$ (Table 1). At day 76, the influent sulfate concentration was further increased to 1.2 $g.L^{-1}$ (Table 1), leading to a decrease of the COD/SO_4^{2-} to 5 (period II). Between days 104 and 124, the influent sulfate concentration was increased to 10.8 $g.L^{-1}$, leading to a decrease of the COD/SO_4^{2-} to 0.5 (period III). Therefore, all methanol could be, theoretically, degraded via sulfate reduction during period III. It is worth to note that the addition of 10.8 $g.L^{1-}$ of SO_4^{2-} implied in an increase of the influent Na^+ concentration to 6.25 $g.L^{1-}$ of Na^+ (Table 1). From day 125 till the end of the experiment (period IV), the influent sulfate concentration was brought back to 1.2 $g.L^{-1}$, resetting the COD/SO_4^{2-} to 5.

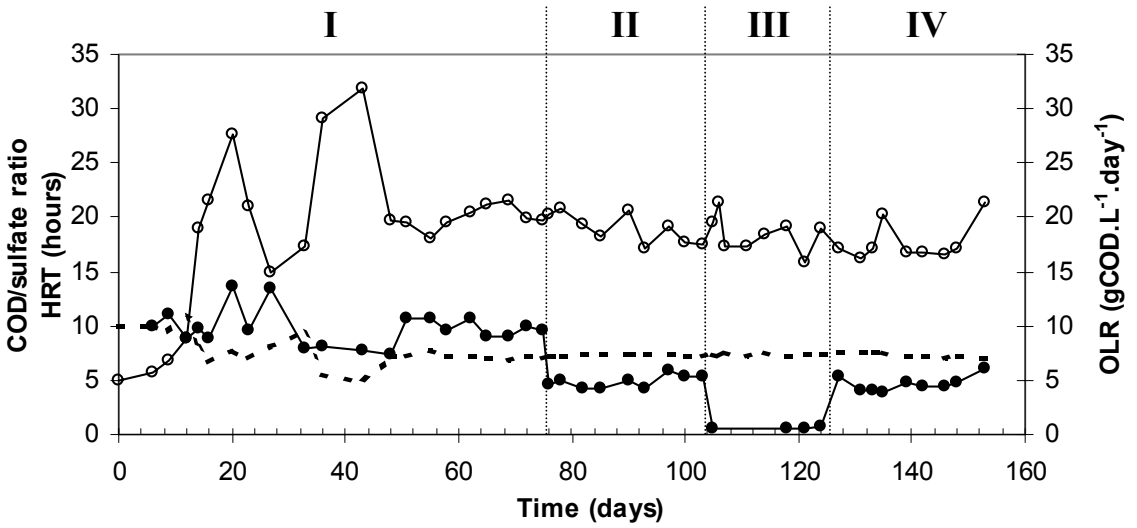


Figure 2. Evolution of the organic loading rate (o), COD/SO_4^{2-} (•) and hydraulic retention time (- - -) applied to the UASB reactor.

Inoculum

The UASB reactor was inoculated with sludge growing in a thermophilic (55ºC) lab-scale (5.1 L) UASB reactor treating methanol-rich wastewater (Paulo *et al.*, 2001), that was originally inoculated with a thermophilic (55ºC) granular sludge from a pilot plant UASB reactor treating paper mill wastewater (Paques bv, Balk, the Netherlands). The inoculum sludge converted methanol to methane syntrophically via H_2/CO_2 and was not exposed to sulfate during the 130 days of reactor run (Paulo *et al.*, 2001).

Medium

The reactor was fed with a synthetic influent, containing basal medium, sulfate and methanol as the sole electron donor. The applied concentration of sulfate, added as sodium sulfate, depended on the established COD/SO_4^{2-} (see above). The influent of the reactor was further supplied with a basal medium consisting of ($g.L^{-1}$): NH_4Cl (7.5), K_2HPO_4 (2.10), $MgSO_4.7H_2O$ (1.5), $CaCl_2.2H_2O$ (0.3), yeast extract (0.5) and a trace element solution prepared according to Zehnder *et al.* (1980), adding 4.5 mL per liter of basal medium consisting of ($mg.L^{-1}$): $FeCl_2.4H_2O$ (2000), H_3BO_3 (50), $ZnCl_2$ (50), $CuCl.3H_20$ (38), $MnCl_2.4H_2O$ (500), $(NH_4)_6MoO_{24}.4H_2O$ (50), $AlCl_3.6H_2O$ (90), $CoCl.6H_2O$ (2000), $NiCl_2.6H_2O$ (92), $Na_2SeO_3.5H_2O$ (194), EDTA (1000), resazurine (200) and HCl 36% (1 mL). Basal medium was added to the main flow at a ratio of 2.22 mL of basal medium per gram COD in the influent.

Maximum specific activity tests

Activity tests were performed with sludge samples harvested from the UASB reactor at the end of periods I, II and III to assess the competition between SRB, MPA and AB for methanol, hydrogen and acetate at different COD/SO_4^{2-}. Activity tests were carried out either in 117 mL vials (methanol or acetate fed) or in 250 mL vials (hydrogen-fed) with 50 mL of mineral medium containing (in $g.L^{-1}$): NH_4Cl (0.28), K_2HPO_4 (0.33), $MgSO_4.7H_2O$ (0.1), $CaCl_2.2H_2O$ (0.01), yeast extract (0.1) and a trace element solution (1 mL/L of mineral medium) prepared according to (Zehnder *et al.*, 1980). Sodium bicarbonate (6.7 $g.L^{-1}$) was added as buffer and the final pH neutralized to 7.0 by adding a concentrated HCl solution. In order to assess the effect of sodium (added to the reactor via sodium sulfate) on methanol degradation, activity tests were also performed with 6 $gNa^+.L^{-1}$ at the end of period III (sodium added as NaCl).

The sludge bed was gently mixed before sampling the sludge in order to get a representative sludge sample. Sampled sludge was rinsed with anaerobic pre-heated (55ºC) medium to remove fine particles from the granules and remaining carbon source. In order to elucidate the pathways of methanol degradation 30 mM of sodium 2-bromoethanesulfonate (BES), 2.5 mM sodium molybdate and 0.17 mM vancomycin were applied to specifically block, respectively, MPA, SRB and AB for vials fed with all substrates, viz. methanol, hydrogen and acetate. Vials containing mineral medium were pre-incubated in a waterbath-shaker (TUV, GLF 1083, Germany) at 55ºC and 55 rpm before addition of washed sludge (about 1.5 g VSS) and either methanol or acetate (2 g $COD.L^{-1}$) as the sole substrate. When using hydrogen as the substrate, the headspace was filled with a H_2/CO_2 gas mixture (80/20 v/v) till the overpressure reached the calculated equivalent of 2 g $COD.L^{-1}$. To assure strict anaerobic conditions, 0.25 mL 0.2 M of Na_2S was introduced in the vials. Assays were performed both in the presence and absence of sulfate. Sulfate was added as sodium sulfate to provide a COD/SO_4^{2-} of 0.5 (excess of sulfate). After closing the bottles with a butyl rubber septum (Rubber bv, Hilversum, the Netherlands) and aluminum cap, the headspace was flushed for 5 minutes with an excess of oxygen-free N_2/CO_2 (70/30 v/v).

Samples were taken from the liquid (0.5 mL for sulfate-fed vials and 0.4 mL for the remaining vials) and gas (0.2 mL) phases for analysis of methanol (methanol-fed vials), hydrogen (hydrogen-fed vials), acetate, sulfide and methane. The methanol, hydrogen and acetate depletion rates and the methanogenic, sulfidogenic and acetogenic activities were calculated from the linear increase or decrease of the different compounds in the vials. Specific rates were obtained by dividing the maximal conversion rates by the amount of VSS, measured upon the completion of the assay. The sulfide concentration in the headspace was calculated from the sulfide concentration in the liquid using Henry's law and the proportionality constant at 55^0C (9.57). The pH was measured at the end of the assays. All experiments were performed in duplo.

Analysis

VSS was analyzed according to standard methods [12]. Sulfide was determined photometrically as described by Weijma *et al.* (2002). Methanol, VFA and methane were measured by gas chromatography (GC), and sulfate by high-pressure liquid chromatography (HPLC), as described by Weijma *et al.* (2000). The volume of biogas produced in the UASB reactor was measured with a wet-type precision gas meter (Schlumberger Industries, Dordrecht, the Netherlands), after passing through a waterlock filled with 3 N NaOH solution and a column filled with soda lima pellets to remove, respectively, H_2S and CO_2 from the gas.

RESULTS

Reactor performance with low sulfate concentration (period I)

Complete methanol degradation was observed within 5 days after the start-up of the UASB reactor (Fig. 3A). Although the methane production was not measured in the first 12 days, the measurements from day 13 onwards show that all methanol was converted to methane at an OLR of 20 g $COD.L^{-1}.day^{-1}$ (Fig. 3D). Sulfide started to be produced from day 14 onwards (Fig. 3C) and steadily increased till complete sulfate removal was observed at day 20 (Fig. 3B), resulting in a sulfate removal rate of 2 g $SO_4^{2-}/L^{-1}.day^{-1}$. At a COD/SO_4^{2-} of 10, the sulfate removal efficiency exceeded 95 % (Fig. 3B), resulting in an average total sulfide concentration of 105 $mg.L^{-1}$ (Fig. 3C). Acetate production remained low during the whole period I, with exception of day 33, on which an acetate concentration as high as 277 mg $COD.L^{-1}$ was measured (Fig. 3C). During period I, methane corresponded to about 92 % of the consumed methanol-COD, whereas sulfide and acetate accounted for only 6 % and 2 % of the electron flow, respectively.

After increasing the OLR to about 32 g $COD.L^{-1}.day^{-1}$ between day 36 and 53, the methanol removal efficiency dropped to about 52 % (Fig. 3A). After resetting the OLR to 20 g $COD.L^{-1}.day^{-1}$ and the HRT to 7.5 h (Fig. 2A), the methanol removal efficiency immediately increased to about 92 % (Fig. 3A). The harvest of 30 g wet sludge from the UASB reactor for the activity tests at day 57 resulted in a drop of the methane production rate from 11.5 g $COD.L^{-1}.day^{-1}$ (measured between days 51-55) to 7.4 g $COD.L^{-1}.day^{-1}$ (measured between days 58-69), corresponding to a decrease in the methanol removal efficiency to about 70 % (Fig 3A). This drop in the methanol removal efficiency, however, did not affect the sulfidogenic activity, as evidenced by the complete sulfate removal in the last 20 days of period I (Fig. 3B).

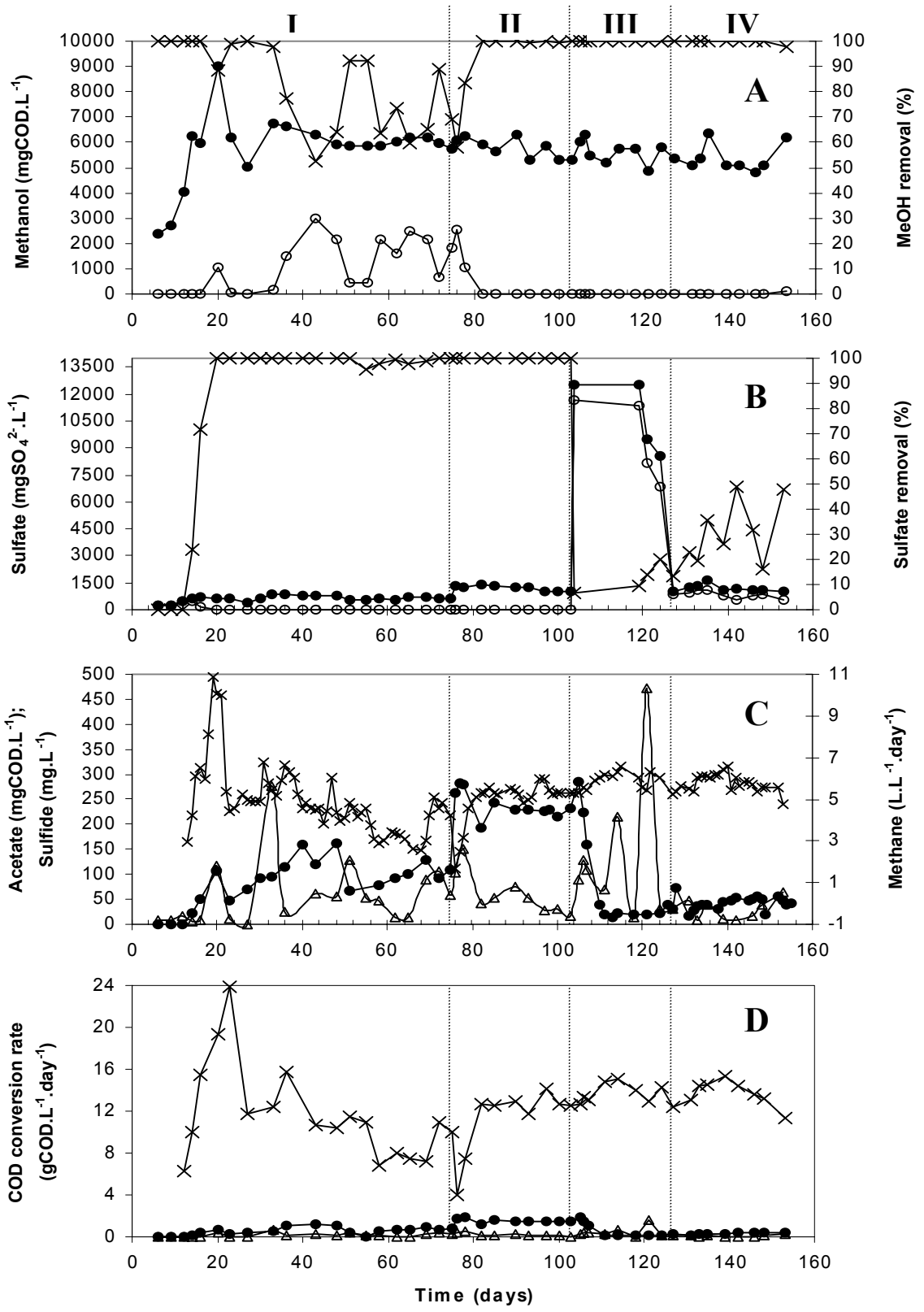


Figure 3. Process performance of the UASB reactor. (A) Evolution of the methanol concentration in the influent (•), effluent (o) and methanol removal efficiencies (x). (B) Evolution of the sulfate concentration in the influent (•), effluent (o) and sulfate removal efficiencies (x). (C) Evolution of the sulfide (•), methane (x) and acetate (△) concentration. (D) Evolution of the COD conversion rate to sulfide (•), methane (x) and acetate (△).

Reactor performance with medium sulfate concentration (period II)

A complete sulfate removal was observed after the increase of the influent sulfate concentration from 0.6 $g.L^{-1}$ to 1.2 $g.L^{-1}$ at day 76 (Fig. 3B), resulting in a sulfate removal rate of about 4 g $SO_4^{-2}.L^{-1}.day^{-1}$. Obviously, the sulfide production promptly increased to an average sulfide concentration of 240 $mg.L^{-1}$ (Fig. 3C). The methane production steadily increased after the decrease of the COD/SO_4^{2-} to 5 (Fig. 3C), resulting in a complete methanol removal from day 82 till the end of the experiment (Fig. 3A). During period II, methane corresponded to about 85 % of the consumed methanol-COD, whereas sulfide and acetate accounted for about 13 % and 2 % of the electron flow, respectively. In contrast to period I, the harvest of 40 g of wet sludge from the UASB reactor at day 101 (end of period II) did not affect the methane production rate (Fig. 3D).

Reactor performance with high sulfate concentration (period III)

The COD/SO_4^{2-} was further decreased to 0.5 at day 104 through a 10 times increase in the influent sulfate concentration from 1.2 $g.L^{-1}$ to 12.5 $g.L^{-1}$ (Fig. 3B). Surprisingly, the sulfide production decreased gradually from 285 $mg.L^{-1}$, measured just before increasing the influent sulfate concentration at day 104, to 13 $mg.L^{-1}$, measured at day 113 (Fig. 3C). After day 105, the methane production rate increased at the expense of the sulfide production rate (Fig. 3D) and full methanol removal from the influent was obtained when operating at an excess of sulfate (COD/SO_4^{2-} of 0.5). During period III, methane corresponded to about 93 % of the consumed methanol-COD, whereas sulfide and acetate accounted for only about 4 % and 3 % of the electron flow, respectively. The highest acetate concentration during the whole experiment (472 $mg.L^{-1}$) was measured at day 121, just after harvesting 40 g wet sludge from the UASB reactor for the activity tests. However, effluent acetate concentrations remained far below from this highest concentration throughout period III and IV (Fig. 3C).

Reactor performance after resetting the COD/SO_4^{2-} back to 5 (period IV)

The poor sulfate reduction efficiency of the UASB reactor during period III might have been related to either a high sodium or a high sulfate concentration due to the Na_2SO_4 addition. To alleviate problems of high ion concentrations, the COD/SO_4^{2-} was reset to 5 on day 125, resulting in a 10 times lower influent sulfate concentration (from 12.5 $g.L^{-1}$ to 1.2 $g.L^{-1}$). The sulfide production did not resume to the concentrations measured during period III (Fig. 3C). Although a slight increase in the sulfide production was observed, the sulfate removal efficiency remained below 30 % till the end of the experiment. During period IV, methane corresponded to about 97 % of the consumed methanol-COD, whereas sulfide and acetate accounted for only about 2 % and 1 % of the electron flow, respectively.

Metabolic characteristics of the sludge

Methanol: A remarkable increase of both the methanol depletion rate and the methanogenic activity was observed during the experiment (Table 2). In contrast, the sulfidogenic activity decreased as a function of time (Table 2), despite the fact that the sulfide production in the UASB reactor during period II was the double of that during period I (Fig. 3C). Methanol was completely converted to methane in the absence of sulfate (Fig. 4A), whereas about 43 % of the methanol was converted to sulfide in the presence of sulfate (COD/SO_4^{2-} of 0.5) by the sludge sampled at the end of period I (Fig. 4B). This strong competition for methanol between SRB and MPA, however, did not occur anymore in the activity tests performed at the end of periods II and III (Figs. 4D and 4F), where methane was the main mineralization product of methanol degradation in excess of sulfate at 55ºC.

Table 2. Maximal specific methanol depletion rate and maximal specific methanogenic, sulfidogenic and acetogenic activities (g $COD.gVSS^{-1}.day^{-1}$) for the sludge sampled at the end of Periods I, II and III. Activity tests were also performed in the presence of 6 g $Na^+.L^{-1}$ for the sludge sampled at the end of Periods III. Standard deviation is given between brackets.

	Absence of sulfate			**COD/SO_4^{2-} of 0.5**			
	MeOH depletion rate	Methano-genic activity	Aceto-genic activity	MeOH depletion rate	Methano-genic activity	Sulfido-genic activity	Aceto-genic activity
Period I	0.87 (0.18)	0.87 (0.10)	0	0.70 (0.14)	0.30 (0.01)	0.30 (44)	0
Period II	3.04 (0.96)	2.62 (0.54)	0.02 (0.01)	3.07 (0.16)	1.71 (0.06)	0.13 (0.01)	0.01 (0.01)
Period III	6.87 (1.10)	5.35 (0.33)	0.14 (0.02)	6.85 (0.32)	4.75 (0.05)	0.01 (0.01)	0.09 (0.01)
Period III (6 g $Na^+.L^{-1}$)	5.18 (0.50)	4.13 (0.07)	0.08 (0.02)	5.80 (0.32)	4.76 (0.06)	0.01 (0.01)	0.11 (0.01)

The rather slow methanol consumption, independently of the presence of sulfate, in incubations with vancomycin in the absence of CO_2 as well as in incubations with excess of H_2/CO_2 and in incubations in the absence of CO_2, strategies that specifically block the AB (Paulo *et al.*, 2001), suggest that the direct methanol conversion by either MPA or SRB was not important (data not shown).

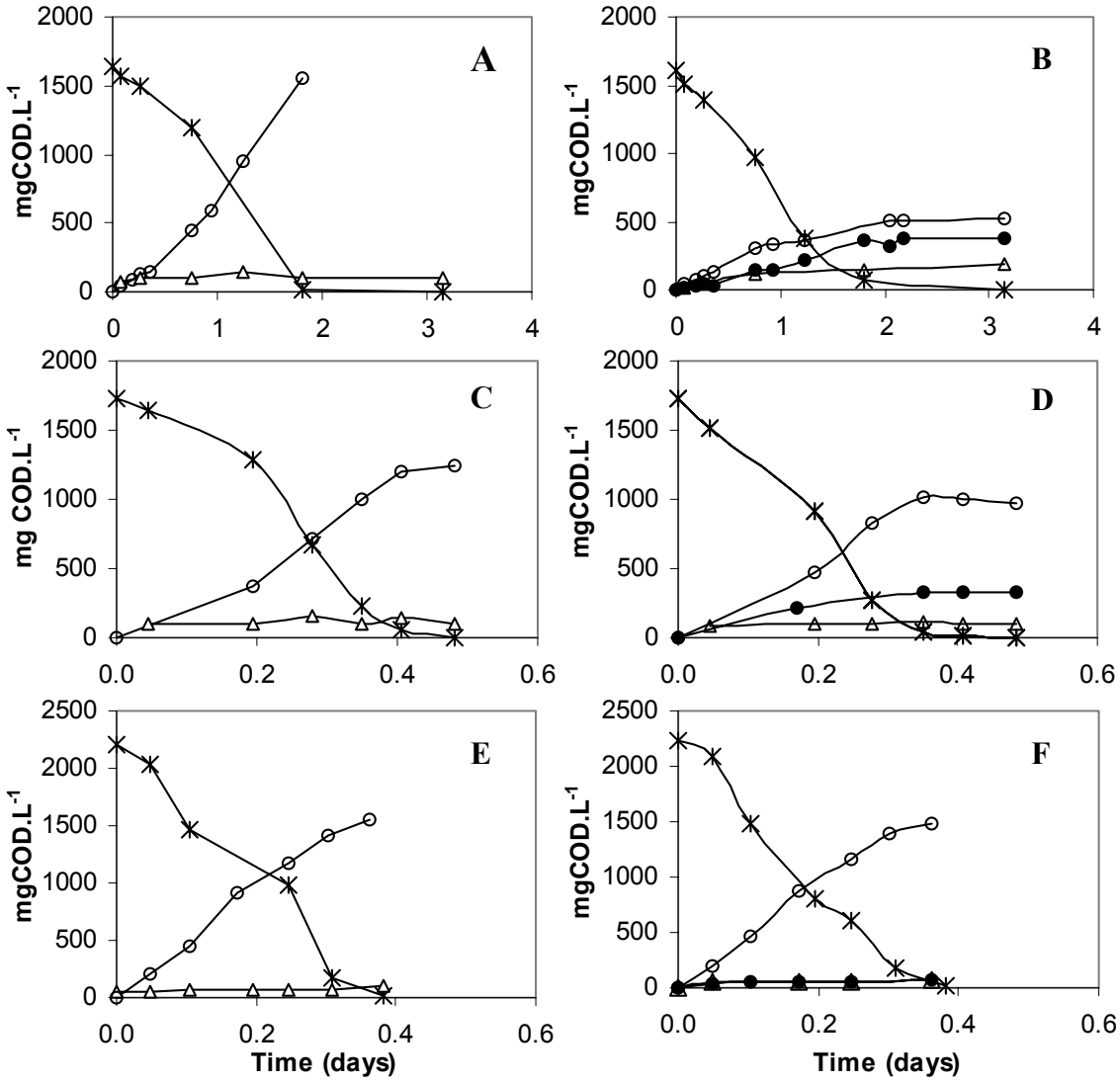


Figure 4. Evolution of the methanol depletion and acetate, methane and sulfide (when sulfate was added) formation during activity assays performed with the sludge harvested from the UASB reactor when operating at different COD/SO_4^{2-}: 10 (Figs. A and B), 5 (Figs. C and D) and 0.5 (Figs. E and F). Activity tests were performed either in the absence (Figs. A, C and E) or the presence (Figs. B, D and F) of an excess of sulfate. Methanol (x), sulfide (•), methane (x) and acetate (△). Note the difference in the time scale of Figs. 4A and 4B compared to the other figures.

Activity tests were also performed in the presence of 6 g $Na^+.L^{-1}$ at the end of period III (Table 2). This activity test aimed to assess the effect of high sodium concentrations on the sulfidogenic activity when operating the UASB reactor at a COD/SO_4^{2-} of 0.5 (period III). No sulfidogenic activity could be detected either in the presence or the absence of additional NaCl (Table 2). Note that the high Na^+ concentration did not affect the methanol depletion rate or the methanogenic activity (Table 2). This suggests that SRB in the sludge were either strongly inhibited by the relatively high sodium concentrations (6 $g.L^{1-}$) or that they were

present in the reactor as individual cells or small particles, thus susceptible to wash-out during the sludge sample rinsing prior to the activity test.

Hydrogen: A remarkable increase in the overall hydrogenotrophic activity of the sludge was observed from period I to period II (Table 3). In contrast to the methanol-fed vials, similar hydrogen depletion rates and methanogenic activities were measured at the end of period II and period III.

Table 3. Maximal specific hydrogen depletion rate and maximal specific methanogenic, sulfidogenic and acetogenic activities (g COD.gVSS^{-1}.day^{-1}) for the sludge sampled at the end of Periods I, II and III. Standard deviation is given between brackets.

	Absence of sulfate			**COD/SO_4^{2-} of 0.5**			
	H_2/CO_2 depletion rate	Methano-genic activity	Aceto-genic activity	H_2/CO_2 depletion rate	Methano-genic activity	Sulfido-genic activity	Aceto-genic activity
Period I	nd	0.24 (0.05)	nd	nd	0.19 (0.02)	0.06 (44)	nd
Period II	3.48 (0.19)	1.94 (0.20)	0.02 (0.01)	2.78 (0.25)	1.39 (0.09)	0.38 (0.02)	0.01 (0.01)
Period III	3.37 (0.96)	1.94 (0.01)	0.28 (0.06)	2.44 (0.60)	1.47 (0.02)	0.35* (0.04)	0.07 (0.09)

*Activity measured after 7 hours of lag phase
nd – not determined

About 70 and 30 % of the hydrogen was converted to, respectively, methane and sulfide in the vials supplied with sulfate for the periods I and II (Figs. 5B and 5D), whereas methane, sulfide and acetate accounted for, respectively, 78, 15 and 7 % of the electron flow (Fig. 5C) in the assay performed after period III. Sulfide was only detected after a lag-phase of seven hours (Fig. 5F), in contrast to the tests performed after period I and II (Figs 5B and 5F). In the absence of sulfate, hydrogen was completely converted to methane with sludge sampled at the end of periods I and II (Figs. 5A and 5C). Acetate formation (16 % of the electron flow) was observed only with sludge sampled at the end of period III (Fig. 5E).

Acetate: The methanogenic activity measured with acetate as the substrate was much lower compared with that measured with methanol or hydrogen as the substrate (Table 4 vs. Tables 2 and 3). Only acetotrophic methanogenic archaea were active in the sludge at the end of periods I and II, both in the absence (Figs. 6A and 6C) and presence (Figs. 6B and 6D) of sulfate. No sulfide formation was detected in activity tests with acetate as the substrate at the end of all periods (Figs. 6B, 6D and 6F). The methanogenic activity decreased sharply in the assays performed at the end of period III (Table 4) and almost no acetate consumption occurred during four days of incubation (Figs. 5E and 5F).

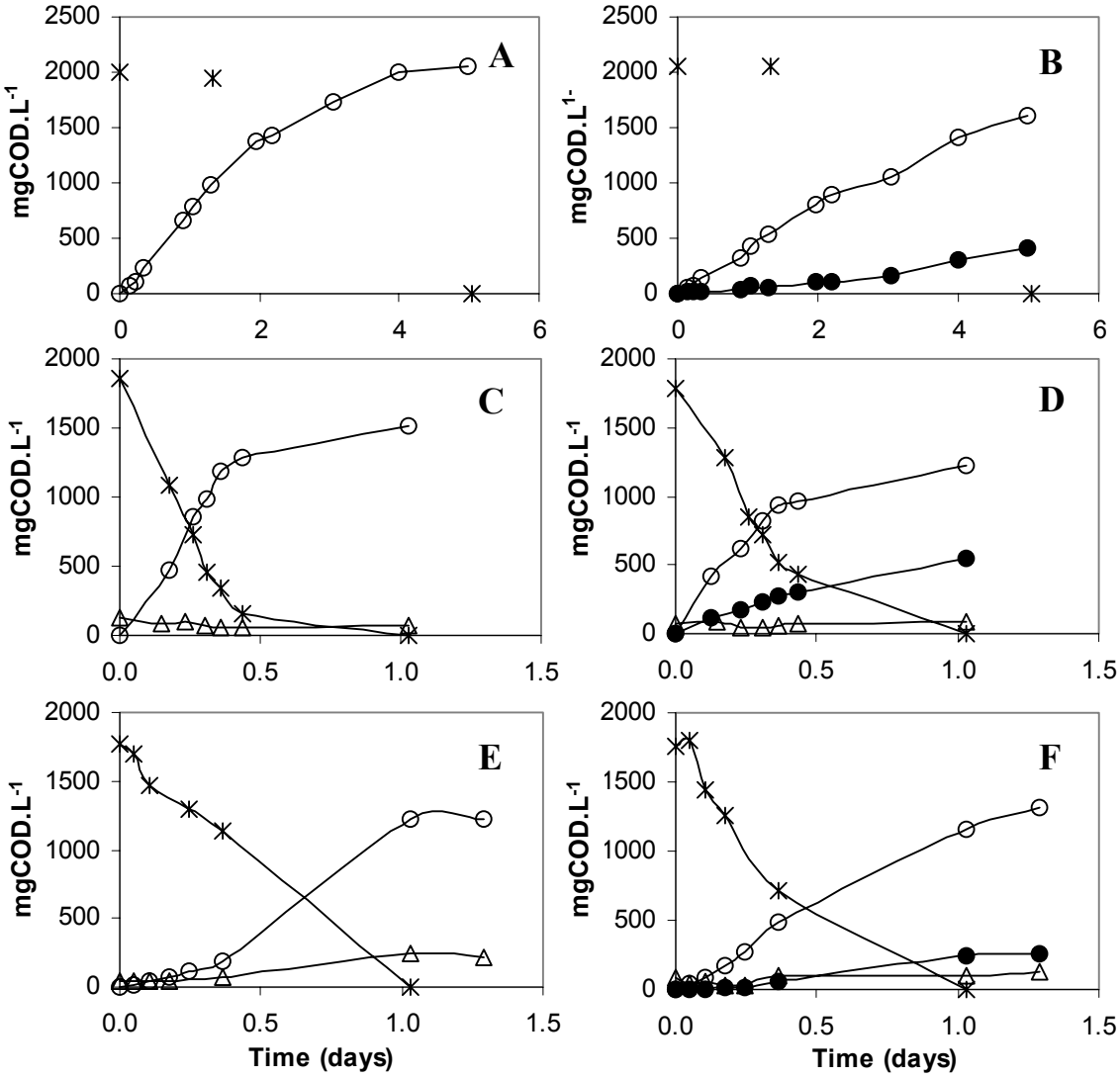


Figure 5. Evolution of the hydrogen depletion and acetate, methane and sulfide (when sulfate was added) formation during activity assays performed with the sludge harvested from the UASB reactor when operating at different COD/SO_4^{2-}: 10 (Figs. A and B), 5 (Figs. C and D) and 0.5 (Figs. E and F). Activity tests were performed either in the absence (Figs. A, C and E) or the presence (Figs. B, D and F) of an excess of sulfate. Hydrogen (x), sulfide (•), methane (x) and acetate (△). Note the difference in the time scale of Figs. 5A and 5B compared to the other figures.

Sludge characteristics

The seed sludge inoculated in the UASB reactor consisted of well-shaped granules with diameters ranging from 2 to 4 mm (Fig. 7A). The granules of the seed sludge had a light brownish color with some white clusters in the outer layer (Fig. 7A). Exposure of the sludge to a COD/SO_4^{2-} of 10 did not alter the good settleability properties of the seed sludge. The granules still had a light brownish color with some white clusters in the outer layer. During

period II, the granules became black colored and a strong disintegration of the granules was observed (Figs. 7C and 7D). Granules were coated with a black layer, probably consisting of metal-sulfide precipitates, whereas the core of the granules retained their light brownish color (Fig. 7D). The disintegration of the sludge led to the formation of a high amount of blackish suspended biomass particles. Despite the presence of biomass particles, the sludge partially granulated again during period III, when the UASB reactor was operated at a COD/SO_4^{2-} of 0.5 (Figs. 7E and 7F). Although some black aggregates still remained in the sludge bed when the UASB reactor was operated again at a COD/SO_4^{2-} of 5 (period IV), the granules partially became again light brownish colored (Fig. 7E and 7F).

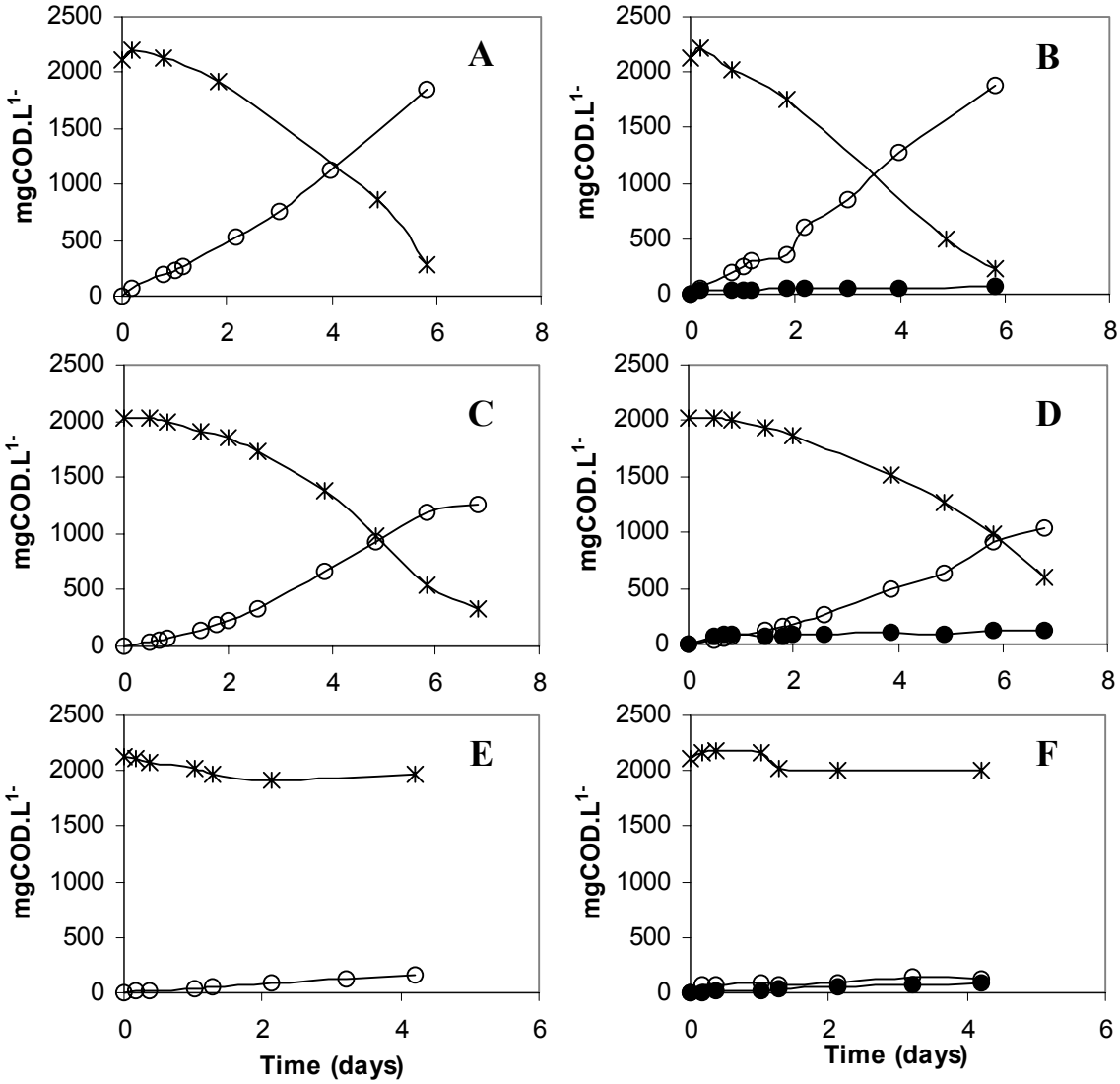


Figure 6. Evolution of the acetate depletion and methane and sulfide (when sulfate was added) formation during activity assays performed with the sludge harvested from the UASB reactor when operating at different COD/SO_4^{2-}: 10 (Figs. A and B), 5 (Figs. C and D) and 0.5 (Figs. E and F). Activity tests were performed either in the absence (Figs. A, C and E) or the presence (Figs. B, D and F) of an excess of sulfate. Methanol (x), sulfide (•), methane (x) and acetate (△).

Table 4. Maximal specific acetate depletion rate and maximal specific methanogenic and sulfidogenic activities (g COD.gVSS^{-1}.day^{-1}) for the sludge sampled at the end of Periods I, II and III. Standard deviation is given between brackets

	Absence of sulfate		**COD/SO_4^{2-} of 0.5**		
	Acetate depletion rate	Methano-genic activity	Acetate depletion rate	Methano-genic activity	Sulfido-genic activity
Period I	0.16 (0.07)	0.13 (0.01)	0.20 (0.02)	0.16 (0.01)	0.01 (0.01)
Period II	0.34 (0.01)	0.18 (0.02)	0.17 (0.01)	0.12 (0.01)	0
Period III	0.07 (0.01)	0.01 (0.01)	0.07 (0.03)	0.01 (0.01)	0.01 (0.01)

*Activity measured after 7 hours of lag phase
nd – not determined

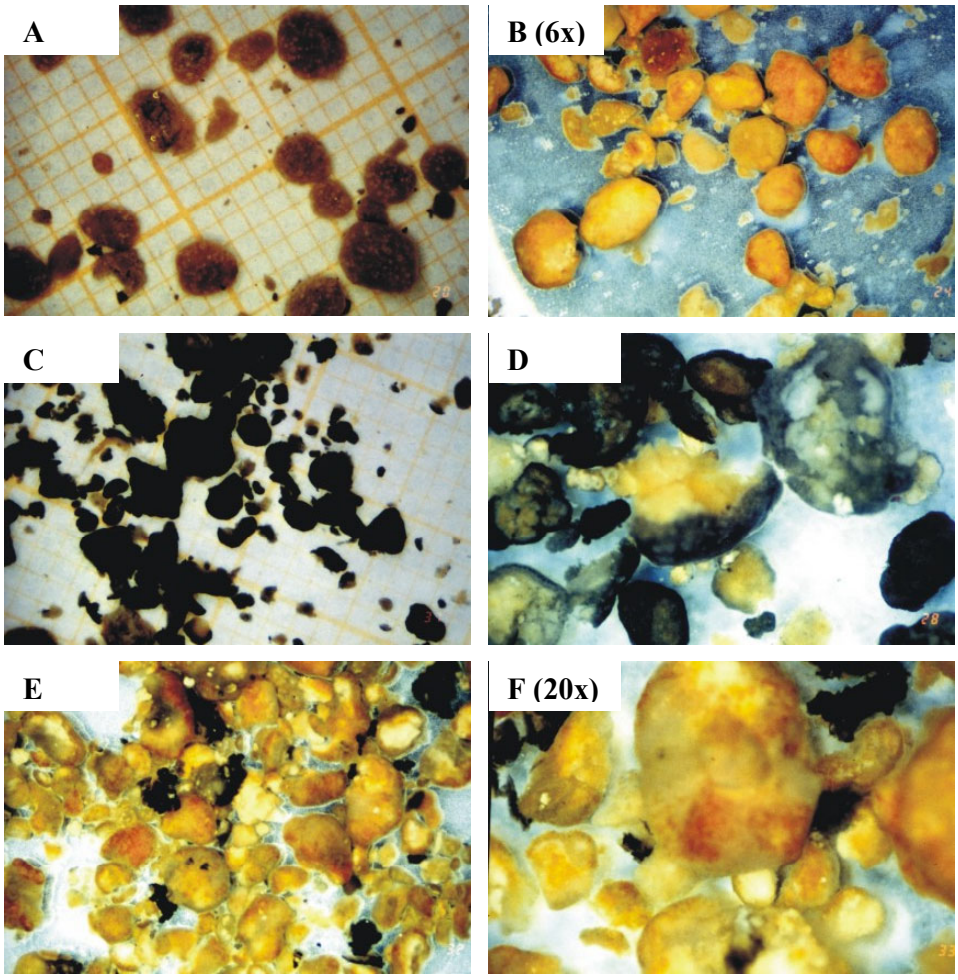


Figure 7. Macroscopic pictures of the seed sludge (Fig. A) and the sludge harvested from the UASB reactor when operating at different COD/SO_4^{-2}: 10 (Fig. B), 5 (Figs. C and D) and 0.5 (Figs. E and F). Size of the smallest orange square under the granules (Figs. A, C and E) indicate 1 mm.

DISCUSSION

This study showed that wastewaters containing methanol and medium sulfate concentrations can be treated in thermophilic (55ºC) anaerobic bioreactors at an OLR up to 20 g $COD.L^{-1}.day^{-1}$ and a HRT of 7.5 h (Figs. 2 and 3A), with methanol being removed both via methanogenesis and sulfate reduction. Complete methanol removal, coupled to a full sulfate removal, was achieved when operating at COD/ SO_4^{2-} of 10 (0.6 g $SO_4^{2-}.L^{-1}$) and 5 (1.2 g $SO_4^{2-}.L^{-1}$), corresponding to sulfate removal rates of 2 g $SO_4^{-2}.L^{-1}.day^{-1}$ and 4 g $SO_4^{-2}.L^{-1}.day^{-1}$, respectively. The possibility of methanization of methanol-rich wastewaters at thermophilic (55ºC) conditions is also confirmed by the strong increase in both the methanol depletion rates and methanogenic activities from period I to period III (Table 2). This increase in activity also demonstrates the smooth recovery of the methylotrophic activity after storing the inoculum sludge at 4ºC for 3 months.

Methanol was not directly used as electron donor for both MPA and SRB, as shown by the activity tests performed with specific inhibitors (data not shown). The negligible sulfidogenic activity on acetate as the substrate (Table 4) in addition to a high sulfidogenic activity on H_2/CO_2 (Table 3) suggests that SRB in this sludge used mainly H_2 as electron donor. A similar pattern was observed for the MPA, with high hydrogenotrophic methanogenic activity measured during the course of the experiment (Table 3). The observed key role of hydrogen in the methanol degradation under thermophilic (55ºC) conditions either in the absence (inoculum sludge) (Paulo *et al.*, 2001) or presence of sulfate (this study) confirms previous findings at 65ºC (Weijma *et al.*, 2000). Methanol degradation occurs in syntrophic association of AB (converting methanol to H_2/CO_2) and hydrogenotrophic SRB at 65ºC (Davidova and Stams, 1996). The lack of acetate degradation by SRB in high rate sulfidogenic reactors has been reported previously, either in thermophilic (Weijma *et al.*, 2000) or mesophilic (Lens *et al.*, 1998; O'Flaherty and Colleran, 1999a; O'Flaherty and Colleran, 1999b) conditions.

Compared to methane and sulfide, acetate was always formed as a minor side-product from methanol (Fig. 3C), accounting for less than 4 % of the electron flow in the UASB reactor. The low acetate production from methanol was confirmed in the activity tests, where acetate accounted for less than 5 % of the electron flow (Fig. 4). The low acetate effluent concentration during periods I and II might be partly related due to its consumption by MPA, as the sludge had a low acetotrophic methanogenic activity (0.12 and 0.18 g $COD.L^{-1}.day^{-1}$; Table 4). This contrasts with the rather high acetotrophic methanogenic activity (0.52 g $COD.L^{-1}.day^{-1}$) of the inoculum sludge measured in a thermophilic (55ºC) methanol-fed UASB reactor operated at an OLR up to 47 g $COD.L^{-1}$ (Paulo *et al.*, 2001). The sludge even lost completely its acetate utilization capacity at the end of period III (Fig. 6E and 6F). The loss of the acetate production capacity of the sludge can be attributed to the absence of

bicarbonate (HCO_3^-) in the influent and the lack of (HCO_3^- generating) sulfate reduction (Fig. 3C). Indeed, bicarbonate is necessary for the formation of acetate out of methanol under anaerobic conditions (Davidova and Stams, 1996). However, low acetate production was also observed in the activity tests supplemented with 6.7 $g.L^{-1}$ of HCO_3^- (Figs. 4, 5 and 6), which suggests a suppression of the acetate producing homoacetogenic population.

A complete sulfate reduction was obtained within 20 days, which indicates that methanol utilizing SRB or hydrogen utilizing SRB (in syntrophic relation with the hydrogen producer homoacetogens) were already present in the seed sludge, despite that this sludge had not been exposed to sulfate for over than 130 days (Paulo *et al.* 2001). The presence of significant quantities of butyrate, ethanol and H_2/CO_2 utilizing SRB species in sludges previously never exposed to sulfate has been reported previously (O'Flaherty and Colleran, 1999a; O'Flaherty and Colleran, 1999b). This might be due to the fermentative and acetogenic growth of SRB in the absence of an electron acceptor (Oude Elferink *et al.*, 1994; Widdel, 1988) or to survival strategies of SRB, e.g. via spores (Widdel, 1988). Total sulfide concentrations as high as 286 $mg.L^{-1}$, corresponding to about 145 $mg.L^{-1}$ of unionized H_2S at pH 7, did not inhibit the methanol degrading consortia (Fig. 3C vs. Fig. 3A). This is in agreement with Weijma *et al.* (2002), who found that inhibition only occurred at total sulfide concentrations as high as 1200 $mg.L^{-1}$ (300 $mg.L^{-1}$ of unionized H_2S at pH 7.5) in a methanol-fed EGSB reactor operated at 65ºC and at a COD/SO_4^{2-} of 3.

The contrasting result of the decrease in the sulfidogenic activity with methanol as the substrate in period II (Table 2) as opposed to the increase of sulfide production in the UASB reactor during period II (Fig. 3C) is most probably due to the handling of the sampled sludge for the activity vials. The suspended biomass particles present in the sludge during period II (Fig. 7C) were removed from the sampled sludge during rinsing prior to inoculation and thus were not inoculated in the activity tests serum vials. The black layer coating the granules (Figs. 7C and 7D) consisted presumably of metal sulfide precipitates, indicating that newly formed biomass around the granules contained a substantial SRB population. This black layer tended to move apart from the granules during period II (Fig. 7D), suggesting that the SRB layer lacked good immobilization properties, as previously reported (Omil *et al.*, 1996; Alphenaar *et al.*, 1993). Weijma *et al.* (2001) found that a methanol fed EGSB reactor showed poor biomass retention, likely due to the flocculent nature of the sludge developed in the reactors, as opposed to the very well settleable granular sludge (20 to 30 g $VSS.L^{-1}$) present in methanogenic EGSB reactors (Rebac *et al.*, 1995). The shear forces present in UASB reactors, e.g. high gas loading rates (Fig. 3C) or elevated liquid upflow velocities (Omil *et al.*, 1996) lead to the detachment of SRB from granules (Alphenaar *et al.*, 1993; Isa *et al.*, 1986a; Isa *et al.*, 1986b). In a UASB reactor working in parallel at the same operational conditions, but in the absence of sulfate, the structure of the sludge remained granular (data not shown). Therefore, further research is needed to find practical ways to improve the retention of active

consortia of SRB and hydrogen producing AB in bioreactors, e.g. by the addition of polymers (Uyanik *et al.*, 2000) or by the use of different reactor configurations, such as sulfate reducing membrane bioreactors (Fedorovich *et al*., 2000).

The partial wash out of SRB probably resulted in a decrease of the sulfidogenic activity, but still enough SRB biomass particles or suspended SRB cells remained present in the reactor to reduce all the supplied sulfate during period II, when the reactor was operated at a COD/SO_4^{2-} of 5 (Fig. 3B). Similarly, the washout of SRB (or hydrogen producer homoacetogens) can not solely explain the metabolic deficiency for methanol and reduced equivalents (viz. H_2 and acetate) when operating at high influent sulfate concentrations (12.5 g $SO_4^{2-}.L^{-1}$), as evidenced by the considerable hydrogenotrophic sulfidogenic activity measured at the end of period III (Table 3). It is worth of note, however, that this hydrogenotrophic sulfidogenic activity was measured only after seven hours of lag phase (Fig. 5F). As the reactor operated at pH 7.0, the suppression of the sulfate reduction can not be attributed to a too low pH (6.5), as reported by Minami *et al*. (1988), who demonstrated that at pHs between 6.2 and 6.8, sulfate reduction was suppressed by 40 % in a 1 m^3 thermophilic (53ºC) fixed-film packed-bed (pumice stone) reactor fed with methanol (10 $g.L^{-1}$) and sulfate (1 $gSO_4^{2-}.L^{-1}$). Therefore, the sudden drop in the sulfide production during period III (Fig. 3C) might also be related to the presence of relatively high sodium concentrations (about 6 g $Na^+.L^{-1}$) following the addition of sodium sulfate as the sulfate source (leading to 12.5 g $SO_4^{2-}.L^{-1}$). Presumably, the deleterious effects of sodium towards SRB were not reversed in the last 30 days of the experiment, as the sulfide production rates did not resume (Fig. 2) upon the SLR decrease (Figs 3C). This warrants further research to elucidate the effect of salt on the anaerobic degradation of methanol under sulfate reducing conditions as well as to find practical ways to overcome the salt stress. These are essential for the adoption of SRB-based bioprocesses in closed water cycles (e.g. certain effluent from the chemical industry), as the deliberate reduction of the bleed in bioreactors leads to salt accumulation (Lens and Kuenen, 2001).

CONCLUSIONS

The results obtained in this research allow to conclude that:

(1) Methanol was completely removed when operating a lab-scale (0.92 L) UASB reactor at an OLR of 20 g $COD.L^{-1}.day^{-1}$ and a HRT of 7.5, with methanol being converted both via sulfate reduction (up to 13 % when operating at a COD/SO_4^{2-} of 5) and methanogenesis (85 %).

(2) Activity tests at 55ºC suggested that methanol was syntrophically converted via H_2/CO_2 by homoacetogenic bacteria, in combination with either SRB or MPA.

(3) Complete sulfate reduction was achieved when operating at a COD/SO_4^{2-} of 10 (0.6 g $SO_4^{2-}.L^{-1}$) and 5 (1.2 g $SO_4^{2-}.L^{-1}$), corresponding to sulfate removal rates of 2 g $SO_4^{-2}.L^{-1}.day^{-1}$ and 4 g $SO_4^{-2}.L^{-1}.day^{-1}$, respectively.

(4) The sulfate reduction efficiency strongly deteriorated when operating at a COD/SO_4^{2-} of 0.5 (12 g $SO_4^{2-}.L^{-1}$), due to improper immobilization of sulfate reducing bacteria in the sludge bed or the presence of relatively high sodium concentrations (about 6 g $Na^{+}.L^{-1}$) due to the supply of sodium sulfate as sulfate source to the influent.

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Chapter 4

Thermophilic (55 - 65°C) and extreme thermophilic (70 - 80°C) sulfate reduction in methanol- and formate-fed UASB reactors

The feasibility of thermophilic (55 to 65°C) and extreme thermophilic (55 to 80°C) sulfate reducing processes was investigated in four lab-scale upflow anaerobic sludge bed (UASB) reactors fed with either methanol or formate as the sole substrates and inoculated with mesophilic granular sludge previously not exposed to high temperatures. Full methanol and formate degradation at temperatures up to, respectively, 70 and 75°C, were achieved when operating UASB reactors fed with sulfate rich (COD/SO_4^{2-} = 0.5) synthetic wastewater. Methane producing archaea (MPA) outcompeted sulfate reducing bacteria (SRB) in the formate-fed UASB reactor at all temperatures tested (65 - 75°C). In contrast, SRB outcompeted MPA in methanol-fed UASB reactors at temperatures equal or exceeding 65°C, whereas strong competition between SRB and MPA was observed in these reactors at 55°C. A short term (5 days) temperature increase from 55 to 65°C was an effective strategy to suppress methanogenesis in methanol-fed sulfidogenic UASB reactors operated at 55°C. Methanol was found to be a suitable electron donor for sulfate reducing processes at a maximal temperature of 70ºC, with sulfate reduction rates as high as 14.4 g $SO_4^{2-}.L^{-1}.day^{-1}$ and with sulfide as the sole mineralization product of methanol degradation at that temperature. An influent NaCl concentration of 10 $g.L^{-1}$, however, inhibited the sulfate reduction process at 70°C.

A modified version of this chapter was submitted to Process Biochemistry.

INTRODUCTION

Anaerobic processes are so far mainly applied for the treatment of industrial wastewaters almost exclusively under mesophilic conditions (Frankin, 2001). In many manufacturing processes, the process water and wastewater temperatures range from 50 to 70°C and in certain processes may even exceed 90°C (Lepisto and Rintala, 1999). Thermophilic (50 to 65ºC) anaerobic processes offer some advantages over mesophilic (25 to 38ºC) operation, such as higher organic loading and conversion rates, shorter retention times and less excess sludge production (van Lier *et al.*, 2001). In the past ten years, several studies have shown that anaerobic thermophilic systems (55 to 65°C) are stable and highly efficient, both in methanogenic (Lepistö and Rintala, 1996; van Lier *et al.* 1996; Ahring *et al.*, 2001) and sulfidogenic (Visser *et al.*, 1993; Weijma *et al.,* 2000a) reactors.

Extending sulfate reduction processes to temperatures equal or over 70ºC is of great interest for relevant industries because the treatment of hot process waters at discharge temperatures omits the cooling of the process waters and allows direct reuse of the treated water without additional re-heating. The only report available so far on sulfate reducing processes at extreme temperatures (equal or over 70°C) shows that sulfate reducing bacteria (SRB) readily consume acetate at 70°C in a sulfate reducing acetate-fed (acetate/SO_4^{2-} of 1.6) UASB reactor operating at an organic loading rate (OLR) of 2.5 g $COD.L^{-1}.day^{-1}$ and a hydraulic retention time (HRT) of 16 to 19 hours (Rintala, 1997). The latter author reported a dominance of SRB over methane producing archaea (MPA) in acetate-fed UASB reactors at 70°C.

Research on extreme thermophilic (equal or over 70°C) sulfate-reducing processes in anaerobic reactors is of paramount importance, as it may allow the direct treatment of hot industrial wastewaters or off-gases. There are few reports showing that the methanization of volatile fatty acids (VFA) (in the absence of sulfate) is feasible and stable in time (over a year) at very high temperatures of 70°C and even 80°C (Rintala and Lepisto, 1992; Lepistö and Rintala, 1996, Rintala, 1997; Lepistö and Rintala, 1999). The aim of the research described in this paper was to study the performance of thermophilic (55 to 65°C) and extreme thermophilic (70 to 80°C) sulfate reducing UASB reactors using methanol or formate as sole substrates. These substrates were chosen as they are inexpensive electron donors and readily utilized by SRB (Nazina *et al.*, 1988; Zhilina and Ilarinov, 1984). The effect of non-feed (starvation) and pH shocks on the performance of the UASB reactors was also investigated. Moreover, the effect of temperature shocks on the process stability and metabolic pathways were studied. In addition, the effect of NaCl (up to 10 $g.L^{-1}$) on the performance of a methanol-fed UASB reactor operated at 70°C was assessed. Finally, the metabolic characteristics and temperature dependence of the granular sludges developed in these bioreactors were also assessed using batch activity tests.

MATERIAL AND METHODS

Continuous experiments

Experimental setup

Two bench-scale (6.5 L) UASB reactors (UASB A and UASB D) and two 0.92 L UASB reactors (UASB B and UASB C) were operated during 98 (UASB A), 80 (UASB B and UASB C) and 170 days (UASB D), in order to study the performance of thermophilic (55 to 65°C) and extreme thermophilic (55 to 80°C) sulfate reducing UASB reactors. The pH of the reactor was maintained at 7.5 (± 0.2) and the effluent was recirculated to obtain an upflow velocity of 1 $m.h^{-1}$ in all four reactors. The flow rate was measured by weighing the amount of effluent on an electrical balance. The COD conversion rates (sulfide, methane and acetate) were expressed as the amount produced of the respective compound (in g COD) per liter reactor (expressed as L) per day. A detailed description of the experimental setup of UASB A and UASB D is found in Chapter 5, whereas a description of the setup of UASB B and UASB C is found in Chapter 3. Sampling ports were placed in the influent tube system, on the top of the reactor and in the biogas conduction system in order to obtain samples of the influent, effluent and biogas, respectively.

Inoculum

Granular anaerobic sludge was obtained from a full scale UASB reactor treating paper mill wastewater (Eerbeek, the Netherlands). The seed sludge had been stored at 4ºC for 6 months prior to inoculation in UASB A, which was started-up with about 2.7 L of inoculum sludge, corresponding to approximately 52 g of volatile suspended solids (VSS). After 18 days of cultivation of the inoculum in UASB A at 55°C, about 0.6 L of this sludge was harvested for the inoculation of UASB B and UASB C (0.3 L each), corresponding to approximately 5.8 g VSS per reactor. Due to reactor failure (refer to Results section) UASB B was two times emptied and re-inoculated (on days 39 and 63) with 0.3 L (5.8 g VSS) of sludge cultivated in UASB A. UASB D was inoculated with a granular sludge from a methanol-fed thermophilic (55°C) sulfidogenic UASB reactor operating at similar conditions of UASB A and originally inoculated with Eerbeek sludge (data not shown). Two liters of this inoculum sludge (corresponding to approximately 38 g $VSS.L^{-1}$) was heat treated (at 70°C for 6 hours) prior to inoculation in UASB D. The original inoculum sludge (Eerbeek, the Netherlands), the sludge cultivated during 18 days in UASB A and the heat treated sludge used as inoculum in UASB D consisted mainly of black, well-shaped granules and also of dispersed flocs.

Substrate and medium

During the whole experiment methanol (UASB A, UASB B and UASB D) and formate (UASB C) were used as sole electron donor and carbon source, providing an influent COD concentration of about 2 $g.L^{-1}$. 99 % pure methanol (Merck, Germany) was used to prepare the methanol stock solution. Formate was supplied as a mixture of 70 % NaCOOH and 30 % HCOOH in order to provide enough protons (H^+) for the oxidation of formate, i.e. to prevent a drastic pH increase which would occur when using solely NaCOOH.

All the reactors were supplied with sulfate, added as sodium sulfate, to achieve a COD/SO_4^{2-} of 0.5 (g COD per g SO_4^{2-}), so theoretically all methanol and formate could be degraded via sulfate reduction. In addition, basal medium containing macro and micro nutrients (2.22 ml per g COD fed) as described in Chapter 3 and trace elements solution (4.5 $mL.L^{-1}$ basal medium) as described by Zehnder *et al.* (1980) were supplied to the influent. Both the basal medium and substrate stock solution were prepared using demineralized water. All chemicals were of analytical grade.

Experimental design

The operational parameters imposed to all four reactors are shown in Table 1. UASB A was started up at 55°C while UASB B and UASB C were started up at day 18 at 65°C using the sludge cultivated in UASB A as inoculum (Table 1). The operational temperature of UASB A was increased to 65ºC between days 47 and 55 in order to assess the effect of a short term temperature increase (65°C) on the performance of the reactor at 55°C (Table 1). The operational temperature of both UASB B and UASB C were increased to 70ºC on day 53 and further increased to 75ºC on day 81 (Table 1). The operational temperature of UASB A was increased to 70ºC on day 74 and further increased to 75ºC on day 88 (Table 1) in order to verify the consistency of the results obtained in UASB B. The operational temperature of both UASB B and UASB C were further increased on day 88 at a rate of 1ºC each 12 hours, till reaching a final temperature of 80ºC on day 90 (Table 1).

UASB D was started up at 55°C with a sludge which was heat treated (70°C) for 6 hours prior to inoculation (see section "inoculum"). The operational temperature of UASB D was increased in two occasions (on days 31 and 51) to 70°C (during two days) in order to assess the effect of a short term temperature increase (70°C) on the performance of the reactor at 55°C (Table 1). The operational temperature of UASB D was increased to 70°C between days 67 to 106, in order to assess the maximal sulfate reduction rate achievable in a methanol-fed UASB reactor operated at that temperature (Table 1). This was done via the gradual decrease of the HRT till a minimal of 3.8 hours on day 102. Simultaneously, the effect of long term temperature increase (70°C) on the performance of the reactor at 55°C was assessed as

well (Table 1). The operational temperature of UASB D was decreased to 55°C on day 107, and reset back to 70°C on day 138. In order to assess the effect of salinity in the performance of the reactor at 70°C, 5 g $NaCl.L^{-1}$ was added to the influent on day 141. The salinity was further increased to 10 g $NaCl.L^{-1}$ on day 149 and kept till the end of the experiment.

Table 1. Summary of the operational parameters applied to the UASB reactors. HRT = hydraulic retention time; OLR = organic loading rate; COD = chemical organic demand; SLR = sulfate loading rate.

UASB A		**55°C**	**65°C**	**70°C**	**75°C**
Parameter	**Days**	**0 - 46**	**47 - 52**	**74 - 88**	**88 – 98**
Influent flow ($L.day^{-1}$)		17.2 ± 0.6	17.47 ± 0.7	17.57*	17.57*
HRT (hour)		9.1 ± 0.3	8.9 ± 0.4	8.88*	8.88*
OLR ($gCOD.L^{-1}.day^{-1}$)		5.7 ± 0.6	5.6 ± 0.3	6.2 ± 0.4	5.4 ± 0.7
COD ($g.L^{-1}$)		2.1 ± 0.2	2.1 ± 0.1	2.3 ± 0.2	2.01 ± 0.2
SLR ($gSO_4^{2-}.L^{-1}.day^{-1}$)		9.5 ± 1.5	10.7 ± 2.3	8.7 ± 0.2	7.3 ± 0.5
SO_4^{2-} ($g.L^{-1}$)		3.6 ± 0.6	3.9 ± 0.8	3.2 ± 0.1	2.7 ± 0.2
COD/SO_4^{2-}		0.58 ± 0.1	0.52 ± 0.1	0.69 ± 0.1	0.58 ± 0.1
pH		7.66 ± 0.18	7.61 ± 0.12	7.61 ± 0.13	7.58 ± 0.13

UASB B		**55°C**	**65°C**	**70°C**	**75°C**
Parameter	**Days**	**0 - 46**	**47 - 52**	**74 - 88**	**88 – 98**
Influent flow ($L.day^{-1}$)		3.0 ± 0.7	2.4 ± 0.2	2.6 ± 0.2	2.5 ± 0.2
HRT (hour)		7.6 ± 1.6	9.2 ± 0.9	8.6 ± 0.9	8.9 ± 0.9
OLR ($gCOD.L^{-1}.day^{-1}$)		7.1 ± 2.1	5.7 ± 0.6	6.1 ± 0.5	5.9 ± 0.9
COD ($g.L^{-1}$)		2.2 ± 0.3	2.2 ± 0.1	2.2 ± 0.1	2.2 ± 0.2
SLR ($gSO_4^{2-}.L^{-1}.day^{-1}$)		14.9 ± 4.0	14.7 ± 4.9	13.73*	11.78*
SO_4^{2-} ($g.L^{-1}$)		4.6 ± 0.8	5.3 ± 0.8	4.67*	4.95*
COD/SO_4^{2-}		0.50 ± 0.1	0.42 ± 0.08	0.47*	0.42*
pH		7.69 ± 0.30	7.65 ± 0.20	7.59 ± 0.05	7.64 ± 0.26

UASB C		**55°C**	**65°C**	**70°C**	**75°C**
Parameter	**Days**	**0 - 46**	**47 - 52**	**74 - 88**	**88 – 98**
Influent flow ($L.day^{-1}$)		3.3 ± 0.5	2.8 ± 0.3	3.0 ± 0.5	2.8 ± 0.6
HRT (hour)		6.8 ± 1.1	8.0 ± 0.9	7.6 ± 1.5	8.2 ± 2.2
OLR ($gCOD.L^{-1}.day^{-1}$)		7.7 ± 1.2	6.2 ± 0.7	6.6 ± 1.1	6.4 ± 1.3
COD ($g.L^{-1}$)		2.1 ± 0.1	2.1 ± 0.1	2.1 ± 0.1	2.1 ± 0.1
SLR ($gSO_4^{2-}.L^{-1}.day^{-1}$)		15.2 ± 4.2	19.6 ± 2.9	16.09*	10.30*
SO_4^{2-} ($g.L^{-1}$)		4.3 ± 2.3	5.8 ± 0.2	4.78*	4.94*
COD/SO_4^{2-}		0.57 ± 0.29	0.35 ± 0.01	0.43*	0.43*
pH		7.78 ± 0.20	7.59 ± 0.15	7.61 ± 0.07	7.12 ± 0.97

Table 1. continued.

UASB D	55°C	55°C	55°C	70°C	55°C	70°C
Parameter Days	**0** ** **– 31** ***	**33 – 51** ***	**53 – 66**	**67 – 106**	**107 – 137**	**138 – 170**
Influent flow ($L.day^{-1}$)	17.9 ± 0.5	17.4 ± 0.2	17.5 ± 0.6	25.2 ± 8.9	18.6 ± 1.6	18.1 ± 1.0
HRT (hour)	8.7 ± 0.2	8.9 ± 0.1	8.9 ± 0.3	7.0 ± 2.1	8.4 ± 0.6	8.6 ± 0.6
OLR ($gCOD.L^{-1}.day^{-1}$)	6.3 ± 1.90	4.4 ± 1.1	6.1 ± 0.8	7.3 ± 2.8	5.6 ± 0.9	5.4 ± 0.4
COD ($g.L^{-1}$)	2.1 ± 0.20	2.0 ± 0.1	1.9 ± 0.2	2.2 ± 0.1	2.1 ± 0.1	2.1 ± 0.1
SLR ($g\ SO_4^{2-}.L^{-1}.day^{-1}$)	10.3 ± 0.1	8.2 ± 0.2	11.3 ± 0.2	17.5 ± 3.2	11.2 ± 0.1	10.2 ± 0.1
SO_4^{2-} ($g.L^{-1}$)	3.4 ± 0.7	4.2 ± 0.3	4.0 ± 0.1	4.4 ± 0.3	4.2 ± 0.1	4.3 ± 0.1
COD/SO_4^{2-}	0.75 ± 0.29	0.48 ± 0.04	0.47 ± 0.08	0.58 ± 0.08	0.50 ± 0.12	0.42 ± 0.10
pH	7.47 ± 0.10	7.56 ± 0.10	7.55 ± 0.11	7.67 ± 0.10	7.66 ± 0.11	7.55 ± 0.13

* Only one data point of that period is available.
** A six-hour heat treatment (70°C) was applied to the inoculum sludge prior to inoculation.
*** A two-day heat treatment (70°C) was applied on days 31 and 51.

Maximum specific activity test

Maximum specific activity tests were performed in 117 ml serum bottles with methanol, formate, acetate and hydrogen as the substrates in the presence of sulfate as described in Chapter 3. The vials were incubated in a waterbath at the selected temperature and shaken at 50 rpm.

Effect of temperature

The effect of temperature (55, 65, 70, 75 and 80°C) was assessed through activity assays with sludge sampled from UASB A, UASB B and UASB C at the end of each operational period. The assays with methanol and formate as the substrate were also performed in the absence of sulfate. In order to highlight the pathways of formate and methanol degradation as a function of temperature (55, 65, 70, 75 and 80°C), methanogenesis (formate-fed vials) and acetogenesis (methanol-fed vials) were suppressed by specific inhibitors. Vials were supplemented with, respectively, 30 mM of sodium 2-bromoethanesulfonate (BES) and 0.17 mM of vancomycin, which are specific inhibitors of methanogenic archaea and acetogenic bacteria (Oremland and Capone, 1988).

Effect of salinity

The effect of different salts (25 g $NaCl.L^{-1}$, 25 g $NaHCO_3^-.L^{-1}$, and a cocktail of salts composed by 25 g $NaCl.L^{-1}$ + 2.5 g $MgCl_2.L^{-1}$ + 3 g $KCl.L^{-1}$) in the degradation of methanol and hydrogen at 70°C was assessed through activity tests with sludge sampled from UASB D on day 170, when the reactor was operating at an influent NaCl concentration of 10 $g.L^{-1}$.

Analysis

VSS were analyzed according to standard methods (APHA, 1985). Sulfide was determined photometrically as described by Trüper and Schlegel (1964). Methanol, VFA and methane were measured by gas chromatography (GC), as described by Weijma *et al.* (2000a). Formate was analyzed by high pressure liquid chromatography as described in Gonzalez-Gil (2002). Biogas produced in the UASB reactors was measured as described in Chapter 3.

RESULTS

Performance of methanol-fed UASB reactors (UASB A and UASB B)

Performance at 55°C (UASB A)

The start up of UASB A proceeded rapidly at 55°C and from day 11 onwards, the methanol removal efficiency was over 99 % (Fig. 1A). A maximal sulfide production rate of 3.45 g $COD.L^{-1}.day^{-1}$ was obtained on day 32 (Fig. 3A) with sulfide as the main mineralization product of methanol degradation, accounting for about 78 % of the consumed methanol-COD (Fig. 4A). Methane production was detected from day 10 onwards and it steadily increased when operating UASB A at 55ºC (Fig. 2A). The maximal methane production rate (1.19 g $COD.L^{-1}.day^{-1}$) was obtained on day 47, prior to the increase of the temperature to 65°C (Fig. 3A). This corresponds to maximally 27 % of the electron flow (Fig. 4A). The effluent acetate concentration reached a maximum of 280 $mg.L^{-1}$ on day 14 (Fig. 2A), but it dropped to concentrations around 130 $mg.L^{-1}$ just before the increase of the operational temperature to 65°C (Fig. 2A), corresponding to around 9 % of the electron flow (Fig. 4A).

Effect of short-term (5 days) temperature increase to 65°C on the performance of UASB A

Just after the operating temperature of UASB A was increased to 65°C on day 47, the sulfide production increased at the expense of a sharp decrease in the methane production (Fig. 2A) which dropped from 3.26 $L.day^{-1}$ (at day 47) to 0.18 $L.day^{-1}$ (24 hours later, on day 48) and remained low till the end of the experiment (Fig. 2A). Apparently, acetate production was not affected by the 5-day temperature increase to 65ºC, because the concentrations remained at about 95 $mg.L^{-1}$ during this 5-day period. Sulfide was by far the main mineralization product when operating UASB A at 65°C, accounting for around 92 % of the electron flow (Fig. 4A). Acetate and methane accounted for, respectively, 7 % and 1 % of the electron flow (Fig. 4A).

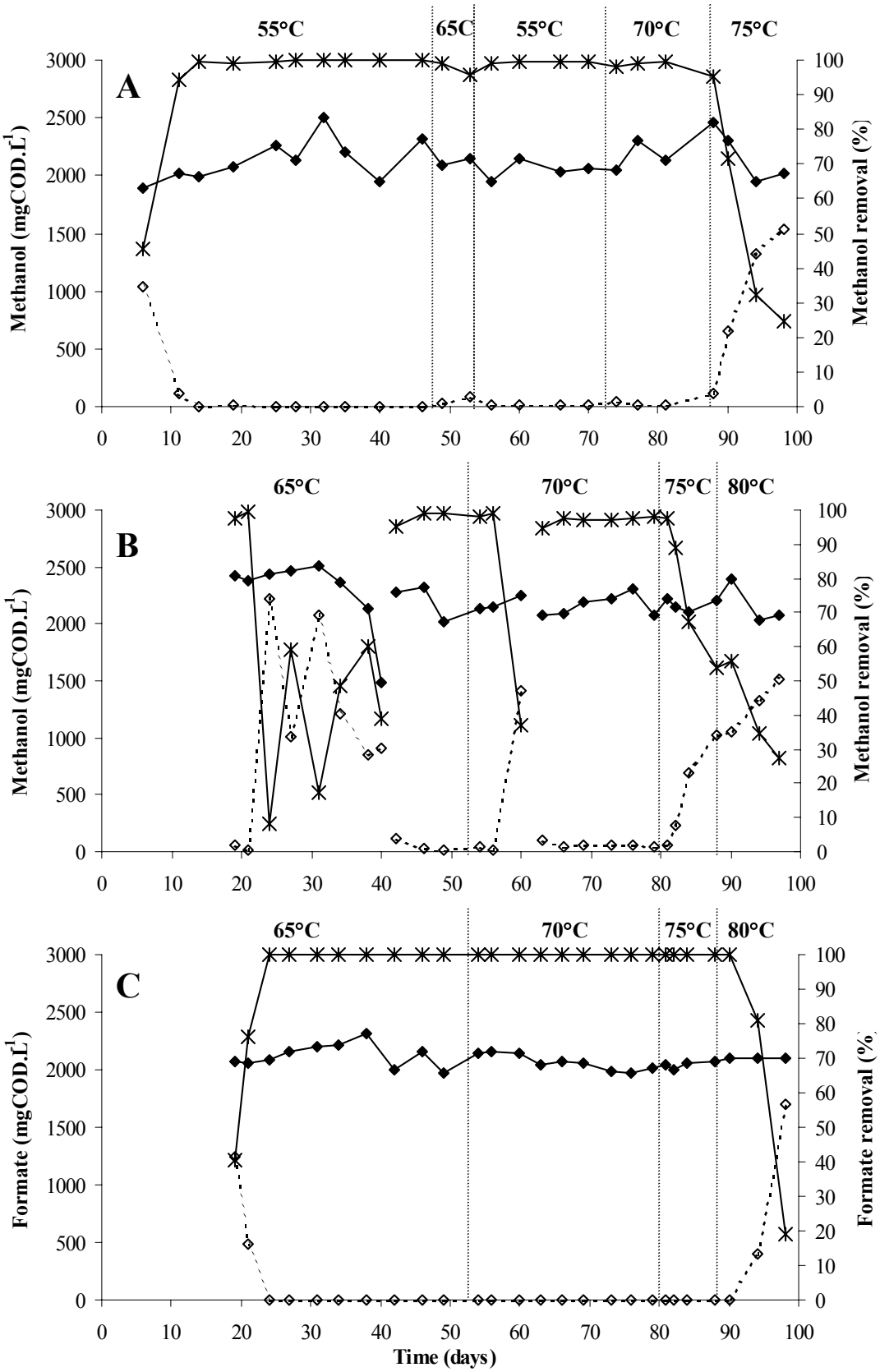

Figure 1. Substrate removal in (A) UASB A, (B) UASB B, (C) UASB C. Substrate concentration in the influent (♦) and in the effluent (✧), substrate removal efficiency (∗).

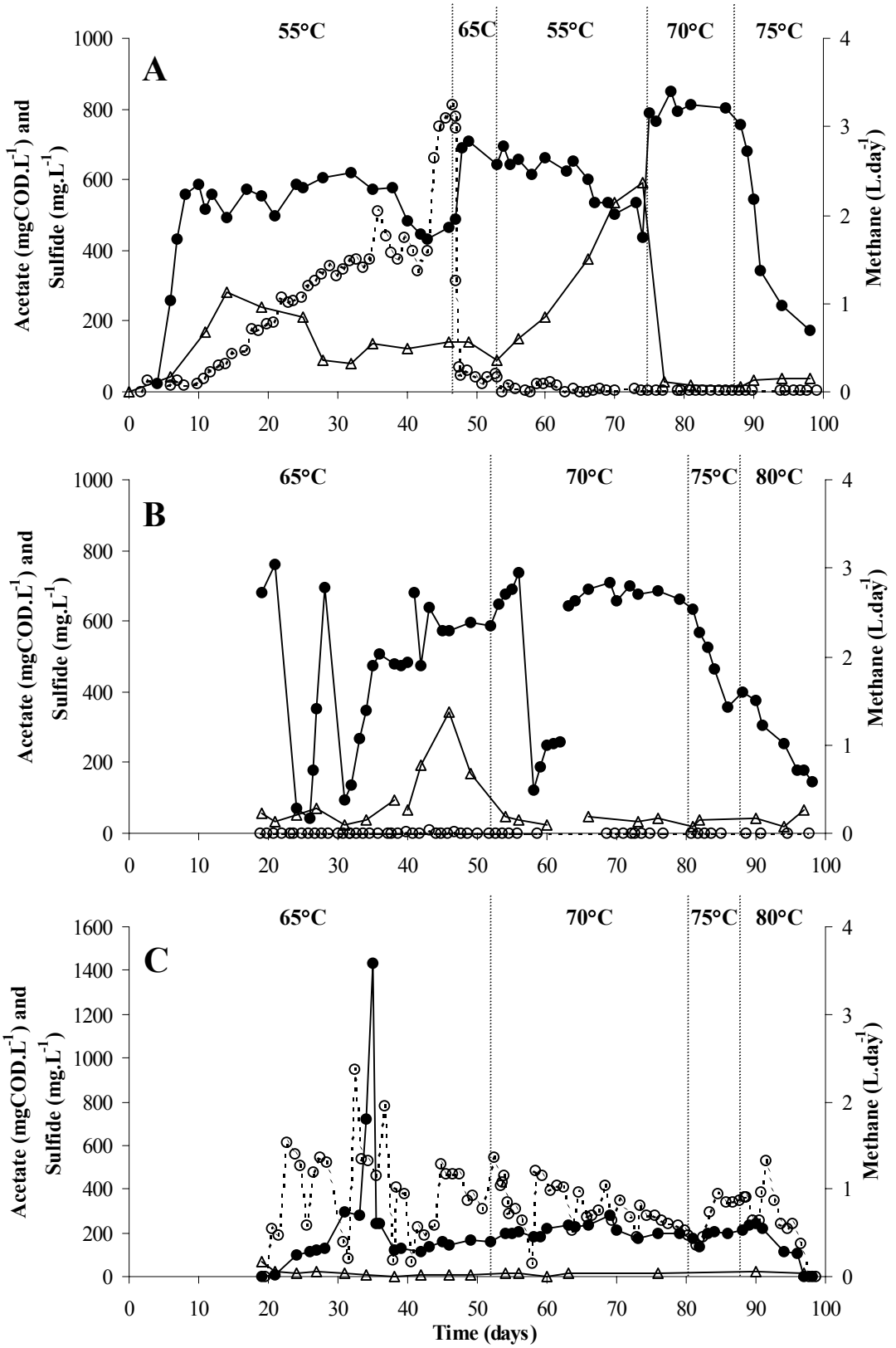

Figure 2. Concentration of products in (A) UASB A, (B) UASB B, (C) UASB C. Acetate (△), sulfide (•) and methane (o) concentrations.

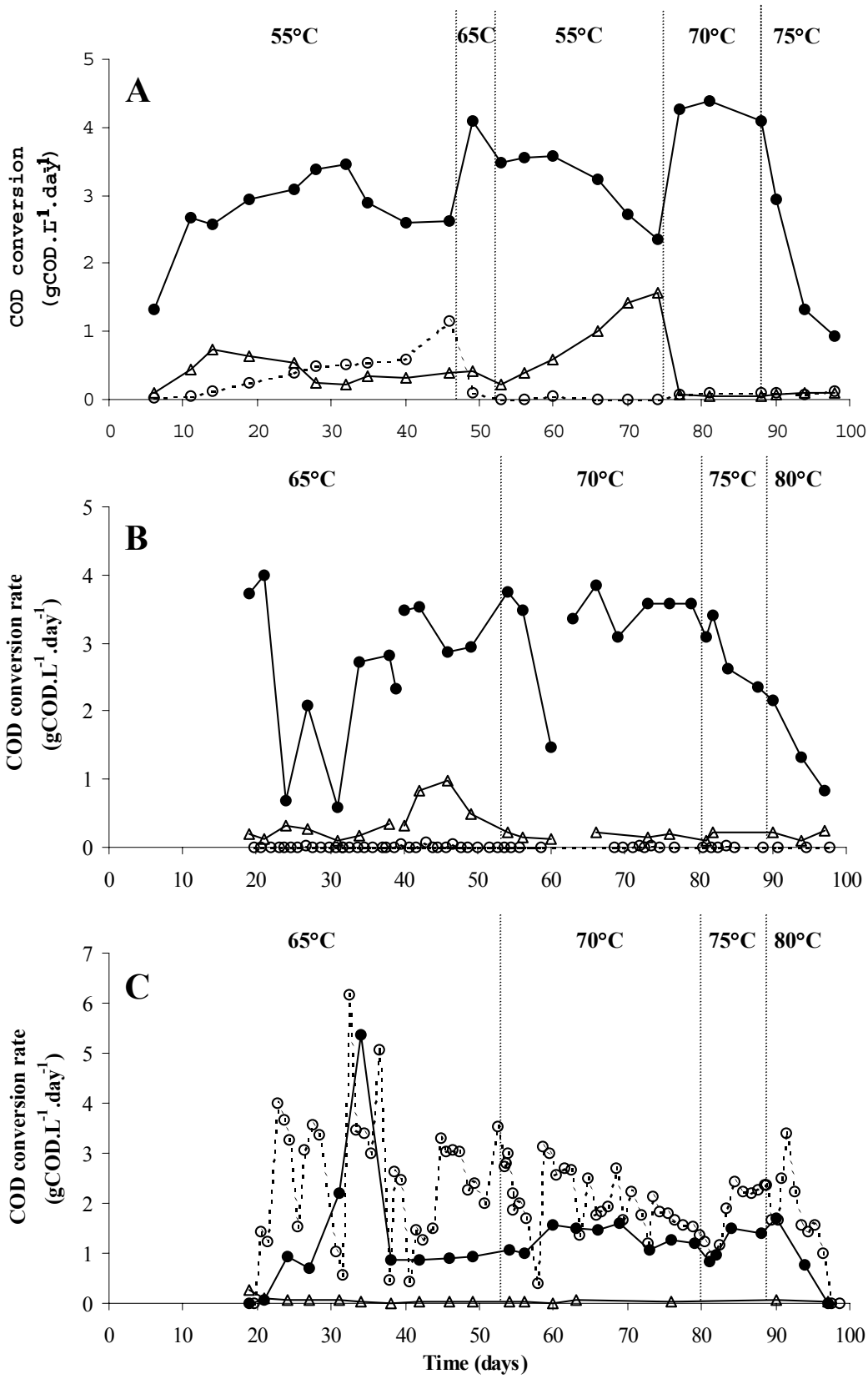

Figure 3. COD conversion rates in (A) UASB A, (B) UASB B, (C) UASB C. Acetate (△), sulfide (•) and methane (o) production rates.

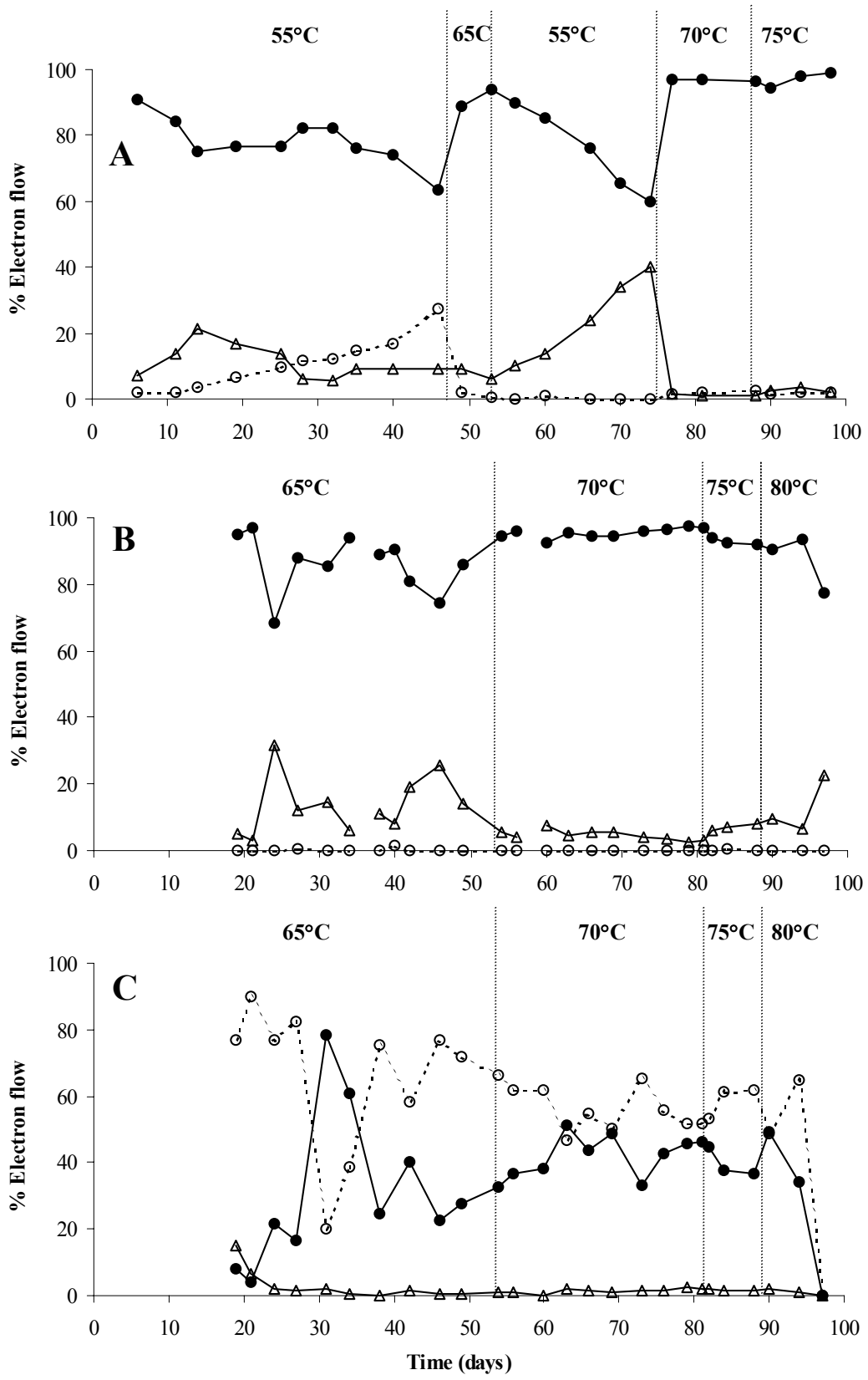

Figure 4. Relative electron flow in (A) UASB A, (B) UASB B, (C) UASB C. Acetate (△), sulfide (•) and methane (o).

Upon switching back the operational temperature to 55°C on day 53, methane production did not resume to the values obtained in the period when UASB A was operated at 55ºC in the first 47 days (Fig. 2A). Acetate production, however, started to increase at the expense of the sulfide production (Fig. 2A) up to concentrations as high as 543 $mg.L^{-1}$ on day 74 (Fig. 2A). The acetate production corresponded to 40 % of the consumed methanol-COD before switching the temperature to 70°C on day 74, whereas sulfide production corresponded to the remaining 60 % of the electron flow (Fig. 4A).

Performance at 65ºC (UASB B)

Already one day after the start-up of UASB B at 65°C, a full methanol removal (Fig. 1B) coupled to a high sulfide production (Fig. 2B) were achieved. Methane production was not detected throughout the reactor run (Fig. 2B), even though the sludge cultivated in UASB A gave a methane production rate of 0.25 $gCOD.L^{-1}.day^{-1}$ on day 18, at the time when part of the sludge was transferred from UASB A to UASB B (Fig. 2C). As observed in UASB A (i.e. after increasing the temperature to 65ºC during 5 days), the main product of methanol degradation in UASB B was sulfide, accounting for 86 % of the electron flow, while acetate production at its maximum corresponded to 14 % of the consumed COD-methanol (Fig. 4B).

The performance of UASB B sharply deteriorated upon exposing the reactor to a one day non-feed (starvation), accompanied by a pH increase to 8.4 (due to a malfunctioning of the pH controller) on day 24 (Figs. 1A and 1B). UASB B rapidly recovered from this disturbance, reaching a sulfide production rate of 4.30 $gCOD.L^{-1}.day^{-1}$ on day 28 (Fig. 3B). Another one day non-feed period on day 29 provoked the sulfide production rate to drop promptly to 0.58 $gCOD.L^{-1}.day^{-1}$ (Fig. 3B). The methanol removal efficiencies did not exceed 60 % anymore and no recovery trend of the sulfide production concentration occurred (Figs. 1B and 2B). UASB B was then re-inoculated with the sludge harvested from UASB A, which was still operating at 55ºC on day 39 (Figs. 1A and 1B). Similarly as found in the first start up of UASB B, full methanol removal coupled to high sulfide concentrations were obtained just after re-inoculation (Fig. 2B), but opposed to the first start up, the acetate production rate started to increase at the expense of the sulfide production rate, reaching a value of 0.98 $gCOD.L^{-1}.day^{-1}$ on day 46 (Fig. 3B). However, the acetate production dropped to around 0.50 $gCOD.L^{-1}.day^{-1}$ on day 49 (corresponding to about 10 % of the electron flow) till the day when the temperature of the reactor was increased to 70°C (Fig. 4B).

Performance at 70ºC (UASB A and UASB B)

Methanol was fully removed in both UASB A and UASB B when operating at 70°C (Fig. 1A and 1B) and the sulfide production further increased in both reactors (Figs. 2A and

2B). A remarkable decrease of the acetate production occurred in UASB A upon increasing the temperature from 55ºC to 70ºC on day 74 (Fig. 2A). A one day non-feed (starvation) period simultaneous to a temperature drop from 70ºC to 55ºC on day 58 caused the methanol removal efficiency of UASB B to drop from almost 100 % to around 37 % (Fig 1B). Re-inoculation of UASB B (70°C) with sludge harvested from UASB A on day 62 (still operating at 55ºC on this day) re-established the complete methanol removal coupled to sulfide production on day 64 (Fig. 1B and 2B).

When operating the reactors at 70ºC, acetate production became very low and methane production could not be detected in either UASB A or UASB B (Figs. 2A and 2B). Thus, almost merely sulfide was produced when operating the methanol-fed UASB reactors at 70ºC. Sulfide accounted for 97 % and 96 % of the electron flow in, respectively, UASB A (Fig. 4A) and UASB B (Fig. 4B), whereas acetate accounted for only 3 % and 4 % of the converted methanol-COD in, respectively, UASB A (Fig. 4A) and UASB B (Fig. 4B).

Effect of a short-term (6 hours) temperature shock (70ºC) of the inoculum sludge in the performance of UASB D at 55 ºC

The 6-hour heat treatment (70°C) of the inoculum sludge completely suppressed methane production in the start-up and operation of UASD D (Fig. 5B), even though this inoculum sludge was harvested from a thermophilic (55°C) methanol-fed UASB reactor with an active methanogenic population (data not shown). As a matter of fact, methane production was not observed throughout the reactor run (Fig. 5B). Acetate production was relatively low during the start-up of UASB D, accounting for less than 12% of the electron flow till day 12 (Fig. 5D). Sulfide production was by far the most important metabolic product during the start-up of UASB D, with a maximal production rate of 4.32 g $COD.L^{-1}.day^{-1}$ on day 7 (Fig. 3C). Acetate production, however, increased at the expense of sulfide production and a maximal acetate concentration of 980 $mg.L^{-1}$ was measured on day 29 (Fig. 5B), which corresponds to a production rate of 2.63 g $COD.L^{-1}.day^{-1}$ (Fig. 5C). Prior to the temperature increase on day 31, sulfide and acetate accounted for 50 % each of the electron flow (Fig. 5D).

Effect of short-term (2 days) temperature increase to 70ºC on the performance of UASB D

Just after the operating temperature of UASB D was increased to 70°C on days 31 and 51, the acetate production sharply decreased, from 980 $mg.L^{-1}$ to 32 $mg.L^{-1}$ measured, respectively, on days 31 and 34, and from 270 $mg.L^{-1}$ to 11 $mg.L^{-1}$ measured, respectively, on days 51 and 54 (Fig. 5B). Acetate production, however, resumed within a few days to a maximal concentrations of 290 $mg.L^{-1}$ (after the first temperature shock on day 31) and of 240

mg.L^{-1} (after the second temperature shock on day 51). A moderate drop in sulfide production occurred upon the 2-day temperature increase to 70°C on days 31 and 51, but sulfide production was recovered within a few days (Fig. 5B). Sulfide represented the most important end product of methanol degradation after these 2-day temperature increase to 70°C, accounting for 80 % of the electron flow, whereas acetate accounted for the remaining 20 % (Fig. 5D).

Performance of UASB D at 70ºC: assessing the maximal sulfate reduction rate

As for UASB A and UASB B, almost only sulfide production was observed in UASB D when operating at 70°C (Figs. 5B and 5D). The sulfide reached a maximal concentration of 970 mg.L^{-1} on day 85. On day 102, the imposed low HRT of 3.8 hours (Fig. 6) resulted in the maximal sulfate reduction rate of 14.4 g SO_4^{2-}.L^{-1}.day^{-1} (Fig. 6). On this day, methanol was detected in the effluent, indicating that the reactor was operating at its maximal capacity (Fig. 5A).

Effect of long term (39 days) temperature increase to 70ºC on the performance of UASB D

Acetate started to be produced within a few days after the operational temperature was decreased to 55°C on day 106 (Fig. 5B), indicating that the effects of long term exposition of the reactor sludge to 70°C are similar to that obtained after short term (2 days) exposure (Fig. 5B). At the end of this period, sulfide accounted for 70 % of the electron flow, whereas acetate accounted for the remaining 30 % (Fig. 5D).

Performance of UASB D at 70ºC: assessing the effect of NaCl in the influent

Only sulfide production was observed when the operational temperature of the reactor was increased to 70°C on day 138 (Fig. 5B). The addition of 5 g NaCl.L^{-1} on day 142 did not affect the performance of UASB D, as evidenced by the high sulfide concentration of 830 mg.L^{-1} measured on day 147 (Fig. 5B). The addition of 10 g NaCl.L^{-1} on day 149, however, caused a drop in the methanol removal efficiency (Fig. 5A), which was accompanied by a steady decrease in the sulfide production (Fig. 5B). The sulfide production rate decreased from 4.80 g COD.L^{-1}.day^{-1} on day 147 to 2.25 g COD.L^{-1}.day^{-1} on day 175. Surprisingly, acetate started to be produced after NaCl was added in the influent (Fig. 5B) and a maximal acetate production rate of 0.31 g COD.L^{-1}.day^{-1} was observed on day 175. It is worth to remember that only very low acetate production was observed in the methanol-fed UASB reactors when no additional NaCl was added to the influent (Figs. 2B and 5B). Sulfide was the main mineralization product at the end of the experiment, accounting for 87 % of the electron flow, whereas acetate accounted for the remaining 13 %.

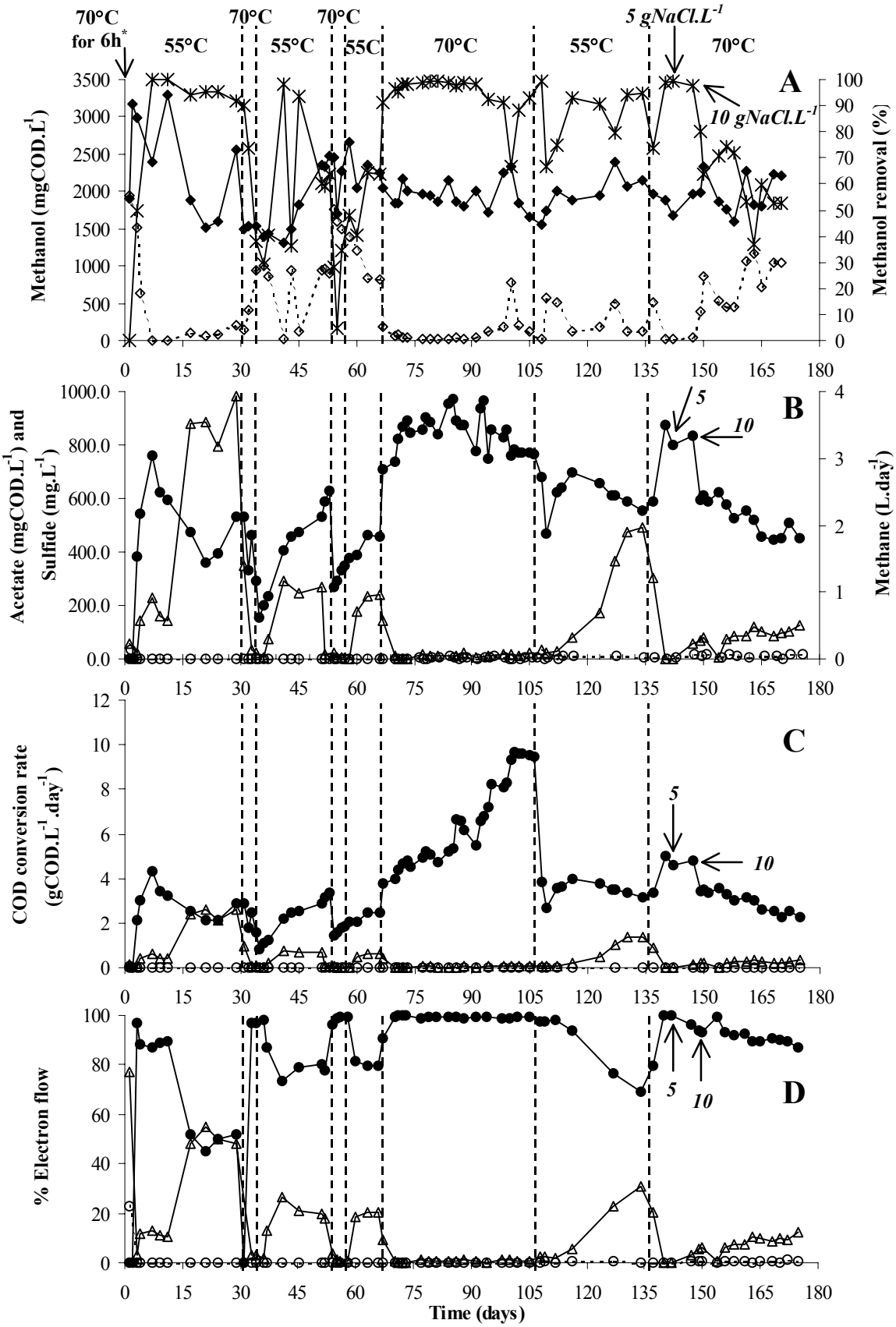

Figure 5. Process performance of UASB D. (A) Methanol concentrations in the influent (♦) effluent (✧) and substrate removal efficiency (∗). (B) Concentrations of sulfide (•), methane (o) and acetate (△). (C) COD conversion rate to sulfide (•), methane (o) and acetate (△). (D) Relative electron flow to sulfide (•), methane (o) and acetate (△).

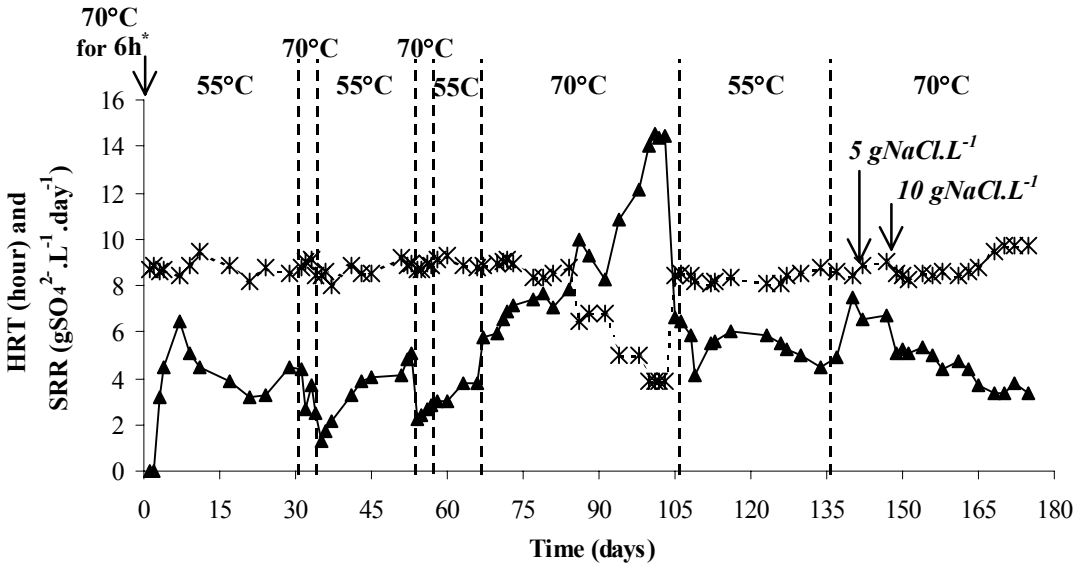

Figure 6. Evolution of the HRT and sulfate reduction rate (SRR) in UASB D.

Performance at 75ºC (UASB A and UASB B)

The methanol removal efficiency steadily decreased in both UASB A and UASB B upon increasing the operation temperature from 70ºC to 75ºC (Figs. 1A and 1B). This was accompanied by a steady decrease of the sulfide production in both UASB reactors (Figs. 2A and 2B). Acetate production remained very low and methane production could not be detected in both reactors (Figs. 2A and 2B).

Performance at 80ºC (UASB B)

An increase of the reactor temperature from 75ºC to 80ºC in UASB B resulted in a further drop of the methanol removal efficiency (Fig. 1B). Sulfide production continued decreasing at this temperature (Fig. 2B) and only a very low acetate production was measured during this period while no methane production could be detected (Fig. 2B).

Performance of the formate-fed UASB reactor (UASB C)

Performance at 65ºC

A complete formate removal was observed from day 24 onwards (3 days after the start-up) in UASB C (Fig. 1C). In contrast to the methanol-fed reactors, methane was the main product of the formate degradation, with average methane production rates of 2.54 g $COD.L^{-1}.day^{-1}$ (Fig. 3C). The sulfide production rate steadily increased after the start up of UASB C, reaching within 12 days a maximal sulfide concentration of 1430 $mg.L^{-1}$ on day 35 (Fig. 2C). This high sulfide concentration was apparently toxic to the SRB, as evidenced by

the sharp drop in the sulfide concentration to 242 $mg.L^{-1}$ one day after the sulfide concentration peak (Fig. 2C) and even dropped to 125 $mg.L^{-1}$ on day 38, remaining at about 140 $mg.L^{-1}$ until the temperature was increased to 70°C (Fig. 2C). Methane was the main mineralization product from formate degradation at 65ºC, accounting for 72 % of the electron flow. Sulfide accounted for the remaining 28 % of the electron flow, if the peak in sulfide production is neglected. The acetate production remained negligible throughout the run of UASB C, corresponding to a maximum of 2 % of the formate-COD removed (Figs. 2C and 4C).

Performance at 70ºC

The sulfide production rate slightly increased to values of around 1.10 g $COD.L^{-1}.day^{-1}$ during the period that UASB C operated at 70°C, whereas the methane production rate slightly decreased to values of around 1.79 g $COD.L^{-1}.day^{-1}$ during the same period (Fig. 3C). Methane was still the main mineralization product of formate oxidation when operating UASB C at 70°C. In contrast to the methanol-fed UASB B, the formate-fed UASB C was not affected by the one day non-feed period and the temperature drop from 70ºC to 55ºC on day 58 (Fig. 1B vs. Fig. 1C). Methane and sulfide production accounted for, respectively, 57 % and 42 % of the electron flow, whereas acetate accounted for only 1 % of the consumed formate-COD (Fig. 4C).

Performance at 75ºC

The formate removal efficiency was not affected by the temperature increase to 75°C in UASB C (Figs 1A and 1B vs. Fig. 1C). The electron flow distribution in UASB C remained similar to that observed when operating the reactor at 70ºC. Methane accounted for 57 % of the electron flow, whereas sulfide accounted for 41 % of the electron flow (Fig 4C). Acetate accounted for only 2 % of the consumed formate-COD (Fig 4C).

Performance at 80ºC

Two days after increasing the operational temperature to 80°C (day 94), a steady decrease of the formate removal efficiency occurred (Fig. 1C). Malfunctioning of the pH controller caused a pH drop to 4.5 on day 97, which lead to a drop of the formate removal to 18 %. From this day onwards, there was neither sulfide nor methane production in UASB C (Fig. 2C).

Effect of temperature on the metabolic characteristics of the sludges

Methanol grown thermophilic granular sludges

The highest methanol depletion rates for the vials inoculated either in the presence or absence of sulfate were obtained for the sludge cultivated at 65ºC with methanol as the substrate (Table 2). It is worth to note that the maximum specific activities of this sludge using either methanol or hydrogen as the substrate were higher at 70°C than at 55°C (Table 2). Very low activities were found at 75°C for all substrates tested, except for the still considerably high sulfidogenic activity on hydrogen as the substrate (Table 2). No activity was found at 80°C for all substrates tested, during the 14 days of incubation (Table 2).

The strong competition between SRB and MPA for methanol and hydrogen in the batch vials inoculated at 55°C (Table 3) are in line with the results of UASB A operating at that temperature (Fig. 2A and 2B). As observed in UASB A, acetate was also a minor secondary product in the vials, either in the absence (18 %) or the presence (9 %) of sulfate (Table 3). As for the continuous reactor when operating at 65ºC or higher (Figs. 2A and 2B), no methane production was observed in the vials inoculated with methanol and excess of sulfate at temperatures equal or higher than 65ºC (Table 3). Thus, the SRB clearly outcompete the MPA for methanol degradation at temperatures equal or higher than 65ºC (Table 3), with a maximal specific sulfidogenic activity at 65°C (Table 2). Direct methanol conversion via methanogenesis (vials amended with vancomycin) was only found at 55°C (Table 2). In contrast, direct methanol conversion via sulfate reduction was possible till a temperature of 75°C (Table 2).

Interestingly, sulfide accounted for almost all of the electron flow when using acetate as the substrate, independently of the tested temperature (Table 3). However, acetate was not an important substrate for the SRB present in the sludge cultivated in the methanol-fed reactors, as evidenced by the very low sulfidogenic activities at all temperatures tested (Table 2). In contrast, the high sulfidogenic and methanogenic activities using hydrogen as the substrate at all temperatures tested clearly suggests that hydrogen plays a key role as an intermediate in the methanol breakdown under thermophilic conditions (Table 2). As for the methanol-fed vials, the highest activities using hydrogen as the substrate were obtained at 65°C (Table 2). In addition, no methane production was observed when using hydrogen as the substrate both at 70°C and 75°C (Table 3), which contrasts to the high methanogenic hydrogenotrophic activity for the formate-grown sludge at that temperature (Table 3).

Table 2. Maximal specific substrate depletion rates and maximal specific acetate, methane and sulfide production rates (g COD.gVSS^{-1}.day^{-1}) for the sludge sampled from UASB A, UASB B and UASB C reactors at different temperatures. For the methanol-fed reactors, sludges sampled from UASB A (55 and 70°C) and from UASB B (65, 75 and 80°C).

T	Methanol-fed (UASB A and UASB B)					Formate-fed (UASB C)				
	Condition	Substrate depletion rate	Acetate production rate	Methane production rate	Sulfide production rate	Condition	Substrate depletion rate	Acetate production rate	Methane production rate	Sulfide production rate
55°C	*MeOH*	1.15 (0.31)	nd	0.43 (0.08)	nd					
	MeOH + SO_4^{2-}	0.53 (0.01)	nd	0.22 (0)	0.20 (0.04)					
	Acetate + SO_4^{2-}	0.07 (0.01)	nd	0	0.08 (0.02)					
	MeOH + SO_4^{2-} + Vc	0.48 (0.19)	nd	0.24 (0.02)	0.08 (0.01)					
	H_2/CO_2 + SO_4^{2-}	nd	nd	0.35 (0.02)	0.34 (0.02)					
65°C	*MeOH*	1.25 (0.13)	nd	618 (135)	nd	*Formate*	2.92 (0.23)	nd	2.74 (0.34)	-
	MeOH + SO_4^{2-}	1.22 (0.18)	nd	0	0.76 (0.18)	Formate + SO_4^{2-}	2.97 (0.21)	nd	2.60 (0.32)	0.15 (0.03)
	Acetate + SO_4^{2-}	0.07*	nd	0	0.16*	Acetate + SO_4^{2-}	0.03 (0.01)	nd	0.02 (0.01)	0.01 (0.01)
	MeOH + SO_4^{2-} + Vc	0.45 (0.08)	nd	0	0.56 (0.10)	Form. + SO_4^{2-} + Bs	0.57 (0.01)	nd	0.10 (0.02)	0.20 (0.04)
	H_2/CO_2 + SO_4^{2-}	nd	nd	1.26 (0.11)	1.17 (0.10)	H_2/CO_2 + SO_4^{2-}	nd	nd	1.38 (0.04)	0.36 (0.04)
70°C	*MeOH*	0.91 (0.17)	0.11 (0.02)	0.75	nd	*Formate*	1.67 (0.23)	0	2.14 (0.31)	nd
	MeOH + SO_4^{2-}	0.73 (0.06)	0	0	0.53 (0.10)	Formate + SO_4^{2-}	1.81 (0.22)	0	1.93 (0.20)	0.38 (0)
	Acetate + SO_4^{2-}	0.01 (0)	0	0	0.01 (0)					
	MeOH + SO_4^{2-} + Vc	0.16 (0.01)	0.03 (0.01)	0	0.12 (0.01)					
	H_2/CO_2 + SO_4^{2-}	nd	0	0	0.81 (0.08)	H_2/CO_2 + SO_4^{2-}	nd	0	1.24 (0.21)	0.43 (0.12)
75°C	*MeOH*	0.08 (0.02)	nd	0.09 (0.01)	nd	*Formate*	0.85 (0.18)	nd	1.12 (0.28)	nd
	MeOH + SO_4^{2-}	0.06 (0.01)	nd	0	0.05 (0.1)	Formate + SO_4^{2-}	0.90 (0.15)	nd	0.91 (0.08)	0.16 (0.04)
	Acetate + SO_4^{2-}	0	nd	0	0					
	MeOH + SO_4^{2-} + Vc	0.02 (0.01)	nd	0	0.02 (0.01)					
	H_2/CO_2 + SO_4^{2-}	nd	nd	0	0.63 (0.12)	H_2/CO_2 + SO_4^{2-}	nd	nd	0.84*	0.28*
80°C	*MeOH*	0	nd	0	0	*Formate*	0	nd	0	nd
	MeOH + SO_4^{2-}	0	nd	0	0	Formate + SO_4^{2-}	0	nd	0	0
	H_2/CO_2 + SO_4^{2-}	nd	nd	0	0	H_2/CO_2 + SO_4^{2-}	nd	nd	0	0

MeOH – Methanol; SO_4^{2-} - Sulfate; Vc – Vancomycin; Bs – sodium 2-bromoethanesulfonate (BES).
(*) Results only from one vial. (nd) not determined. Standard deviation is given within parenthesis.

Table 3. Electron flow (%) for the sludge sampled from the UASB reactors at different temperatures. For the methanol-fed reactors, samples were harvested from UASB A (55 and 70°C) and from UASB B (65, 75 and 80°C).

T	Methanol-fed (UASB A and UASB)				Formate-fed (UASB C)			
	Condition	CH_4	H_2S	Acetate	Condition	CH_4	H_2S	Acetate
55°C	MeOH	82 (2)	nd	18 (2)				
	MeOH + SO_4^{2-}	38 (2)	53	9 (2)				
	Acetate + SO_4^{2-}	2 (0)	98 (1)	nd				
	MeOH + SO_4^{2-} +Vc	72 (4)	17 (3)	11 (1)				
	H_2/CO_2 + SO_4^{2-}	42 (1)	53 (2)	5 (1)				
65°C	MeOH	81(1)	nd	19 (1)	Formate	100	nd	0
	MeOH + SO_4^{2-}	0	96 (1)	4 (1)	Formate + SO_4^{2-}	91 (1)	9 (1)	0
	Acetate + SO_4^{2-}	0*	100*	nd	Acetate + SO_4^{2-}	48 (7)	52 (7)	nd
	MeOH + SO_4^{2-} +Vc	0	97 (1)	3 (1)	Formate + SO_4^{2}+Bs	52 (3)	48 (3)	0
	H_2/CO_2 + SO_4^{2-}	48 (3)	47 (4)	5 (1)	H_2/CO_2 + SO_4^{2-}	66 (9)	22 (7)	12 (2)
70°C	MeOH	68 (5)	nd	32 (5)	Formate	100	nd	0
	MeOH + SO_4^{2-}	0	88 (5)	12 (5)	Formate + SO_4^{2-}	90 (2)	10 (2)	0
	Acetate + SO_4^{2-}	7 (2)	93 (2)	nd				
	MeOH + SO_4^{2-} +Vc	0	76 (7)	24 (7)				
	H_2/CO_2 + SO_4^{2-}	0	90 (1)	10 (1)	H_2/CO_2 + SO_4^{2-}	81 (2)	13 (1)	6 (2)
75°C	MeOH	65 (7)	nd	35 (7)	Formate	100	nd	0
	MeOH + SO_4^{2-}	0	84 (6)	16 (6)	Formate + SO_4^{2-}	88 (1)	12 (1)	0
	Acetate + SO_4^{2-}	0	0	nd				
	MeOH + SO_4^{2-} +Vc	0	72 (9)	28 (9)				
	H_2/CO_2 + SO_4^{2-}	0	88 (2)	12 (2)	H_2/CO_2 + SO_4^{2-}	78*	14*	8*
80°C	MeOH	0	nd	0	Formate	0	nd	0
	MeOH + SO_4^{2-}	0	0	0	Formate + SO_4^{2-}	0	0	0
	H_2/CO_2 + SO_4^{2-}	0	0	0	H_2/CO_2 + SO_4^{2-}	0	0	0

MeOH – Methanol; SO_4^{2-} - Sulfate; Vc – Vancomycin; Bs – sodium 2-bromoethanesulfonate (BES).
(*) Results only from one vial. (nd) not determined. Standard deviation is given within parenthesis.

Formate grown thermophilic granular sludges

The highest formate depletion rates for the vials inoculated either in the presence or the absence of sulfate were obtained for the sludge cultivated at 65ºC grown on formate (Table 2). No activity was found with this sludge at 80°C for all substrates tested, during the 14 days of incubation (Table 2).

The high methane content upon the completion of the batch tests (Table 3) are in line with the observed methanogenic behavior of the sludge cultivated in the formate-fed UASB C (Fig. 2C). This indicates that the MPA outcompete the SRB when using formate as the substrate for all temperatures tested. No acetate production could be detected when using formate as electron donor at all temperatures tested (Table 3). Sulfide accounted for 9, 10 and 12 % of the electron flow for vials inoculated at, respectively, 65, 70 and 75°C (Table 3).

Although a high methanogenic activity was obtained when using hydrogen as the substrate this is still two times lower than the formate consumption rates at 65°C (Table 2). The more evident competition between SRB and MPA for hydrogen probably indicates that the sulfide production observed in UASB C might be exclusively attributed to hydrogenotrophic sulfate reduction (Table 3). Even though methane production was temporarily obstructed in the vials amended with BESA, methane production started to be detected within three days (Table 2).

Effect of different salts on the metabolic characteristics of the sludge from UASB D

Obviously, the highest production rates for methanol and hydrogen supplied vials were obtained for vials without the amendment of salts (Table 4). In the absence of additional salt, the methanol depletion rate and the sulfide production rate (methanol-fed vial) were similar to that obtained for the sludges harvested from UASB A at 70°C (Table 2 vs Table 4). However, the sulfide production rate at low salinity (hydrogen-fed vial) was two times lower than that obtained for the sludges from UASB A at 70°C (Table 2 vs Table 4).

The results of Table 4 shows that high salt concentrations are very toxic for the sulfate reducing sludge cultivated in UASB D at 70°C. Very low methanol depletion rates were observed for the vials amended with NaCl and the cocktail of salts (Table 4). The results of Table 4 also shows that the SRB are the most sensitive microorganism to high salt concentrations, as almost no sulfide production was observed in salt amended vials. On the other hand, acetate accounted for more than 95 % of the electron flow in these vials (Table 4), indicating that the acetogenic bacteria are the most tolerant microorganism to high salinity. In addition, the hydrogen utilizing microorganisms are strongly inhibited by high salinity, as no hydrogen oxidation occurred in salt-amended vials during the 107 days of incubation, except for the vials amended with a cocktail of salts, where a very low hydrogen consumption was observed after 52 days of lag-phase (Table 4).

Bicarbonate appears to be the salt which caused the most inhibitory effect on the granular sludge harvested from UASB D, as only a small fraction of methanol was converted in this period (Table 4). It must be remembered, however, that the initial pH in the

bicarbonate amended vials was 8.2, so that this extra inhibitory effect must also be taken into account.

Table 4. Maximal specific methanol depletion rate, maximal specific acetate, methane and sulfide production rates (g COD.gVSS^{-1}.day^{-1}) and electron flow (%) for the sludge sampled from UASB D on day 170 (when the reactor was operating at an influent NaCl concentration of 10 g.L^{-1}). The experiments were conducted at a pH of 7.5.

T = 70°C	**Maximal specific activity (g COD. gVSS^{-1}.day^{-1})**				**Mass Balance (%)**		
Condition	**Subst. deplet. rate**	**Acetate prod. rate**	**Methane prod. rate**	**Sulfide prod. rate**	**HS^-**	**CH_4**	**Acetate**
MeOH + SO_4^{2-}	0.90 (0.19)	0.04 (0.01)	0	0.62 (0.13)	80 (1)	0	19 (1)
MeOH + SO_4^{2-} + NaCl [(a)]	0.07 (0.05)	0.05 (0.02)	0	0	4 (1)	0	96 (1)
MeOH + SO_4^{2-} + $NaHCO_3$ [(b,c)]	0.01	0.01	0	0	12 (2)	0	23 (1)
MeOH + SO_4^{2-} + cocktail [(a)]	0.08 (0.01)	0.05 (0.01)	0	0	3 (2)	0	97 (2)
H_2/CO_2 + SO_4^{2-}	nd	0.07 (0.02)	0	0.36 (0.06)	74 (1)	0	26 (1)
H_2/CO_2 + SO_4^{2-} + NaCl	nd	0	0	0	0	0	0
H_2/CO_2 + SO_4^{2-} + $NaHCO_3$[c]	nd	0	0	0	0	0	0
H_2/CO_2 + SO_4^{2-} + cocktail [(d)]	nd	0.01	0	0.01	42 (6)	0	58 (6)

(a) Lag phase of approx. 10 days.
(b) Almost all methanol remained in the bottles after 107 days of incubation (which was considered in the calculations of the electron flow).
(c) The initial pH in these bottles was 8.2.
(d) Lag phase of approximately 52 days and hydrogen not fully consumed after 107 days of incubation.

DISCUSSION

Operation of (extreme) thermophilic methanol-fed sulfidogenic reactors

This work clearly shows that methanol is a suitable electron donor for sulfate reduction in UASB reactors inoculated with mesophilic granular sludges at a maximal temperature of 70ºC. At this maximal temperature, the substrate methanol is exclusively used

for sulfate reduction, thus minimizing the loss of substrate by other anaerobic conversions (Figs. 4A, 4B and 5D). From the results of the sulfide production rates (Table 2) and total amount of biomass present in UASB A and UASB B upon the completion of the experiment (about 9 g VSS.L^{-1}), it can be calculated that sulfate reduction rates as high as 20.5 g SO_4^{2-}.L^{-1}.day^{-1} can be achieved at 65°C in methanol-fed UASB reactors. However, the maximal sulfate reduction rate of 14.4 g SO_4^{2-}.L^{-1}.day^{-1} obtained in UASB D operated at 70°C (Fig. 6) and the complete absence of methane and acetate production in UASB A, UASB B and UASB D at that temperature (Fig. 3A, 3B and 5B) suggests that 70°C is the most attractive operational temperature for methanol-fed sulfate reducing reactors. The maximal sulfate reduction obtained at 70°C (14.4 g SO_4^{2-}.L^{-1}.day^{-1}) is comparable to that reported by Weijma *et al.* (2000b), who found a sulfate reduction rate of 14 gSO_4^{2-}.L^{-1}.day^{-1} in a methanol-fed expanded granular sludge bed (EGSB) reactor operated at 65°C. In agreement with this work, Weijma *et al.* (2000b) also reported that acetate accounted for 10 to 13 % of the electron flow at 65°C.

This work showed that a 6-hour exposure of the inoculum sludge to a temperature of 70°C is an effective strategy to suppress methanogenesis for the start-up of methanol-fed UASB reactors operated at 55°C (Fig. 5B). If existent, the production of methane can be easily suppressed in thermophilic methanol fed reactors, by either running the reactor at temperatures equal or higher than 65°C (Figs. 2A and 2B) or by subjecting 55°C operated reactors to a short (2 days) temperature (65 – 70°C) shocks (Figs. 2A and 5B). It seems, however, that the production of acetate, except in methanol fed reactors operated at 70°C, is unavoidable in thermophilic reactors (Figs. 2A, 2B and 5B).

Operation of (extreme) thermophilic formate-fed sulfidogenic reactors

Provided that methanogenesis is suppressed, the use of formate as electron donor for sulfate reducing reactors can potentially confer an advantage compared to other substrates, as no acetate is formed at all temperatures (65-75°C) tested in this work. Apparently, the SRB were irreversibly affected on day 35 by the exposure to the high sulfide concentration of 1400 mg.L^{-1} in UASB C (Fig. 2C). In an extensive review about sulfur problems in anaerobic digestion, O'Flaherty and Colleran (2000) underscored that there is a considerable variation among different groups of SRB with respect to sulfide inhibition. O'Flaherty *et al.* (1998) reported a 50 % inhibition of the activity of the hydrogen oxidizer *Desulfovibrio vulgaris* at a sulfide concentration of 1340 mg.L^{-1} (215 mg.L^{-1} undissociated H_2S at pH 7.6). A total sulfide concentration of 977 mg.L^{-1} (156 mg.L^{-1} undissociated H_2S at pH 7.6) caused a 50 % inhibition in a granular sludge cultivated in a mesophilic lab-scale adapted reactor (O'Flaherty *et al.*, 1998). To the best of our knowledge so far there are no reports on the sensitivity of (extreme) thermophilic formate and hydrogenotrophic SRB to sulfide toxicity. Therefore,

research must be orientated to assess the effect of high (toxic) sulfide concentrations in granular sludges at (extreme) thermophilic conditions. This work showed that sulfide removal technologies, e.g. by stripping (Yamaguchi *et al*., 1999) or extractive membrane reactors (de Smul and Verstraete, 1999), have to be applied for high rate formate-fed sulfate reducing reactors.

Temperature thresholds of high rate anaerobic sulfate-reducing reactors

Both continuous (Fig. 1) and batch experiments (Tables 2 and 3) clearly demonstrate that methanol and formate degradation occurs at temperatures up to, respectively, 70 and 75°C. The reason for the higher temperature threshold of formate and hydrogen degradation compared to methanol degradation (Table 2) might be related to a higher temperature tolerance of thermophilic formate and hydrogen utilizing bacteria that are likely present in mesophilic sludges. The observed lower temperature threshold for methanol is in agreement with the results of Balk *et al.* (2002), who showed that the temperature range for growth of the syntrophic methanol-oxidizing, hydrogen-producing bacterium *Thermotoga lettingae*, isolated from a thermophilic (65°C) methanol-fed sulfate reducing reactor, lays between 50 to 70°C, with an optimum growth at 65°C.

This work shows that MPA and SRB able to utilize hydrogen and formate at 75°C were present in the sludge, whereas SRB and MPA able to utilize methanol and acetate at 75°C were most probably absent in the sludge. Extreme thermophilic SRB and MPA capable to oxidize formate have been isolated from a variety of inoculum sources, as for instance, the methanogenic archaea *Methanobacterium thermoformicicum* (Zhilina and Ilarinov, 1984) and the SRB counterpart *Thermodesulfobacterium mobile* (Rozanova and Pivovarova, 1988). Several hydrogen utilizing sulfate reducers as well as methanogens have been reported to grow at temperatures above 70°C (Madigan *et al.*, 1997; Huber and Stetter, 1998). The lack of sulfate reduction in the methanol-fed reactors at temperatures exceeding 70°C might be due to an insufficient time for the proliferation of a population of extreme thermophilic sulfate reducers present in the inoculum sludge. Indeed, Lepisto and Rintala (1996) demonstrated that a period of 36 days was needed to detect methane production after start up of an acetate-fed UASB reactor at 76°C. However, one can expect that extreme thermophilic microorganisms might not survive in non-specific environments, such as in mesophilic anaerobic bioreactors, from which the inoculum sludge originated. Indeed, Huber and Stetter (1998) reported that, in contrast to thermophiles, extreme thermophiles are unable to grow below 60ºC. Nonetheless, the same authors emphasize that extreme thermophiles may survive for long times at ambient temperature, which obviously is essential for their dissemination in cold atmospheres and hydrospheres (Huber and Stetter, 1998).

Temperature effect on the substrate degradation pathway

The sharp drop in methane production from methanol at 65°C found in this work (Figs. 2A, 2B and 5B) was also observed by Ahring (1994) and Ahring *et al.* (2001) in batch tests with glucose, acetate, propionate or butyrate (no sulfate added) with a sludge grown in an anaerobic digester treating manure. Only methane formation from hydrogen was enhanced at 65°C with this sludge (Ahring, 1994; Ahring *et al.*, 2001). These results are in contrast with those of Lepisto and Rintala (1996), who found that acetate and butyrate are still converted to methane at temperatures up to 80°C in UASB reactors. This discrepancy can be explained by the predominance of *Methanosarcina* related microorganisms in the anaerobic digesters of Ahring *et al.* (2001), a methanogen with an upper temperature of 60°C (Zinder and Mah., 1979). *Methanosarcina* like species, known to metabolize methanol (Zinder, 1990), could also be observed in optical microscopy of crushed granular sludge samples harvested from UASB A operated at 55°C (data not shown). Thus, the sudden steep drop in the methanogenic activity of the methanol-fed reactors found in the current work at operational temperatures exceeding 55°C can be related to a predominance of *Methanosarcina* related methanogens (Figs. 2A, 2B and 5B).

The very slow acetate degradation in the current work at all temperatures tested (Table 2) is in contrast with the acetate oxidation observed in extreme thermophilic methanogenic reactors found either via sulfate reduction at 70°C (Rintala, 1997) or via methanogenesis up to 80°C (Lepisto and Rintala, 1996). The absence of acetate degradation by SRB in high rate sulfidogenic reactors has been reported previously in thermophilic (Weijma *et al.*, 2000a, Chapter 3) and mesophilic (Omil *et al.*, 1997; Lens *et al.*, 1998) systems. The acetate utilizing population in the inoculum sludge is very small and their growth rate is rather slow, so that acetate becomes an end product rather than an intermediate in sulfidogenic reactors. According to Omil *et al.* (1998), based on theoretical calculations with the growth kinetics of acetotrophic SRB, a long time (1000 days) is needed for the development of a substantial population of acetate oxidizing SRB. Alternatively, it might be that the acetate oxidizing SRB are negatively affected by the high sulfide concentrations prevailing in the reactors (O'Flaherty and Colleran, 2000). It has been shown that mesophilic acetotrophic SRB are much more sensitive to sulfide toxicity than the hydrogenotrophic SRB (O'Flaherty *et al.*, 1998).

Although the results of batch vials amended with vancomycin show that SRB are able to oxidize methanol directly at 55 to 75°C (Table 2), the high methanogenic (at 55 and 65°C) and sulfidogenic (at 55, 65, 70 and 75°C) activity on H_2/CO_2, suggests that both SRB and MPA used mainly hydrogen as electron donor. The observed key role of hydrogen in the methanol degradation (Table 2) confirms previous findings at 55°C either in the absence (Paulo et *al*., 2001) or presence of sulfate at 55°C (Chapter 3) and at 65°C (Weijma *et al.*, 2000a). Working with an enrichment culture obtained from anaerobic granular sludge of a

sulfate reducing reactor at 65°C, Davidova and Stams (1996) demonstrated that methanol degradation occurs at 65ºC in a syntrophic association of acetogenic bacteria (converting methanol to H_2/CO_2) and hydrogenotrophic SRB. This corroborates with the process fundamentals, as hydrogenotrophic SRB gain more energy from the consumption of molecular hydrogen and have a higher substrate affinity than the methanogenic counterparts, decreasing the hydrogen concentration below the threshold value of hydrogenotrophic MPA (Oude Elferink *et al*., 1994). Indeed, the measured hydrogen threshold of methanogenic cultures was about ten times higher than that of the sulfate reducing cultures (Davidova and Stams, 1996).

In contrast to methanol as the substrate, the strong competition between MPA and SRB for formate was apparently not influenced by the different operational temperatures, viz. 65, 70 and 75°C (Fig. 4C). The very high formate utilization rates measured (Table 2) suggest that direct formate consumption is more important than the syntrophic formate consumption via hydrogen. In contrast, the H_2/CO_2 utilization rates of SRB were higher than those obtained with formate as the substrate (Table 2), suggesting that the SRB are able to compete with the MPA for the latter substrate in the sludge cultivated in the formate-fed bioreactor. The reason for the observed strong competition for hydrogen between MPA and SRB in this work might be related to the low number of the SRB population present in the inoculum sludge (from a methanogenic reactor) or the H_2S toxic shock on day 35 (Fig. 2C).

Salt effect on the performance of thermophilic (70°C) methanol-fed sulfidogenic reactors

This study showed that the acetogenic bacteria (AB) displayed a higher sensitivity to NaCl than the SRB in a methanol-fed sulfate reducing reactor operated at 70°C, as sulfide production in the UASB D steadily decreased after the addition of 10 g $NaCl.L^{-1}$, whereas acetate started to be produced at this influent NaCl concentration (Fig. 5B). The reason why the SRB were the most affected trophic group in the biomass can be explained by their dependence on the substrate hydrogen (see previous sections), as the batch activity tests showed that no hydrogenotrophic was found for the salt-amended vials during 107 days of incubation (Table 4).

Batch activity tests also showed that the addition antagonistic salts (magnesium and potassium) did not counteract the inhibitory effects of NaCl, as postulated by Feijoo *et al.* (1995). In addition, batch tests activity tests suggested that bicarbonate is the most inhibitory salt for the biomass cultivated in the methanol-fed reactor, as almost no methanol was consumed after 107 days of incubations (Table 4) . However, the high pH (8.2) prevailing in the bicarbonate-amended vials are likely to have contributed to the process inhibition.

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Chapter 5

Effect of NaCl on thermophilic (55ºC) methanol degradation in sulfate reducing granular sludge reactors

The effect of NaCl on thermophilic (55ºC) methanol conversion in the presence of excess of sulfate (COD/SO_4^{2-} = 0.5) was investigated in two 6.5 L lab-scale upflow anaerobic sludge bed (UASB) reactors inoculated with granular sludge previously not adapted to NaCl. Methanol was almost completely used for sulfate reduction in the absence of NaCl when operating at an organic loading rate of 5 $gCOD.L^{-1}.day^{-1}$ and a hydraulic retention time of 10 hours. The almost fully sulfidogenic sludge consisted of both granules and flocs developed after approximately 100 days in both reactors. Sulfate reducing bacteria (SRB) outcompeted methane producing archaea (MPA) for methanol, but acetate represented a side-product, accounting for maximal 25 % of the total COD converted. Either MPA or SRB did not use acetate as substrate in activity tests. High NaCl concentrations (25 $g.L^{-1}$) completely inhibited methanol degradation, whereas low salt concentrations (2.5 $gNaCl.L^{-1}$) provoked considerable changes in the metabolic fate of methanol. The MPA were most sensitive towards the NaCl shock (25 $g.L^{-1}$). In contrast, the addition of 2.5 $g.L^{-1}$ of NaCl stimulated MPA and homoacetogenic bacteria.

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INTRODUCTION

The characteristics of industrial wastewaters such as temperature and salinity are determined by the production process, and can be far from the physiological optima of microorganisms. With the current trend to close water cycles in industry, there is a need for hot and salt tolerant wastewater treatment processes. These parameters impose the need for adapted treatment processes, as high temperatures denature enzymes of mesophilic bacteria (Madigan *et al.,* 1997), whereas high osmolarity environments triggers rapid fluxes of cell water, thus causing a reduction in turgor and dehydration of the cytoplasm (Kempf and Bremer, 1998).

The effect of sodium on methanogenic digestion has been studied extensively. Research was mainly oriented towards the feasibility of anaerobic digestion treating seafood wastewater (Feijoo *et al.,* 1995; Méndez *et al.,* 1995; Soto *et al.,* 1993) or concentrated effluents from reverse osmosis (de Baere *et al.,* 1984). Mesophilic and thermophilic (up to 55ºC) high-rate methanogenic treatment of seafood wastewater proceeds successfully at NaCl concentrations ranging from 15 and 25 $g.L^{-1}$.

Methanol is a constituent of many sulfate-rich wastewaters, such as in the widely used "Kraft" process for wood pulping in the paper industry. Also, methanol is often relied as an inexpensive and efficient electron donor for inorganic produced wastewaters, such as in the thermophilic (65ºC) biodesulfurization of flue-gases (Weijma *et al.,* 2000). The presence of sulfate does not exert considerable effects on the methanol conversion under mesophilic conditions (Weijma, 2000) with just a small fraction of the methanol being converted via sulfate reduction (Glombitza, 2001). In contrast, the presence of sulfate greatly affects methanol conversion under thermophilic (65ºC) conditions. In a methanol-fed expanded granular sludge bed (EGSB) reactor operated at 65ºC and pH 7.5, sulfate reduction outcompeted methanogenesis and sulfide accounted for more than 80 % of the consumed methanol-COD (Weijma *et al.,* 2000). This temperature (65ºC) was chosen to prevent growth of methanol consuming *Methanosarcina* species, which do not grow at or beyond a temperature of 65ºC (Zinder *et al.,* 1984).

So far, no studies have been carried out that determine the range of the salt concentration on which methanol can support sulfate reduction. Whether sulfate reduction or methanogenesis prevails in bioreactors operating at 55ºC with methanol as the sole electron and carbon source and how this is affected by increasing salinities are still unclear. The aim of this work was to verify the feasibility of thermophilic (55°C) upflow anaerobic sludge bed (UASB) reactors fed with methanol as the sole electron donor and a high sulfate concentration (COD/SO_4^{2-} of 0.5) and to assess the effect of high influent NaCl (25 $g.L^{-1}$) on a granular sludge inoculum previously not adapted to NaCl. The effect of low influent NaCl (2.5 $g.L^{-1}$) on the performance the same UASB reactor was assessed as well.

MATERIAL AND METHODS

Experimental set-up

To investigate the aims of this work, two bench-scale (6.5 L) UASB reactors (Fig. 1) were operated under identical operational conditions during 230 days, except for the influent salt concentration. One reactor (UASB I) was operated as control reactor, whereas the influent of the second reactor (UASB II) was supplemented with different sodium chloride concentrations. The UASB reactors were operated at a temperature of 55°C, upflow velocities between 1 (first 112 days) and 2 $m.h^{-1}$ (remaining of the experiment) and a pH of 7.0 ± 0.2 (first 64 days) to 7.5 ± 0.2 (remaining of the experiment).

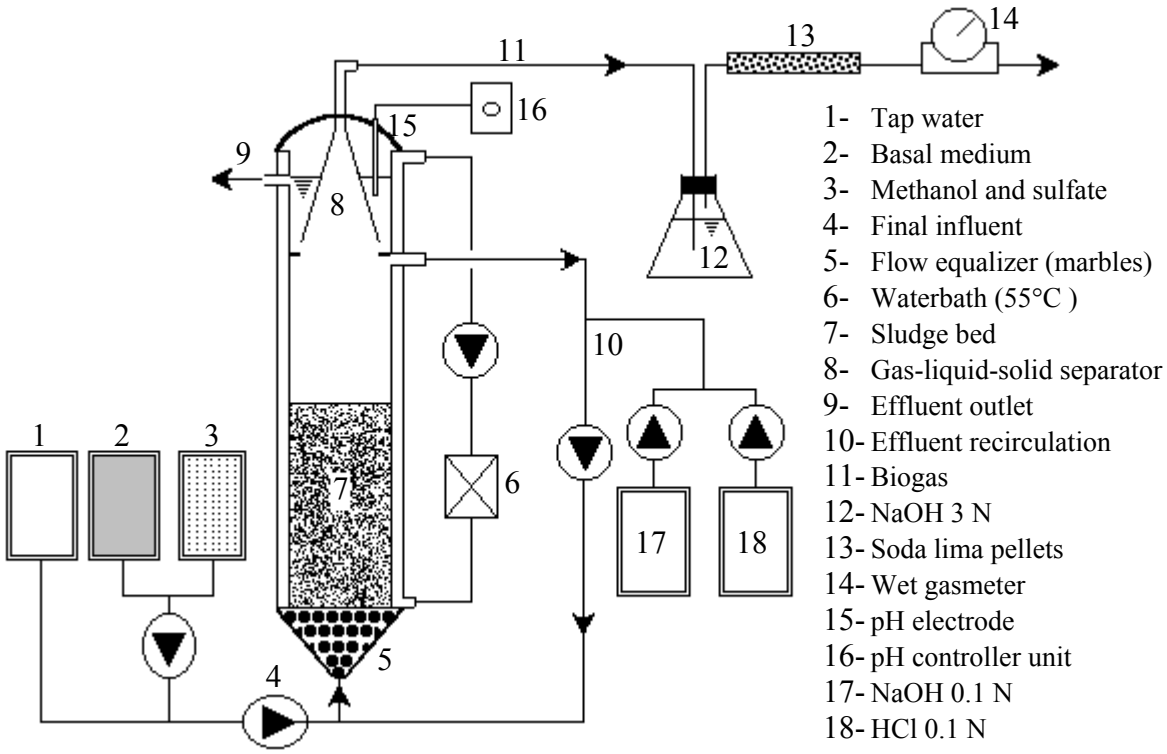

Figure 1. Schematic representation of the UASB reactor.

In order to keep the reactor temperature at 55ºC, the reactors were equipped with a double wall, through which water, heated in a thermostatic waterbath (Julabo, Seelbach, Germany), was recirculated. Effluent recycling was applied to obtain a superficial liquid upflow velocity (V_{up}) to 1 $m.h^{-1}$. Both reactors were fed using peristaltic pumps (Watson Marlow 501 U and 505 S, Falmouth, Cornwall, UK). Concentrated stock solutions and basal medium were added to the main influent flow using vertical axis peristaltic pumps (Gilson Minipuls 3 and 2, respectively, Villiers, France). The pH in the reactors was controlled by automatic pH control, by adding concentrated NaOH or HCl solutions in the recirculation system. The pH was measured with a sulfide-resistant Flushtrode pH electrode (Hamilton Flushtrode, Hilkomij bv, Rijswijk, the Netherlands) connected to the automatic pH controller with two changeable set-points to adjust the pH.

Inoculum

Both reactors were inoculated with sludge growing in two thermophilic (55ºC) lab-scale (6.5 L) UASB reactors treating a synthetic paper-mill wastewater at pH 6 (Lens *et al.*, 2002). The influent contained starch, sucrose, lactate, propionate and acetate, and had a COD/SO_4^{2-} of 10. The inoculum sludge consisted mainly of black well-shaped granules and also of dispersed flocs. Each reactor started-up with about 2 L of inoculum sludge, corresponding to approximately 40 g of volatile solids (VS) per reactor.

Medium

Both reactors were fed with a synthetic influent, containing methanol as the sole electron donor. Sulfate was added as sodium sulfate to provide a COD/SO_4^{2-} of 0.5 (g COD per g SO_4^{2-}), so theoretically all methanol could be degraded via sulfate reduction. The influent of both reactors was further supplied with a basal medium as described in Chapter 3. Basal medium was added to the main flow at a ratio of 2.22 mL of basal medium per gram COD in the influent.

Experimental design

Table 1 summarizes the operational parameters applied to UASB I and UASB II in the different experimental periods. Both reactors were started at a hydraulic retention time (HRT) of 10 h (Period I), which was decreased to 6 h (Period II-A) at day 43 and increased back to 10 h at day 64 till the end of the experiment (Period II-B and Period III).

The evolution of the organic loading rate (OLR) and the sulfate loading rate (SLR) are given in, respectively, Figs. 2A and 2B. The UASB reactors operated at an OLR of 5 $gCOD.L^{-1}.day^{-1}$ (Periods I, II-B and III) except from days 43 to 64 when the OLR was temporarily increased to 10 $gCOD.L^{-1}.day^{-1}$ (Period II-A). Nonetheless, considerable fluctuations of the OLR were observed, mainly between days 80–104 and days 156–182 for UASB I and between days 80–98 and days 164–178 for UASB II. Several feed interruptions were imposed to both UASB reactors in order to assess the stability of the process for short (16 hours) starvation periods. The feed supply of UASB I was interrupted at both days 185 and 212, whereas the feed supply of UASB II was interrupted at day 176.

UASB II was fed with a NaCl concentration of 25 $g.L^{-1}$ (corresponding to 10 g $Na^+.L^{-1}$) from the start-up till day 13 (beginning of Period I). No NaCl was added anymore till day 163 (Period II-B). From day 164 onwards, 2.5 g $NaCl.L^{-1}$ was again added to the influent flow of UASB II (Period III). In order to verify the reversibility of the observed effects, NaCl was omitted again at day 197 till the end of the experiment (end of Period III).

Table 1. Summary of the average operational parameters applied to the UASB I (A) and UASB II (B) reactors. (HRT = hydraulic retention time; OLR = organic loading rate; COD = chemical organic demand; SLR = sulfate loading rate).

A – Control reactor	**Period I**	**Period II-A**	**Period II-B**	**Period III**
Parameter Days	0 – 42	43 – 63	64 – 162	163 – 230
Influent flow (litre.day^{-1})	16.1	25.0	16.1	15.4
HRT (hour)	9.6	6.2	9.7	10.1
OLR (gCOD.litre^{-1}.day^{-1})	4.4	10.1	4.8	5.6
COD (g.litre^{-1})	1.8	2.6	1.9	2.3
SLR (gSO_4^{2-}.litre^{-1}.day^{-1})	8.9	16.9	9.3	9.9
SO_4^{2-} (g.litre^{-1})	3.5	4.3	3.8	4.3
COD/SO_4^{2-}	0.52	0.62	0.53	0.54
pH	7.0	6.9	7.5	7.6
NaCl (g.litre^{-1})*	0	0	0	0
Na^+ (g.litre^{-1})**	2.1	2.5	2.2	2.5

B – Salt-fed reactor	**Period I**	**Period II-A**	**Period II-B**	**Period III**
Parameter Days	0 – 42	42 – 63	64 – 162	163 – 230
Influent flow (litre.d^{-1})	16.2	24.9	15.8	15.4
HRT (hour)	9.6	6.2	9.7	10.7
OLR (gCOD.litre^{-1}.day^{-1})	4.3	10.1	4.8	5.5
COD (g.litre^{-1})	1.4	2.6	1.9	2.3
SLR (gSO_4^{2-}.litre^{-1}.day^{-1})	8.6	17.0	9.2	10.0
SO_4^{2-} (g.litre^{-1})	3.4	4.4	3.7	4.2
COD/SO_4^{2-}	0.53	0.61	0.54	0.52
pH	7.1	7.0	7.5	7.5
NaCl (g.litre^{-1})*	25	0	0	2.50
Na^+ (g.litre^{-1})**	11.7	2.6	2.2	3.1

* calculated values

** calculated values considering the addition of sodium both from NaCl and Na_2SO_4

Maximum specific activity test

Activity tests were performed to determine the maximum specific activity of the sludge developed in each UASB reactor after 147 days of operation. Activity tests were carried out in 117 mL-vials with 50 mL of mineral medium containing (in g.L^{-1}): NH_4Cl (0.28), K_2HPO_4 (0.33), $MgSO_4.7H_2O$ (0.1), $CaCl_2.2H_2O$ (0.01), $NaHCO_3$ (6.70), yeast extract (0.1) and a trace element solution (1 mL/L of mineral medium) as described in Chapter 3. Methanol was used as sole electron donor and assays were performed at pH 7.0 both in the

presence and absence of sulfate. During the assays, the vials were placed in the waterbath-shaker at 55ºC and 50 rpm. The methanol depletion rate and the methanogenic, sulfidogenic and acetogenic activities were calculated from the linear increase or decrease of the different compounds in the vials. Specific rates were obtained by dividing the maximal rates by the exact amount of volatile suspended solids (VSS), measured upon the completion of the assay. All these experiments were performed in duplo.

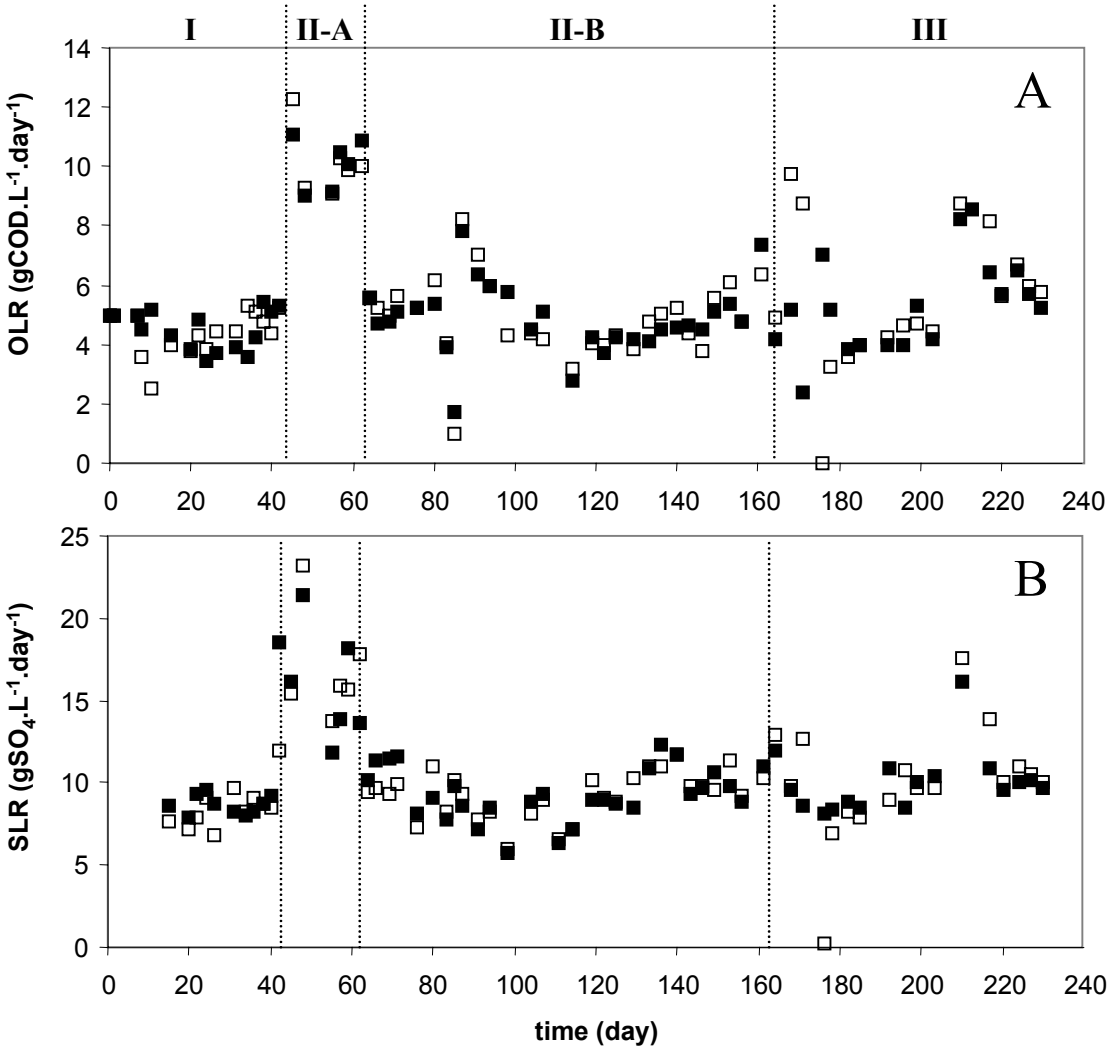

Figure 1. Evolution of (A) the organic loading rate and (B) the sulfate loading rate applied to the UASB I (■) and UASB II (□).

Batch toxicity assay for NaCl

Prior to the addition of 2.5 g $NaCl.L^{-1}$ in the influent of UASB II at day 164 (period III), batch toxicity assays were performed with sludge sampled at day 162 to assess the effect of NaCl on the methanol degradation and to determine the 50 % inhibition concentration (IC_{50}) of NaCl on the methanol depletion rate. Experimental procedures were similar to those applied for the maximum specific activity tests, except that only methanol was measured as a function of time. The same mineral medium was used, supplemented with a gradient series of

NaCl (0, 2.5, 5, 7.5, 10, 15, 20 and 25 g $NaCl.L^{-1}$). If also sodium added as sodium sulfate is considered, these values correspond to 1.9, 2.5, 3.1, 3.7, 4.3, 5.5, 6.7 and 7.9 g $Na^{+}.L^{-1}$. Control vials were assayed without NaCl addition. The percentage of toxicity was determined by comparing the activity of the vials on which NaCl was added with the control vials. The final acetate, sulfide and methane concentrations (in g $COD.L^{-1}$) were determined for each vial, upon completion of the test. All these experiments were performed in duplo.

Analysis

VSS were analyzed according to standard methods (APHA, 1985). Sulfide was determined photometrically as described by Trüper and Schlegel (1964). Methanol, VFA and methane were measured by gas chromatography (GC), whereas sulfate was measured by high-pressure liquid chromatography (HPLC), as described by Weijma *et al.* (2000). The volume of biogas produced in the UASB reactors was measured as described in Chapter 3.

RESULTS

Effect of a salt shock on reactor start-up (Period I – days 0-42)

In contrast to the full methanol removal (with methane production rates up to 3.66 g $COD.L^{-1}.day^{-1}$ at day 10) in the non-salt exposed reactor (UASB I – Figs. 3A-C), high influent sodium chloride concentrations (25 g $NaCl.L^{-1}$) completely inhibited the methanol degradation in the salt exposed reactor (UASB II - Figs. 4A-C). Omission of NaCl from the influent from day 13 onwards resulted in partial recovery of the methanol consumption within seven days (Fig. 4A). At day 32 the sulfide and acetate production rates increased up to 0.91 and 1.09 $gCOD.L^{-1}.day^{-1}$, respectively (Fig. 4C). The MPA were strongly inhibited by the 13 day salt shock in UASB II (Figs. 4B-C). In the non-salt exposed UASB I methane production corresponded to about 87 % of the consumed methanol-COD, whereas sulfide and acetate accounted for about 11 % and 2 % of the electron flow in the non-salt exposed UASB I reactor during Period I (Fig. 3C).

An alkaline pH shock (9.5) during 8 hours at day 32 inhibited sulfide and acetate production in UASB II, but the system recovered within a few days (Fig. 4C). Surprisingly, some methane production was detected immediately after the pH shock, with a maximal methane production rate of 0.67 g $COD.L^{-1}.day^{-1}$ at day 36 (Fig. 4C), which is still a factor 10 lower compared to the methane production rates in UASB I (Fig. 3C).

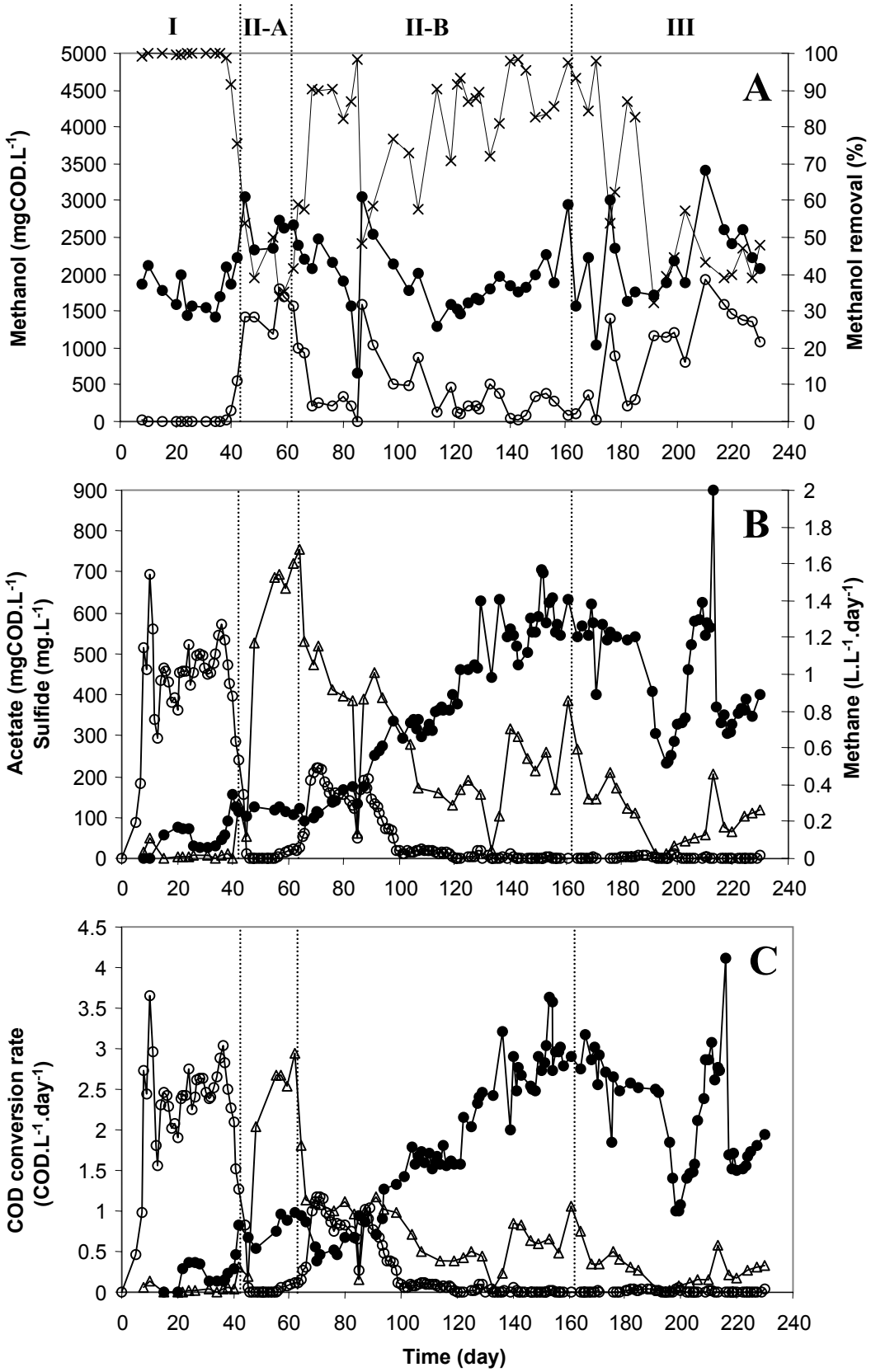

Figure 3. Process performance of UASB I. (A) Evolution of the methanol concentrations in the influent (•), effluent (o) and methanol removal efficiencies (x). (B) Evolution of the concentrations of sulfide (•), methane (o) and acetate (△). (C) Evolution of the COD conversion rate to sulfide (•), methane (o) and acetate (△).

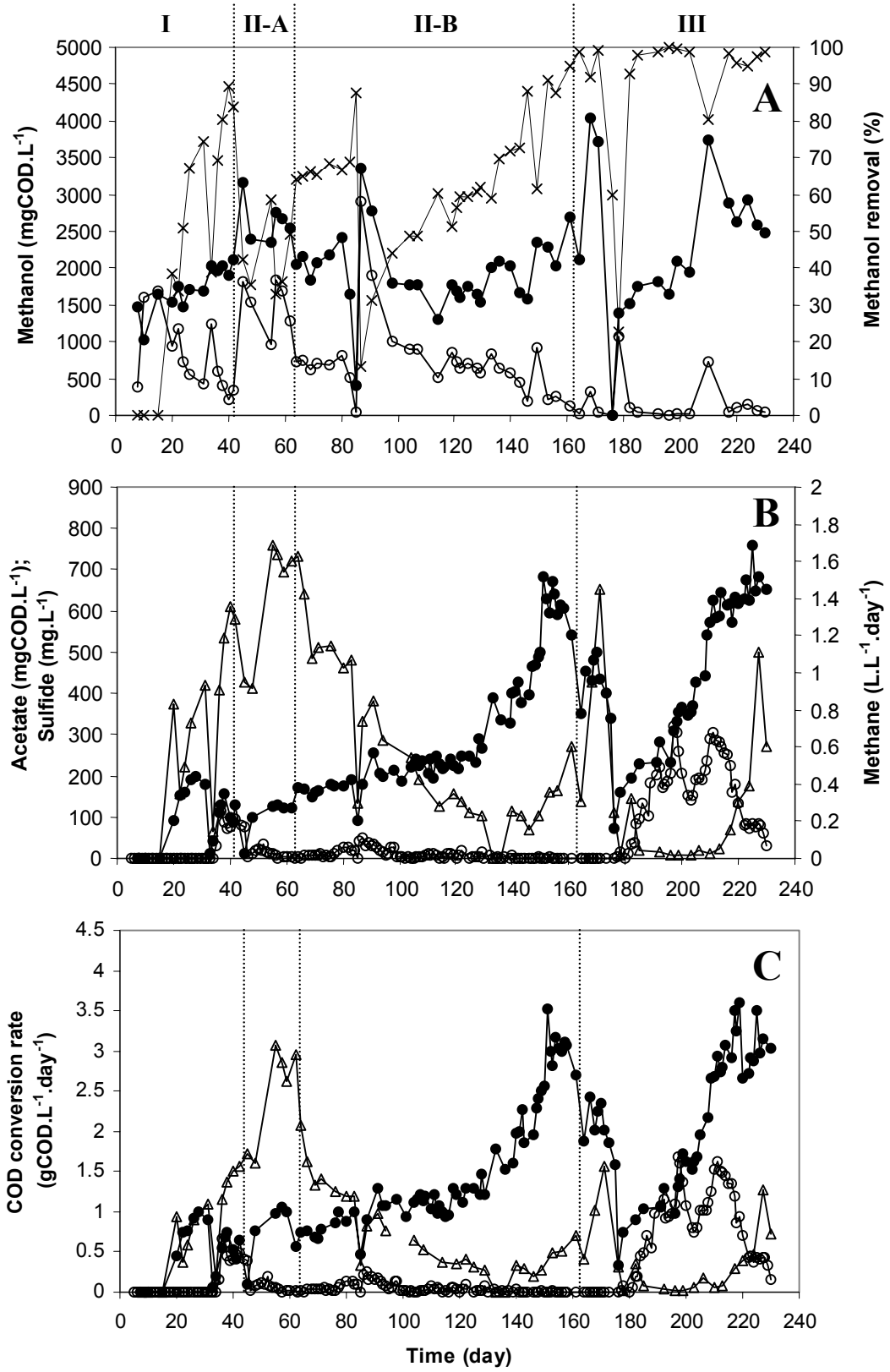

Figure 4. Process performance of UASB II. (A) Evolution of the methanol concentrations in the influent (•), effluent (o) and methanol removal efficiencies (x). (B) Evolution of the concentrations of sulfide (•), methane (o) and acetate (△). (C) Evolution of the COD conversion rate to sulfide (•), methane (o) and acetate (△).

Effect of variations in the operational parameters (Period II – days 43-62)

Effect of increasing the OLR (Period II-A)

Incomplete methanol degradation was observed upon increasing the OLR from 5 to 10 g $COD.L^{-1}.day^{-1}$ in UASB I, with effluent concentrations ranging from 160 to 1700 mg $COD.L^{-1}$ (Fig. 3A). The acetate production rate increased considerably when operating at an OLR of 10 g $COD.L^{-1}.day^{-1}$, reaching values up to 2.94 g $COD.L^{-1}.day^{-1}$ at day 62 (Fig. 3C). Note that the decrease in methane production started even before the OLR was increased to 10 g $COD.L^{-1}.day^{-1}$ at day 43 (Period II-A) and that this was not coupled to the increase of the sulfide or the acetate production (Fig. 3B), but to higher effluent methanol concentrations (Fig. 3A).

Increasing the OLR to 10 g $COD.L^{-1}.day^{-1}$ at day 43 in UASB II caused an immediate inhibition in the methane and sulfide production rates coupled to a steady increase of the acetate production rate to values up to 3.07 g $COD.L^{-1}.day^{-1}$ at day 55 (Fig. 3C). Sulfide production rates resumed within a few days, with an average sulfide production rate of 0.87 g $COD.L^{-1}.day^{-1}$ (Fig. 4C). At an OLR of 10 g $COD.L^{-1}.day^{-1}$, acetate production amounted to 63 % and 76 % of the electron flow for UASB I and UASB II, respectively. Sulfide was the second most important product, accounting for 35 % and 21 % of the electron flow in UASB I and UASB II, respectively. During Period II-A, methanogenesis was rather insignificant, as it consumed less than 3 % of the electron flow in both reactors.

Effect of decreasing the OLR and increasing the pH (Period II-B)

The methanol removal efficiency steadily increased upon resetting the OLR to 5 g $COD.L^{-1}.day^{-1}$ and increasing the pH to 7.5 both in UASB I and UASB II (Figs. 3A and 4A). The methanol removal efficiency in UASB I was well over 80 % while in UASB II it increased from 35 % at the end of Period II-A to almost complete methanol removal (96 %) at day 161 (Fig. 4A).

Methane production partially resumed upon resetting the OLR to 5 g $COD.L^{-1}.day^{-1}$ in UASB I, corresponding to approximately 35 % of the average methane production rate during Period I (Fig. 3C). However, between days 90 to 100 the methane production of UASB I decreased to almost zero and hardly any biogas production was detected till the end of the experiment (Fig. 3B). The sulfide production increased at the expense of the acetate production in both UASB I and UASB II at the beginning of Period II-B. Sulfide concentrations as high as 706 $mg.L^{-1}$ and 670 $mg.L^{-1}$ were measured at the end of Period II-B in, respectively, UASB I and UASB II (Figs. 3B and 4B).

Despite that sulfide was the main mineralization product from methanol degradation at the end of Period II-B, accounting for 79 % and 85 % of the electron flow in, respectively,

UASB I and UASB II, acetate still represented a secondary by-product, accounting for 20 % and 14 % of the electron flow in UASB I and UASB II, respectively (Figs. 3C and 4C). Methanogenesis was rather insignificant in the last days of Period II-B, as it consumed less than 1 % of the electron flow in both reactors (Figs. 3C and 4C).

Effect of bicarbonate omission (within Period II-B)

Although acetate concentrations decreased after day 76 for both reactors (Figs. 3B and 4B), omission of bicarbonate from day 76 onwards had no effect on the acetate production rates in both reactors. Considerable acetate production was measured at the end of Period II-B in both reactors, with acetate production rates up to 1.06 g $COD.L^{-1}.day^{-1}$ and 0.70 g $COD.L^{-1}.day^{-1}$ measured in, respectively, UASB I and UASB II (Figs. 3C and 4C).

Stability of the reactor performance (Period III)

Effect of low influent NaCl concentration

The effluent sulfide concentrations decreased immediately upon the addition of 2.5 $g.L^{-1}$ of NaCl to the influent of UASB II at day 163 (Fig. 4B), from 620 $mg.L^{-1}$ (average of days 143-163) to 420 $mg.L^{-1}$ (average of days 164-175). The high acetate production rates measured at days 168 (1.0 g $COD.L^{-1}.day^{-1}$) and 171 (1.6 g $COD.L^{-1}.day^{-1}$) are probably related to the higher OLR (9.7 and 8.8 g $COD.L^{-1}.day^{-1}$, respectively) applied to UASB II (Fig. 4C), rather than to the salt addition.

To verify if the MPA were stimulated by the supplementary NaCl addition, NaCl was omitted again from the UASB II influent from day 197 onwards. Between days 196 and 210, a rapid increase of the sulfide production rate from 1.0 to 2.7 g $COD.L^{-1}.day^{-1}$ (Fig. 4C) was observed, reaching again the values obtained prior to salt addition (end of Period II-B). Till day 211, no indication of a decrease of the methane production was evident in UASB II, perhaps due to the oscillating OLR (Fig. 4B). However, from day 211 onwards, the acetate production rate increased at the expense of the methane production rate, reaching acetate production rates up to 1.3 g $COD.L^{-1}.day^{-1}$ (Fig. 4C). At the end of the experiment, sulfide accounted for 78 % of the electron flow in UASB II, whereas acetate and methane production amounted to 18 % and 4 % of the electron flow, respectively.

Effect of feed interruption (within Period III)

The methanol removal efficiency resumed within a few days after a 16-hour non-feed period at day 176 in UASB II, returning to values close to 100 % methanol elimination till the end of the experiment (Fig. 4A). Acetate production did not recover to concentrations found

prior to the feed interruption and remained low till day 196 (Fig. 4C) whereas sulfide concentrations increased from 70 $mg.L^{-1}$ at day 176 to about 240 $mg.L^{-1}$ between days 178-196 (Fig. 4B). Methane was detected one week after the non-feed period and 20 days after salt was added to the influent. The methane production rate of UASB II gradually increased to 0.71 g $COD.L^{-1}.day^{-1}$ at day 196 (Fig 4C).

Unlike UASB II, methanol removal efficiencies did not improve in UASB I after the two 16-hour non-feed periods at days 185 and 212 (Fig. 3A vs. Fig. 4A), whereas sulfide production fully recovered in 20 days, reaching values up to 626 $mg.L^{-1}$ at day 209 (Fig. 3B). The effluent acetate concentration remained low after the starvation period (Fig. 3B). Immediately after restarting the reactor at the end of the 16-hour batch mode (day 212), a sulfide concentration as high as 900 $mg.L^{-1}$ was measured (Fig. 3B). The reactor recovered from this feed interruption, as sulfide production increased from 305 $mg.L^{-1}$ at day 218 to 399 $mg.L^{-1}$ measured in the last day of the experiment (Fig. 3B). Acetate production rates remained around 0.20 g $COD.L^{-1}.day^{-1}$, but with a clear increasing trend.

Metabolic characteristics of the sludge

Specific activities with methanol

The methanol depletion rate in activity tests performed with sludge harvested from UASB II increased significantly (73%) between days 147 and 162, whereas a less significant (8 %) increase was observed during this period for UASB I (Table 2), which operated much more stable at the end of Period II-B compared with UASB II.

Table 2. Specific methanol depletion rate and methanogenic, sulfidogenic and acetogenic activity rates (g $COD.gVSS^{-1}.day^{-1}$) for the sludges sampled from each reactor at days 147 and 162. Standard deviation between brackets.

Reactor	Day	Condition	Methanol depletion	Methanogenic activity	Sulfidogenic activity	Acetogenic activity
UASB I	147	- SO_4^{2-}	1.08 (0.16)	0.02 (0.01)	-	0.98 (0.13)
	147	+ SO_4^{2-}	1.13 (0.22)	0.04 (0.00)	0.54 (0.11)	0.29 (0.05)
	162	+ SO_4^{2-}	1.23 (0.02)	-	-	-
UASB II	147	- SO_4^{2-}	1.13*	0.25*	-	0.82*
	147	+ SO_4^{2-}	0.83 (0.07)	0.01 (0.00)	0.38 (0.04)	0.11 (0.02)
	162	+ SO_4^{2-}	1.44 (0.15)	-	-	-

* single measurement
- not measured

Methanol was completely converted to acetate in the absence of sulfate by UASB I sludge (Fig. 5A), whereas methane accounted for 18 % of the electron flow for the sludge sampled from UASB II (Fig. 5C). In the presence of sulfate, no methanogenic activity was detected with sludges harvested from both reactors (Figs. 5B, 5D) and methanol was mainly converted to sulfide and in a minor extent to acetate. Sulfide amounted for 72 and 62 % of the converted-COD for sludges sampled from UASB I and UASB II, respectively. Both SRB and MPA did not consume acetate, even when sulfate was added to the vials after 6 days of incubation (Fig. 5A and 5C).

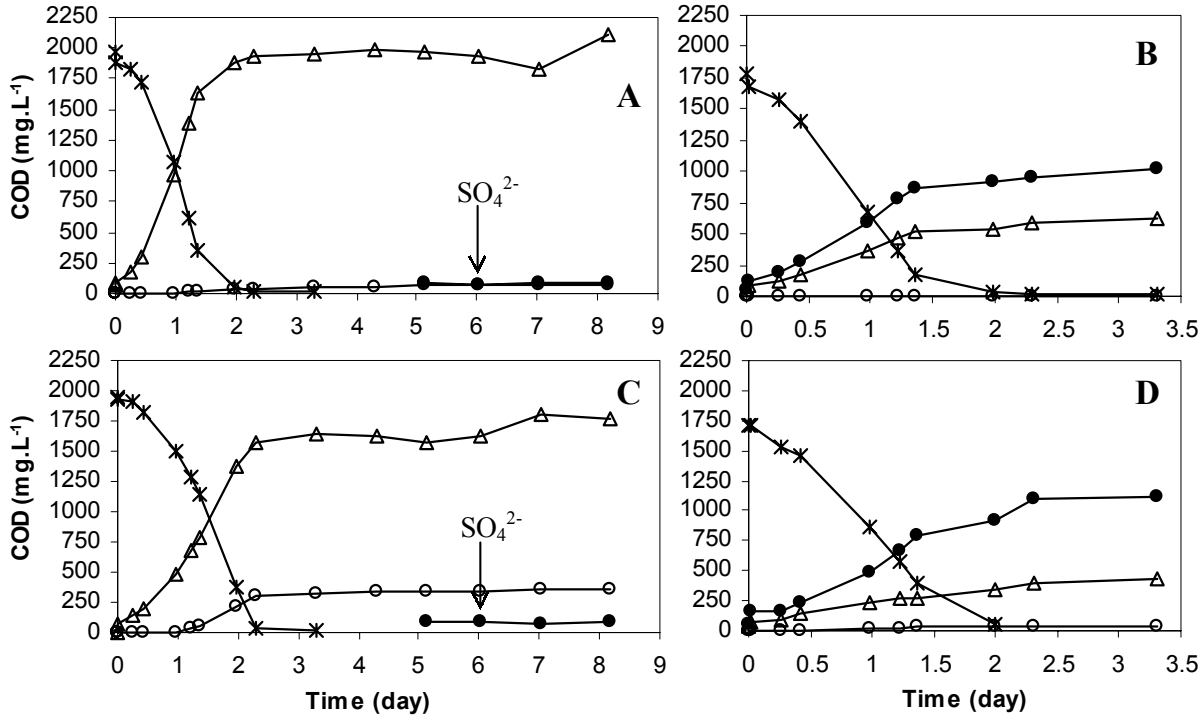

Figure 5. Evolution of methanol depletion (x) and sulfide (•), acetate (△) and methane (o) formation during the activity test performed at day 147 for the sludges sampled from UASB I (A,B) and UASB II (C,D) in the absence (A,C) and presence (B,D) of sulfate. Sulfate was added in the non-sulfate fed vials after 6 days of incubation.

NaCl toxicity tests for the salt treated reactor - UASB II

The IC_{50} concentration of NaCl for UASB II sludge was 9.30 $g.L^{-1}$. This corresponds to an IC_{50} concentration for Na^+ of 5.50 g $Na^+.L^{-1}$ (Fig. 6A) if also sodium introduced to the medium via sodium sulfate is considered. Strong competition between SRB and AB was observed for NaCl concentrations up to 15 $g.L^{-1}$, whereas acetate dominated sulfide formation in the vials containing 20 and 25 $g.L^{-1}$ of NaCl (Fig. 6B). Methane was only detected in the vials with 20 and 25 g $NaCl^{-1}$, although methane production corresponded for only 3 and 7 % of the electron flow, respectively (Fig. 6B). It is worth to note that the experiment lasted 1.8

days for vials with 0 to 5.0 g NaCl.L^{-1}, 4.9 days for vials with 7.5 to 15.0 g NaCl.L^{-1}, 6.8 days for the vial with 20.0 g NaCl.L^{-1} and 7.8 days for the vial with 25.0 g NaCl.L^{-1}.

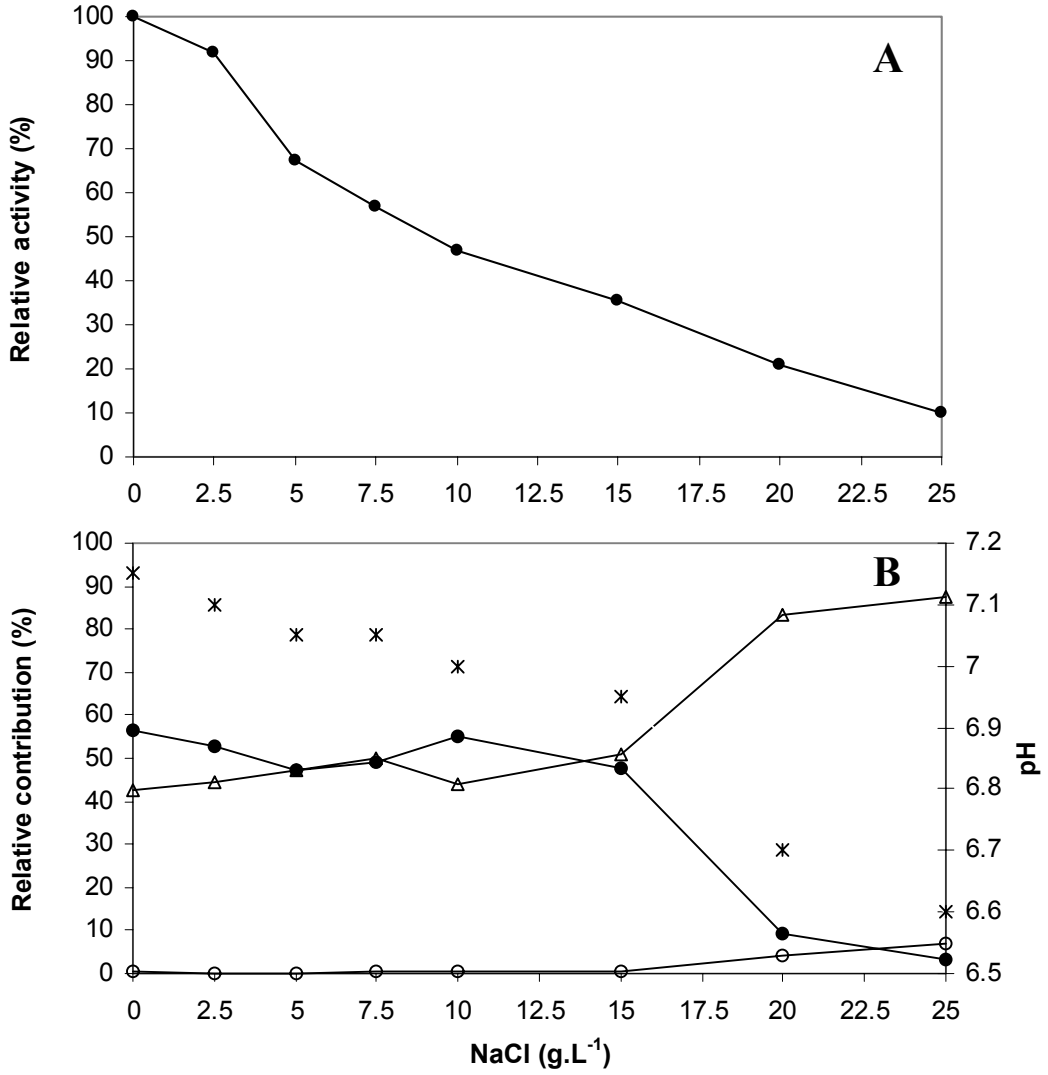

Figure 6. Effect of salt on methanol conversion on the sludge sampled from UASB II at day 162. (A) Relative methanol depletion rates compared to the methanol depletion rate of the incubations in the absence of supplementary NaCl. (B) Relative contribution of sulfide (•), methane (o) and acetate (△) and the pH (✳) upon completion of the NaCl toxicity test.

Sludge characteristics

The seed sludge inoculated in both reactors consisted of well-shaped granules and dispersed flocs. During the course of the experiment, the sludge developed towards aggregates covered with a thin fluffy grey coat, presumably due to the release of exopolymers by the cells. Ultimately, these particles became loosely linked with each other with the same coating material, forming a big voluminous sludge bed. Occasionally, the voluminous sludge bed of both reactors floated entirely as a result of the gas entrapment in the sludge bed voids,

even though the gas production was rather low in both reactors (Figs. 3B and 4B). Immediately after the partial disruption of the links between the aggregates (by gently mixing), and consequent release of the entrapped gas, the sludge bed descended promptly, indicating the good settleability properties of the sludge. Interestingly, the biomass of the salt-fed reactor (UASB II) started to loose the fluffy gray coat after methane gas production restarted at day 183 and ultimately the sludge became granular again. No visual biomass washout was observed throughout the experiment in both reactors. The height of the sludge bed increased over time, but precise measurements were not possible, as the expansion of the bed varied with the degree of gas entrapment in the void spaces.

DISCUSSION

Methanol removal at 55ºC

This study showed that methanol was almost completely used for sulfate reduction at 55ºC in the absence of NaCl (end of Period II-B) in methanol-fed UASB reactors when operating at an OLR of 5 g $COD.L^{-1}.day^{-1}$ and at an HRT of 10 hours. Thus, SRB outcompete MPA for the use of methanol at 55ºC. This is in agreement with the thermodynamics of methanol conversion, which predict that sulfate reduction will be the predominant process at this temperature (Widdel, 1988).

It took, nevertheless, a long time period (100 days) to turn into an almost fully sulfidogenic sludge. Selective wash-out of methanogens from the reactors is rather unlikely, as high-rate anaerobic reactors are designed for long sludge retention times, which can be as high as 0.5 – 1 year for UASB reactors (Hulshoff Pol, 1989). Also, the decrease in methane production during Period II-B can not be attributed to kinetic limitations of methylotrophic MPA, as reported by Weijma *et al.* (2002), as methanol was still present in excess (Fig. 3A). In addition to a direct conversion of methanol to H_2S and CO_2 (Nanninga and Gottschal, 1987; Nazina *et al.*, 1988), SRB can grow in syntrophic associations, either with H_2/CO_2 (Davidova and Stams, 1996) or acetate (Lowe *et al.,* 1993) producing organisms. The SRB dominance in both reactors might be due to syntrophic degradation of methanol via H_2/CO_2 as an intermediate, as previously reported either in the absence (Paulo *et al.*, 2001) or in excess (Weijma *et al.*, 2000) of sulfate. In the latter case, H_2 utilizing kinetics determine the outcome of the competition and it is well known that SRB outcompete MPA for hydrogen in the presence of sulfate (Davidova and Stams, 1996; Oude Elferink *et al.,* 1994).

Despite that the SRB outcompeted MPA in both UASB reactors, acetate represented a side-product, accounting for up to 25 % of the total COD converted (Figs. 3C and 4C). Such metabolism is undesired, as a further treatment step is needed to remove the acetate from the effluent. Moreover, when methanol is supplied as an external electron donor in SRB based bioprocesses for the removal of sulfur oxyanions, acetate production represents a loss of

electron donor. Acetate was not used by either MPA or SRB as the substrate in activity tests (Figs. 5A and 5C). This might be due to the inoculum sludge, which also had no activity with acetate, both in the presence or absence of sulfate (Lens *et al.,* 2002). It is, however, unlikely that this is the only reason, as the lack of acetate degradation has been observed in both mesophilic (Lens *et al.,* 1998) and thermophilic (Weijma *et al.*, 2000; Lens *et al.*, 2002) high rate sulfate reducing reactors.

Effect of NaCl on thermophilic methanol removal

This paper shows that the effect of NaCl on methanol degradation is highly concentration dependent. After switching from high to no-salt conditions during period I in UASB II, methanogenesis remained lower than sulfidogenesis and acetogenesis (Fig. 4B). Apparently, the MPA were the most sensible trophic group to the NaCl shock (25 $g.L^{-1}$). In contrast, 2.5 g $NaCl.L^{-1}$ was apparently stimulatory for both AB and MPA during period III in UASB II (Fig. 4C). The recovery of methane production in this reactor could also be attributed to the 16 hour non-feed period at day 176. This is, however, rather unlikely, as no methane production was detected in UASB I after two similar non-feed periods (days 185 and 212). Methane production by a methanol-fed mesophilic UASB reactor has been found to be NaCl dependent (Florencio *et al.,* 1993). Moreover, sodium is reported to be an essential ion for methanogens, probably because of its role in a chemiosmotic coupling mechanism (Perski *et al.*, 1982). In this work, the methanogenic process was not sodium limited, as the addition of sulfate as Na_2SO_4 provided a background concentration of 2.5 g $Na^+.L^{-1}$ in both reactors. Further research is needed to elucidate the apparent stimulatory effect of the rather low Na^+ concentration (3.5 $g.L^{-1}$) on methanogenesis.

Distinct effects of low and high salinity were also observed for the SRB. The 31 % decrease of sulfide production (Fig. 3B) after the NaCl addition in the influent (2.5 $g.L^{-1}$) suggests that the SRB are apparently inhibited by rather low NaCl concentrations, which is reversible upon omission of NaCl (Fig. 3B). The apparent inhibitory effects of low NaCl concentrations to the SRB are in contrast with Nilsen *et al.* (1996), who reports that methanol degrading thermophilic sulfate reducers, such as *Desulfotomaculum kuznetsovii* and *Desulfotomaculum saporovans*, grow at NaCl concentrations ranging from 0 to 29.8 $g.L^{-1}$ and from 0 to 35.1 $g.L^{-1}$, respectively. The high sensitivity of UASB II to the 25 g $NaCl.L^{-1}$ suggest that these salt tolerant species were not present in the granular sludge cultivated in the UASB II.

At first sight, the complete inhibition at high salinity (25 g $NaCl.L^{-1}$) contrasts the many reports of successful methanization of high saline (up to 12 $g.L^{-1}$ of Na^+) seafood effluents, either in mesophilic (Méndez *et al.,* 1995; Soto *et al.,* 1993; Feijoo *et al.,* 1995) or thermophilic (Méndez *et al.,* 1995) anaerobic reactors. However, seafood wastewaters have a

rather complex composition containing many different substrates, contrasting the single substrate methanol wastewater applied in this study. Nonetheless, some aspects of methanization of seafood wastewaters in high-rate reactors can not be disregarded, such as the need for a start-up procedure, which aims at acclimation of the biomass (Soto *et al.,* 1993; Omil *et al.,* 1996). Thus, a successful sulfate reducing process could be obtained by a stepwise exposure of the sludge to increasing salt concentration, although the adaptation period can be very time consuming. Therefore, further research is needed to find alternative ways to overcome salt toxicity, e.g. bioaugmentation with salt-tolerant SRB, such as those present in soda lakes and marine and oil sediments (Widdel, 1988); addition of antagonistic cations in order to alleviate Na^+ inhibitory effects (Soto *et al.,* 1993; Feijoo *et al.,* 1995) or addition of compatible solutes, such as betaine and glycerol to promote osmotic adaptation (Yerkes *et al.*, 1997).

Stability of thermophilic methanol removal

The immediate deterioration of the performance of both reactors after a 16-hour non-feed period clearly illustrates the sensitivity of the thermophilic UASB reactors for substrate starvation (Figs 3A, 4A). Thermophilic processes are known to be more sensitive than mesophilic processes (van Lier *et al.,* 2001) and also methylotrophic methanogenic (55ºC) UASB (Paulo *et al.*, 2001) and sulfate-reducing (65ºC) EGSB (Weijma *et al.,* 2000) reactors subjected to a feed interruption were found to be affected. Weijma *et al.* (2000) reported a temporary (few days) inhibition of the sulfide production rate after a 10-hour non-feed period, while the methane and acetate production rates remained unaffected. This paper shows that the degree of process disturbance depends on the reactor operation, as shown by comparing UASB II at day 176 (Fig. 3A) with UASB I at days 185 and 212 (Fig. 2A). In addition to the starvation effects, reactor failure in UASB I can be attributed to sulfide toxicity, as evidenced by the high (900 $mg.L^{-1}$) sulfide concentration, measured just after restarting the reactor at the end of the 16-hour batch mode (Fig. 2B). Such a high concentration can also be expected at day 185, as the system was running in excess of methanol (Fig. 2A). In contrast, no toxic sulfide concentrations could be produced in UASB II, where full methanol degradation occurred on the days prior to the feed interruption.

Bicarbonate omission from day 76 onwards had no effect on the performance of both reactors. Because bicarbonate (HCO_3^-) is necessary for the formation of acetate from the anaerobic degradation of methanol (Davidova & Stams, 1996), its omission can have a significant impact on the methanol degradation. As sulfide production (Figs 3B, 4B) is associated to bicarbonate production, the system was not necessarily free of bicarbonate. This could have supported the bicarbonate-depending conversions.

Sludge characteristics

The formation of a voluminous sludge bed consisting of granules and flocculent particles was also reported by Weijma *et al.* (2000), indicating the negative impact of sulfate on the granular characteristics. In the absence of sulfate, a thermophilic (55ºC) granular sludge can be properly retained in UASB reactors, even when operating at an OLR up to 47.3 g $COD.L^{-1}.day^{-1}$ and a HRT as low as 3.2 h (Paulo *et al.,* 2001). In the present study, the sludge did not wash-out at the applied upflow velocities (1-2 $m.h^{-1}$), which shows that the aggregates can also be successfully retained in sulfate reducing reactors.

Increasing the V_{up} to 2 $m.h^{-1}$ at day 112 could not overcome the sludge aggregation resulting in a big voluminous bed. This is in agreement with Weijma *et al.* (2000), who achieved disruption of such sludge aggregation only when applying a V_{up} as high as 50 $m.h^{-1}$. Application of an artificial gas loading rate, e.g. with nitrogen gas, can be an interesting alternative to induce turbulence inside sulfate-reducing reactors (Lens *et al.,* 2003). The induced hydraulic mixing can prevent the sludge aggregation and thus ensure good biomass medium contact.

CONCLUSIONS

The results obtained in this research allows to conclude that:

(1) Methanol was almost completely used for sulfate reduction at 55ºC in the absence of NaCl in methanol-fed UASB reactors when operating at an OLR of 5 g $COD.L^{-1}.day^{-1}$ and at an HRT of 10 hours.

(2) High NaCl concentrations (25 $g.L^{-1}$) completely inhibited the methanol degradation of the non-adapted granular inoculum sludge at thermophilic (55ºC) conditions. The MPA were the most sensible trophic group to the NaCl shock.

(3) Low NaCl concentrations (2.5$g.L^{-1}$) considerably changed the metabolic fate of methanol at thermophilic (55ºC) conditions. Methanogenesis became a secondary product when adding 2.5 g $NaCl.L^{-1}$.

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Chapter 6

Long term adaptation of a methanol-fed thermophilic (55ºC) sulfate reducing granular sludge reactor to NaCl

A lab scale upflow anaerobic sludge bed reactor (UASB) reactor was operated during 273 days at increasing NaCl concentrations (0.5 to 12.5 g $NaCl.L^{-1}$) to assess whether the stepwise addition of the salt NaCl results in the acclimation of that sludge. The 6.5 L thermophilic (55ºC) sulfidogenic (COD/SO_4^{2-} of 0.5) UASB reactor operated at an organic loading rate of 5 g $COD.L^{-1}.day^{-1}$, a hydraulic retention time of 10 hours and was fed with methanol as the sole electron donor. The results show that the adaptation of thermophilic (55°C) sulfidogenic methanol degrading biomass to a high osmolarity environment is unlikely to occur. Sulfide was the main mineralization product from methanol degradation, regardless of the NaCl concentration added to the influent. However, sulfide production in the reactor steadily decreased after the addition of 7.5 g $NaCl.L^{-1}$, whereas acetate production was stimulated at that influent NaCl concentration. Batch tests performed with sludge harvested from the UASB reactor when operating at different influent salinities confirmed that acetate is the main metabolic product at NaCl concentrations higher than 12.5 $g.L^{-1}$. The apparent order of NaCl toxicity towards the different trophic groups was found to be: sulfate reducing bacteria > methane producing archaea > acetogenic bacteria.

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INTRODUCTION

Constraints in water supply as well as restrictive environmental regulations have been stimulating industrial processes to re-examine their water management strategies. As a result, reuse/recycling of process water is becoming a valuable tool to reduce both fresh water intake and effluent disposal. One of the problems of recycling process water by deliberately closing the water loops is the toxicity that can be exerted due to build up of high salt concentrations in the circuit water. In addition, wastewater treatment processes, such as the biodesulfurization of flue gases, produce hot wastewaters that must be treated before their reuse as scrubbing water. As a consequence, there is a need for hot and salt tolerant biological wastewater treatment processes, which overcome the problems associated with these waters, i.e. enzyme denaturation by high temperatures (Madigan *et al.*, 1997) and cell decay due to osmotic shocks in the high osmolarity environments (Kempf and Bremer, 1998).

The effect of sodium on the methanization of seafood wastewater has been extensively studied (Feijoo *et al.*, 1995; Guerrero *et al.*, 1997). Mesophilic and thermophilic (up to 55ºC) high-rate methanogenic treatment of seafood wastewater proceeds successfully at NaCl concentrations ranging from 15 to 25 $g.L^{-1}$. In contrast to methanogenic reactors, little is known about the effect of sodium salts on the performance of sulfate reducing reactors. The addition of 25 g $NaCl.L^{-1}$ was found to completely inhibit methanol degradation in a thermophilic (55ºC) upflow anaerobic sludge bed (UASB) reactor fed with methanol as the sole electron donor and in excess (COD/SO_4^{2-} of 0.5) of sulfate (Chapter 5).

For the methanization of sea-food wastewaters, many authors suggest the stepwise increase of salt levels as a strategy for the adequate acclimation of the sludge to high salinity (Feijoo *et al.*, 1995; Omil *et al.*, 1995). Thus, gradual selection for salt tolerant microorganisms occurs in an initially non-adapted inoculum sludge. The aim of this work was to assess whether stepwise addition of the salt NaCl is also suitable for the acclimation of the sludge cultivated in a thermophilic (55ºC) sulfidogenic UASB reactor (COD/SO_4^{2-} of 0.5) fed with methanol as the sole electron donor. This was investigated by monitoring the performance of a lab-scale UASB reactor subjected to increasing influent NaCl concentrations and by assessing the maximum specific activity of the sludge harvested from the UASB reactor when operating at different influent salinities.

MATERIAL AND METHODS

Experimental set-up

To investigate the aims of this work, a 6.5 L UASB reactor was operated during 273 days fed with methanol as the sole electron donor and carbon source and in excess of sulfate

(COD/SO_4^{2-} of 0.5). The UASB reactor, described in detail in Chapter 5, had an internal diameter of 0.10 m and height of 1 m. The reactor was equipped with a water jacket, maintaining the reactor temperature at 55ºC. Effluent recycling was applied to obtain a superficial liquid upflow velocity (V_{up}) of 1 $m.h^{-1}$. The reactor was operated at a pH of 7.5 (± 0.2) by using an automatic pH control device.

Inoculum and medium

The inoculum was collected from a thermophilic lab-scale (6.5 L) UASB reactor operating under similar conditions as used in the present work (Chapter 5). The sludge was stored during 3 months at 4°C prior to inoculation. Sulfide was the main mineralization product of methanol degradation in this sludge, with acetate as a secondary product (Chapter 5).

The synthetic influent contained methanol as the sole electron donor. Sulfate was added as sodium sulfate to provide a COD/SO_4^{2-} of 0.5 (g COD per g SO_4^{2-}). Thus, theoretically all methanol could be degraded via sulfate reduction. The influent of both reactors was further supplied with a basal medium and a trace elements solution as described in Chapter 3. Basal medium was added to the main flow at a ratio of 2.22 mL of basal medium per gram COD in the influent. NaCl was selected as model compound to increase the salinity of the influent.

Experimental design

The UASB reactor operated at a hydraulic retention time (HRT) of 10 hours and an organic loading rate (OLR) of 5 g $COD.L^{-1}.day^{-1}$ throughout the whole experiment. The NaCl concentration in the influent was increased stepwise from 0.5 g $NaCl.L^{-1}$ added at day 28 up to 12.5 g $NaCl.L^{-1}$ added between days 201 and 245. The conductivity of the reactor mixed liquor was measured to assess the salinity applied to the reactor (Fig. 1C). At day 246, NaCl was omitted from the influent in order to assess the reversibility of the NaCl induced effects.

Maximum specific activity (MSA)

Sludge samples were harvested from the UASB reactor at days 31, 103, 145, 200 and 241 (corresponding to a influent NaCl concentration of, respectively, 0.5, 3.5, 7.5, 10 and 12.5 g $NaCl.L^{-1}$) and activity tests were performed to assess the effect of NaCl on the MSA. The batch vials were supplemented with a gradient series of NaCl (up to 25 g $NaCl.L^{-1}$) as indicated in Figs. 2, 3 and 4. The MSA was determined as described in Chapter 3.

Batch toxicity assays were performed with sludge sampled at day 241 to assess the effect of NaCl on the methanol degradation and to determine the 50 % inhibition concentration (IC_{50}) of NaCl on the methanol depletion rate. Experimental procedures were similar to those applied for the maximum specific activity tests. Fig. 4 shows the NaCl concentrations amended to the batch vials for the sludges harvested at day 241. Control vials were assayed without NaCl addition. The percentage of toxicity was determined by comparing the activity of the vials on which NaCl was added with the control vials. The final pH and the amount of volatile suspended solids (VSS) for each vial was determined upon completion of the test. All these experiments were performed in duplo.

Analysis

VSS were analyzed according to standard methods (APHA, 1985). Sulfide was determined photometrically as described by Trüper and Schlegel (1964). Methanol, VFA and methane were measured by gas chromatography (GC) as described by Weijma *et al.* (2000). The volume of biogas produced in the UASB reactors was measured as described in Chapter 3.

RESULTS

UASB reactor operation

Reactor start-up (background salinity)

Complete methanol degradation was only obtained during four days after the start-up (Fig. 1A). After that day, methanol started to accumulate in the reactor effluent, indicating that the UASB reactor operated under overloading conditions (Fig. 1A). Methane production rates as high as 1.32 g $COD.L^{-1}.day^{-1}$ were observed in the first days of reactor operation (Fig. 1C), although the reactor was inoculated with a sludge grown in a sulfidogenic methanol fed thermophilic UASB that exhibited no methanogenic activity in the presence of sulfate (Chapter 5). Methane production started to decrease one week after reactor start up and no methane could be detected anymore after day 26 (Fig. 1B). Acetate production accounted for about 57 % of the electron flow in the first days of operation. Sulfide production increased steadily at the expense of methane production one week after reactor start up, reaching a sulfide production rate as high as 2.1 g $COD.L^{-1}.day^{-1}$ at day 18. Sulfide was the main mineralization product prior to the addition of salt in the reactor, accounting for 50 (± 21) % of the electron flow, whereas acetate and methane accounted for 40 (± 10) % and 10 (± 10) % of the consumed methanol-COD, respectively.

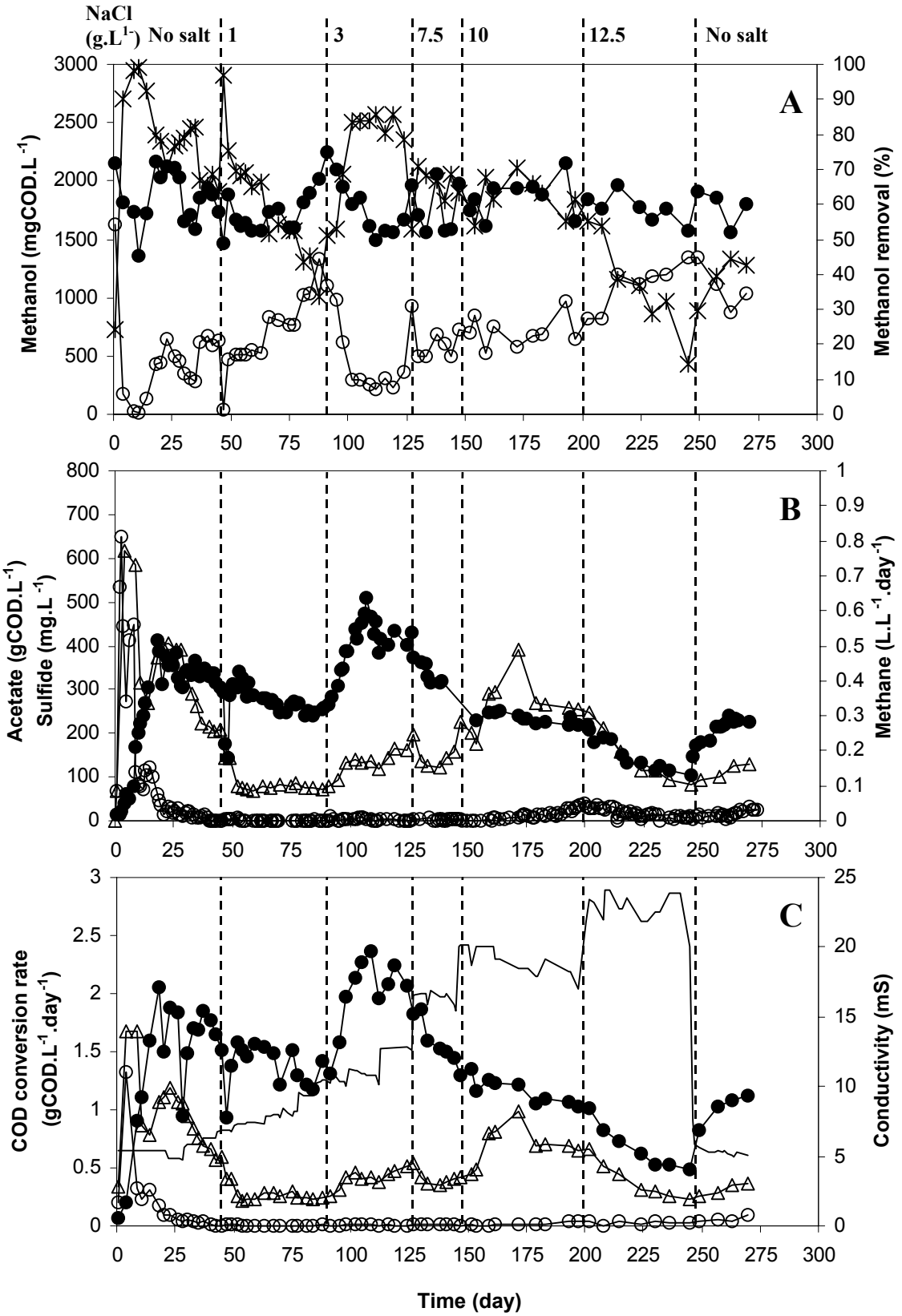

Figure 1. Process performance of the UASB reactor. (A) Evolution of the methanol concentration in the influent (•), effluent (o) and methanol removal efficiencies (x). (B) Evolution of the sulfide (•), methane (o) and acetate (△) concentration. (C) Evolution of the COD conversion rate to sulfide (•), methane (o) and acetate (△) and the evolution of the conductivity (—).

Effect of stepwise increase of the influent salt concentration on the reactor performance

Low salinity (0.25 – 3.5 gNaCl.L^{-1})

When the reactor operated at influent NaCl concentrations up to 2 g NaCl.L^{-1} (day 76), methanol removal efficiencies of around 75 % were obtained (Fig. 1A). It dropped to around 50 % when the reactor was operated up to 3 g NaCl.L^{-1} (days 76-98). The sulfide production rate decreased from 2.05 g COD.L^{-1}.day^{-1} at day 18 to 1.18 g COD.L^{-1}.day^{-1} at day 84 (3 g NaCl.L^{-1} in the influent) (Fig 1C). The acetate production rate also decreased steadily, from 1.19 g COD.L^{-1}.day^{-1} at day 23 to 0.21 g COD.L^{-1}.day^{-1} at day 54 (Fig 1C) and to 0.25 g COD.L^{-1}.day^{-1} at day 98, when the influent NaCl concentration was further increased to 3.5 g.L^{-1}. Sulfide was the main mineralization product during the 69 days (days 28 -97) when the reactor was subjected to low NaCl concentrations, accounting for about 79 (± 7.8) % of the electron flow, whereas acetate and methane accounted for about 20 (± 7.3) % and 1 (± 0.5) % of the consumed methanol-COD, respectively.

Medium salinity (3.5 – 5.0 gNaCl.L^{-1})

The sulfide production rate increased after the reactor was supplied with 3 g NaCl.L^{-1} at day 84 (Fig. 1B), from 1.18 g COD.L^{-1}.day^{-1} at day 84 (low salinity period) to 2.59 g COD.L^{-1}. day^{-1} at day 107, when 4 g NaCl.L^{-1} was applied to the reactor (Fig. 1C). As for the production of sulfide, the acetate production rate also increased after the elevation of the influent salinity to 3 g NaCl.L^{-1}, from 0.23 g COD.L^{-1}.day^{-1} at day 84 to around 0.45 g COD.L^{-1}.day^{-1} between days 98 and 124 (Fig. 1C). The methanol removal efficiency increased as a consequence of the higher sulfide and acetate production, with an average removal efficiency of about 82 % between days 102 and 124 (Fig. 1A). Methane was hardly detected during this period. As for the low salinity concentrations, sulfide was also the main mineralization product when the reactor was subjected to medium NaCl concentrations, accounting for 83 (± 1.5) % of the electron flow. Acetate and methane production accounted for about, respectively, 16 (± 1.5) % and 0.5 (± 0.5) % of the consumed methanol-COD of this period.

High salinity (7.5 – 12.5 gNaCl.L^{-1})

The increase of the influent NaCl concentration of the UASB reactor to 7.5 g.L^{-1} caused a steady decrease in the sulfide production (Fig. 1B), accompanied by a drop in the methanol removal efficiency to around 65 % between days 126 and 145 (Fig. 1A). The sulfide production rate decreased from 2.06 g COD.L^{-1}.day^{-1} at day 124 to 1.45 g COD.L^{-1}.day^{-1} at day 144 and dropped further to 1.03 g COD.L^{-1}.day^{-1} after increasing the influent NaCl concentration to 10 g NaCl.L^{-1} at day 145 (Fig. 1C). Acetate production was not affected by

the presence of 7.5 g $NaCl.L^{-1}$ in the influent, as evidenced by the relatively constant acetate production rate of 0.41 g $COD.L^{-1}.day^{-1}$ (Fig. 1C). Opposed to the decrease of sulfide production, acetate production steadily increased upon the addition of 10 g $NaCl.L^{-1}$ to the influent, increasing up to 0.99 g $COD.L^{-1}.day^{-1}$ at day 172. Low methane production rates were detected when operating the reactor with an influent NaCl concentration of 10 $g.L^{-1}$ (Fig. 1B), never exceeding more than 2 % of the electron flow.

Increasing the influent NaCl concentration to 12.5 $g.L^{-1}$ at day 201 resulted in a steady decrease of both the sulfide and acetate production (Fig. 1B). Sulfide production and acetate production rates as low as, respectively, 0.48 g $COD.L^{-1}.day^{-1}$ and 0.23 g $COD.L^{-1}.day^{-1}$ were measured at day 245 (Fig. 1C). A methanol removal efficiency of only 14 % was measured at day 245 (Fig. 1A). Even though the production of acetate was stimulated by the presence of high NaCl concentrations, sulfide was still the main mineralization product of methanol degradation, accounting for about 62 (± 9) % of the electron flow. Acetate and methane production accounted for the remaining 36 (± 8) % and 2 (± 1.5) % of the consumed methanol-COD, respectively.

Effect of salt omission on the recovery of the reactor performance (background salinity)

Immediately after omission of NaCl from the influent of the UASB reactor (Fig. 1B), sulfide production increased and sulfide production rates as high as 1.12 g $COD.L^{-1}.day^{-1}$ were achieved at the end of the experiment (Fig. 1C). Acetate production remained relatively low after switching to no salt conditions, achieving acetate production rates similar to those observed when the reactor was operating at low (0.25 - 3.0 $g.L^{-1}$) NaCl concentrations (Fig. 1B). Rather low methane production rates were detected in this period, accounting for less than 3 (± 1) % of the electron flow. Sulfide was the main mineralization product at the end of the experiment, accounting for 74 (± 2) % of the electron flow, whereas acetate accounted for the remaining 23 (± 1) % consumed methanol-COD.

Maximum specific activities (MSA)

Evolution of the MSA of the sludges in the absence of salt

The highest methanol degradation rates (1019 $mgCOD.gVSS^{-1}.day^{-1}$) were obtained with sludge harvested at day 31, when only 0.5 g $NaCl.L^{-1}$ was added to the influent of the UASB reactor (Fig. 2A). Surprisingly, the methanol depletion rate dropped to 624 mg $COD.gVSS^{-1}.day^{-1}$ for the sludge sampled at day 103, despite that the methanol removal in the UASB reactor at day 103 was as high as at day 31 (Fig. 1A). The methanol depletion rate increased in time and values as high as 908 mg $COD.gVSS^{-1}.day^{-1}$ were obtained when operating the UASB reactor with an influent NaCl concentration of 10 $g.L^{-1}$ (Fig. 2A). The

increase to 12.5 g NaCl.L^{-1} in the influent of the UASB reactor caused a drop in the methanol depletion rate to 671 mg COD.$gVSS^{-1}$.day^{-1}, despite that the vials were not amended with any supplementary salt (Fig. 2A). Both the acetate and the sulfide production rates followed the same pattern of the methanol depletion rate, except that the sulfide production rate already dropped for the sludge sampled when the UASB reactor operated at a NaCl concentration of 10 g.L^{-1} (Fig. 2A).

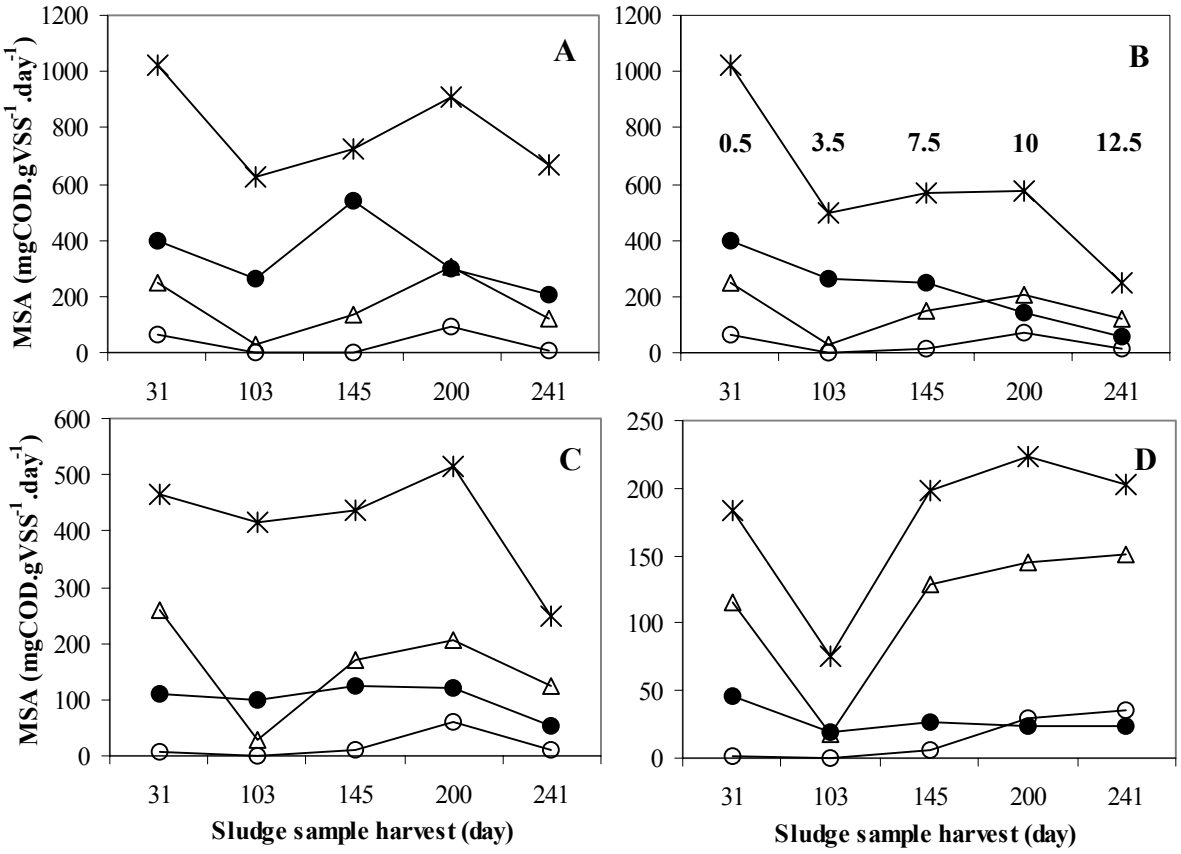

Figure 2. Evolution of the methanol depletion rate and acetate, methane and sulfide formation rates during activity assays performed with the sludge harvested from the UASB reactor at different days and inoculated with different salinities: (A) no salt amended, (B) actual reactor salinity, (C) 12.5 g NaCl.L^{-1} and (D) 25.0 g NaCl.L^{-1}. Methanol (x), sulfide (•), methane (o) and acetate (△). Note the difference in the maximum specific activity scale of the figures.

Sulfide was the main mineralization product in the vials without NaCl amendment, accounting for more than 60 % of the electron flow (Fig. 3A), independently of the salt concentration added to the UASB reactor. Acetate production was as high as the sulfide production only with the sludge sampled at day 200, when the UASB reactor operated at an influent NaCl concentration of 10 g.L^{-1} (Fig. 3A). Methane production was only detected at considerable concentrations at day 200, accounting for 13 % of the electron flow (Fig. 3A). Methane was also detected for the sludges harvested at day 31 and day 241, accounting for 8 % and 4 % of the electron flow, respectively (Fig. 3A).

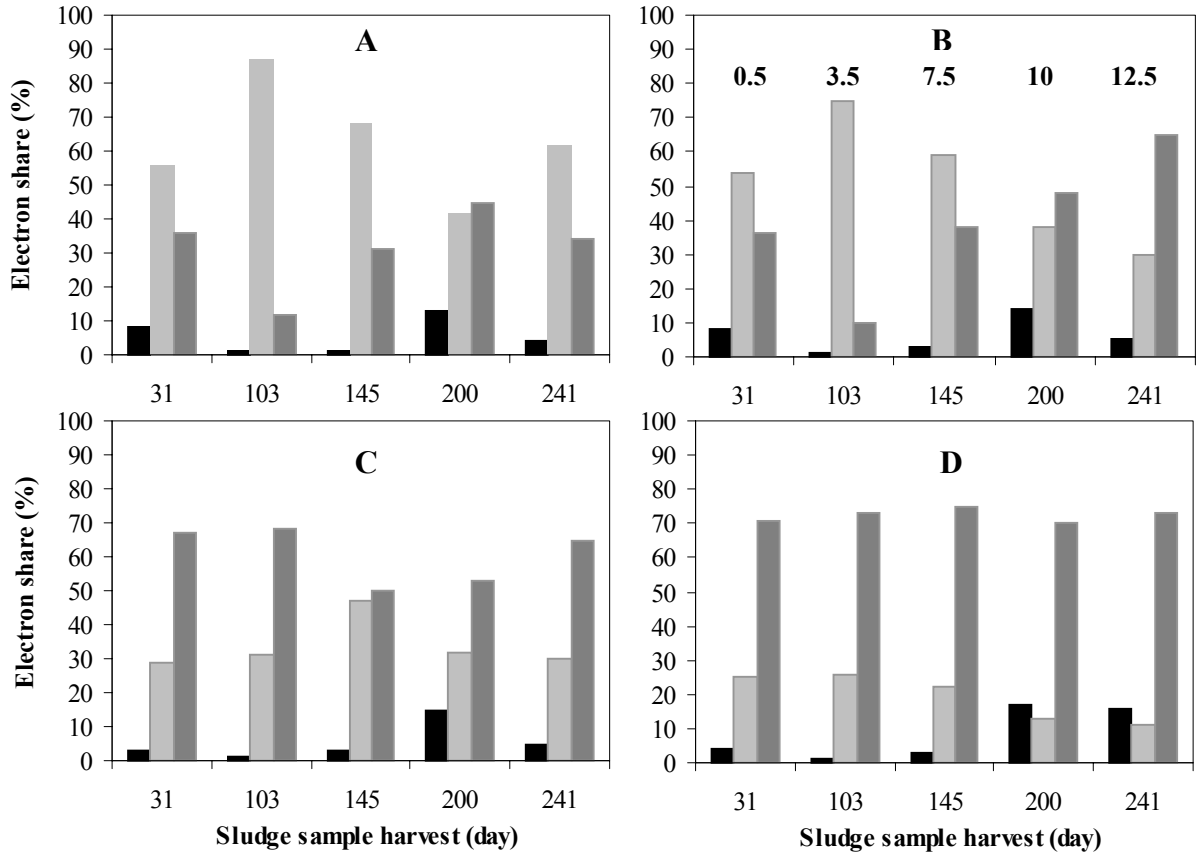

Figure 3. Evolution of the product formation from methanol degradation (%) during activity tests performed with the sludge harvested from the UASB reactor at different days and inoculated with different salinities: (A) no salt amended, (B) actual reactor salinity, (C) 12.5 g $NaCl.L^{-1}$ and (D) 25.0 g $NaCl.L^{-1}$. Sulfide (light grey bar), methane (black bar) and acetate (dark grey bar).

Effect of salt in the SMA of sludges cultivated at different salinities

All rates decreased with the increase of the salinity, independently of the salt concentration on which the sludge was cultivated (Fig. 2B,C and D). As for the vials without NaCl amendment, the methanol depletion rate decreased for the sludge harvested at day 103, despite the good performance of the UASB reactor at that time of sludge sampling (Figs. 3B,C and D). In the vials amended with similar NaCl concentrations, a significant drop of the activity was observed only for the vials inoculated with the sludge harvested at day 241, when the UASB reactor operated at an influent NaCl concentration of 12.5 $g.L^{-1}$ (Figs. 2B, C and D). A similar pattern was found for the sulfide production rate (Figs. 2B and 2C), except for the sludge sampled at day 241 (influent NaCl concentration of 12.5 $g.L^{-1}$), when the acetate production rate exceeded the rate measured with the sludge sampled between days 145 - 200 (influent NaCl concentration of 10 $g.L^{-1}$) (Fig. 2D).

The presence of different NaCl concentrations strongly affected the fate of methanol (Figs. 3B,C and D). Sulfide was the main mineralization product only for the vials amended with less than 7.5 g $NaCl.L^{-1}$ (Figs. 3B,C and D). Acetate became the main fate of methanol degradation for the vials amended with more than 10 g $NaCl.L^{-1}$, independently of the influent NaCl concentrations imposed to the UASB reactor (Figs. 3B,C and D). For instance, acetate production always accounted for more than 70 % of the electron flow in the vials amended with more than 25 g $NaCl.L^{-1}$, independently of the UASB influent sodium concentration (Fig. 3D). Considerable methane production (around 16 %) was only detected in the vials inoculated with sludge sampled at day 200 (10 g $NaCl.L^{-1}$ UASB reactor influent), independently of the salt concentration amended to the vials (Figs. 3B,C and D). In addition, considerable methane production (about 17 %) was detected in the vials amended with 25 g $NaCl.L^{-1}$ and inoculated with sludges sampled at day 200 (10 g $NaCl.L^{-1}$ in the influent of the UASB reactor) and at day 241 (12.5 g $NaCl.L^{-1}$ in the influent of the UASB reactor).

IC_{50} of NaCl of sludge cultivated at high salinity (12.5 $gNaCl.L^{-1}$)

The IC_{50} concentration of NaCl of the sludge sampled at day 241 (12.5 g $NaCl.L^{-1}$ in the UASB reactor influent) was 6.90 $g.L^{-1}$ (Fig. 4A). This corresponds to an IC_{50} concentration for Na^+ of 5.1 $g.L^{-1}$ if also the sodium introduced in the medium both via sodium sulfate and sodium bicarbonate is considered. In the absence of sulfate, higher acetate and methane production rates occurred (Fig. 4A), with acetate and methane accounting for, respectively, 70 % and 30 % of the electron flow (Fig. 4B). As observed for the sludges harvested at previous days, the presence of NaCl in different concentrations strongly affected the fate of methanol under thermophilic conditions (Fig. 4B). Acetate was the main mineralization product in vials amended with more than 7.5 $g.L^{-1}$ NaCl (Fig. 4B), whereas sulfide predominated as end product in vials amended with 5 g $NaCl.L^{-1}$ or less (Fig. 4B). The methane concentration upon termination of the experiment increased with increasing NaCl concentrations (Fig. 4B). However, methane accounted for maximally 16 % of the electron flow for the vials amended with 25 $g.L^{-1}$ of NaCl (Fig. 4B).

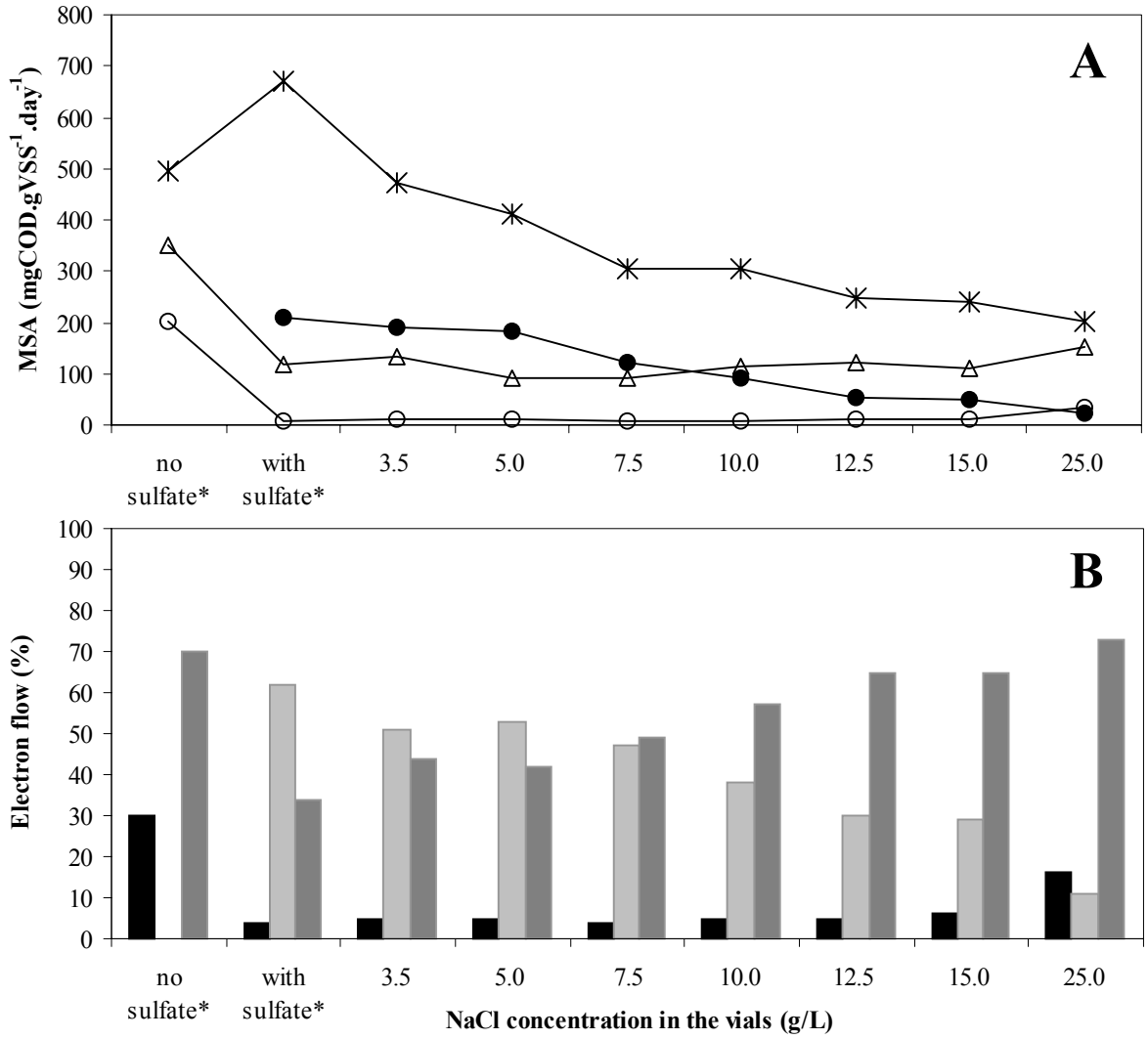

Figure 4. Effect of salt concentration on the maximum specific activity (A) and electron flow (B) on day 241 (influent NaCl concentration of 12.5 $g.L^{-1}$). (A) Methanol depletion rate (x) and acetate (△), methane (o) and sulfide (•) formation rates. (B) Product formation from methanol degradation (%): sulfide (light grey bar), methane (black bar) and acetate (dark grey bar).

DISCUSSION

Long term adaptation to NaCl stress

Stepwise acclimation of the biomass to NaCl was found to be an ineffective strategy to treat saline sulfate-rich wastewaters using methanol as electron donor (Fig. 1). Thus, the adaptation of thermophilic (55°C) sulfidogenic methanol degrading biomass to high osmolarity environment is unlikely to occur. The results obtained in this work contrast with previous papers who affirm that the stepwise exposure of the sludge to increasing salt concentration is a necessary procedure for the successful treatment of salt-rich wastewaters

(Gharsallah *et al.*, 2002; Omil *et al.*, 1996; Soto *et al.*, 1993). These authors worked, however, with the anaerobic treatment of seafood wastewaters, which have a rather complex composition containing many different substrates, contrasting the single substrate methanol applied in this study. In addition, seawater was used as process water and it may have contained synergetic cations (e.g. K^+, Mg^{2+}) or provoked the inoculation and growth of halotolerant seawater microorganisms in the sludge.

In a previous work, a higher IC_{50} value of 9.30 g $NaCl.L^{-1}$ was obtained in a UASB reactor operating at low influent salt concentrations (Chapter 5). The lower IC_{50} value of 6.90 g $NaCl.L^{-1}$ obtained in this work suggests a limited extent of adaptation of the sludge to high salinity (Fig. 4A). However, one must consider that only a partial recovery of the activity might have occurred when re-suspending the sludge cultivated in the UASB reactor at 12.5 g $NaCl.L^{-1}$ in a fresh medium (no NaCl), so that the control vials probably had a lower activity compared to the sludge cultivated in fresh medium (Fig. 1 and Fig. 4A). The only partial reversibility of the deleterious effect of salt towards the granular sludge suggest that a period of time is needed to re-establish stable performance when switching from high salt to low salt influent concentrations. This was confirmed by the only partial recovery of the UASB reactor even 26 days after switching the influent NaCl concentration from 12.5 $g.L^{-1}$ to no salt at day 246 (Fig. 1).

The long term adaptation might have failed because of the lack of good attachment properties of the salt tolerant microorganisms, leading to their washout from the UASB reactor. In order to verify the existence of salt tolerant microorganisms in the sludge, a parallel UASB reactor was run at a HRT of around 80 hours using the same inoculum (data not shown). Very low methanol removal efficiencies (around 25 % of methanol was converted to acetate) were obtained when operating the reactor at a conductivity of 30 $mS.cm^{-1}$ (corresponding to about 20 g $NaCl.L^{-1}$ in the reactor bulk), despite that sufficient hydraulic retention time (80 h) allowed the growth of halotolerant species present in the granules or as cell suspension (data not shown). The absence of halotolerant microorganism in the sludge cultivated in this work is further confirmed by the batch tests. Indeed, higher activities were obtained for the batch vials amended with lower salt concentrations (Fig. 4A), as also found in the activity tests performed with sludge cultivated in the UASB reactor at lower salinities (Figs. 2A and B).

In view of the problems because of high salinity, viz. the limited extent of adaptation and reduction in methanol degradation kinetics coupled to a suppression of sulfidogenesis, further research must be oriented towards alternative ways to overcome salinity stress in sulfidogenic bioreactors. For this, the potential application of compatible solutes, i.e. organic compounds accumulated by halotolerant microorganisms in response to the increase in the salinity (Kempf and Bremer, 1998), or special microorganisms adapted to high salinity, the so-called halophiles, need to be applied for the anaerobic treatment of saline sulfate-rich

waste streams. Recently, a whole range of autotrophic and heterotrophic sulfate reducing species have been described that are able to grow under high salinity, such as the halophile *Desulfobacter halotolerans* (Brandt and Ingvorsen, 1997). To the best of our knowledge, there are so far no reports on the successful immobilization of these halophilic sulfate reducing bacteria (SRB) in bioreactor sludges. Such an approach will become essential, as it would push the current operation limits of sulfate reducing bioreactors to more extreme working conditions.

Effect of NaCl on the fate of methanol degradation

This study showed that, in agreement with Chapters 4 and 5, the SRB displayed a higher sensitivity to NaCl than the acetogenic bacteria (AB), as sulfide production in the reactor steadily decreased after the addition of 7.5 g $NaCl.L^{-1}$, whereas acetate production was stimulated at this influent NaCl concentration (Figs. 1B and 1C). This study further confirms that sulfide is the main mineralization product from methanol degradation in a thermophilic (55°C) UASB reactor operated at an OLR of 5 g $COD.L^{-1}.day^{-1}$ and a HRT of 10 hours, regardless of the NaCl concentration added to the influent. Despite that sulfide was the predominant metabolic product of methanol degradation when operating the reactor at an influent NaCl concentration up to 12.5 $g.L^{-1}$ (about 62 % of the electron flow), batch experiments showed that acetate is the main metabolic product at higher NaCl concentrations (Figs. 3C, 3D and 4B).

Although methane production by methane producing archaea (MPA) was very low in the UASB reactor (Fig. 1B and 1C), a methanogenic population was cultivated in the bioreactor when operating at higher salinities, as considerable methane production was detected in the batch vials amended with 25 g $NaCl.L^{-1}$ at days 200 and 241, even exceeding the production of sulfide (Fig. 3D). The apparent order of toxicity of NaCl towards the different trophic groups was found to be: SRB > MPA > AB. Thus, acetate can be expected to be the main mineralization product of methanol degradation for influent NaCl concentrations higher than 12.5 $g.L^{-1}$.

In principle, it is expected that the produced acetate would be further converted to H_2S or CH_4, as a number of both SRB (Widdel, 1988) and MPA (Clarens and Moletta, 1990; Nozhevnikova and Chudina, 1984) are able to oxidize acetate to CO_2 at thermophilic conditions. However, neither methane nor sulfide production were detected in batch tests inoculated with acetate as single substrate (data not shown). The lack of acetate degradation in sulfate reducing thermophilic bioreactors is well reported, even under low salinity conditions (Chapter 5; Weijma *et al.*, 2000). As such, the production of acetate is undesired in sulfate reducing bioreactors, as it induces the need for further treatment steps, either when the water is meant for reuse or sulfide is to be biologically converted to elemental sulfur (Janssen *et al.*, 1997).

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Chapter 7

Assessment of the use of compatible solutes to overcome salinity stress in thermophilic (55ºC) methanol-fed sulfate reducing granular sludges

High NaCl concentrations (25 g.L $^{-1}$) considerably decreased the methanol depletion rates for sludges harvested from two lab-scale sulfate reducing UASB reactors. In addition, 25 gNaCl.L^{-1} strongly affected the fate of methanol degradation, with clear increase in the acetate production at the expense of sulfide and methane production. The addition of different osmoprotectants, viz. glutamate, betaine, ectoine, choline, a mixture of compatible solutes and K^+ and Mg^{2+}, slightly increased methanol depletion rates for UASB reactors sludges. However, the acceleration in the methanol uptake rate favored the homoacetogenic bacteria, as the methanol breakdown was steered to the formation of acetate without increasing sulfate reduction and methane production rates. Thus, the compatible solutes used in this work were not effective as osmoprotectants to alleviate the acute NaCl toxicity on sulfate reducing granular sludges developed in methanol degrading thermophilic (55ºC) UASB reactors.

A modified version of this chapter will be published in Water Science and Technology.

INTRODUCTION

The characteristics of industrial wastewaters such as temperature and salinity are determined by the production process, and can be far from the physiological optima of microorganisms. With the current trend to close water cycles in industry, there is a need for wastewater treatment processes effective under hot and saline conditions. These parameters impose the need for adapted wastewater treatment processes, as high temperatures denature enzymes of mesophilic bacteria (Madigan *et al.*, 1997), whereas high osmolarity environments trigger rapid fluxes of cell water, thus causing a reduction in turgor and dehydration of the cytoplasm (Kempf and Bremer, 1998).

Thus far, anaerobic treatment processes use as much as possible endogenous microorganisms, commonly present in granular sludges. Granular sludge based processes have been used extensively to treat an enormous range of substrates in a big range of environmental conditions (Frankin, 2001). However, current research is evolving the borders of the metabolic capacity of these natural populations for the treatment of industrial wastewaters. For instance, 25 g $NaCl.L^{-1}$ (10 g $Na^{+}.L^{-1}$) completely inhibited methanol degradation in a thermophilic (55ºC) upflow anaerobic sludge bed (UASB) reactor in the presence of excess of sulfate (COD/SO_4^{2-} of 0.5). Even sodium concentrations as low as 3.0 g $Na^{+}.L^{-1}$ provoked considerable changes in the metabolic fate of methanol under thermophilic conditions (Chapter 5).

In search of alternative ways to overcome salt toxicity in anaerobic bioreactor systems, one can be inspired by strategies that microorganisms use themselves. The successful occupancy of what are often hostile environments (such as elevated osmolarity environments), uncongenial to other life forms, can be attributed at least in part to the development of complex stress management strategies, which have evolved to allow the bacterial cell to sense and respond to changes in its external environment (Sleator and Hill, 2001). Two fundamentally different strategies exist within the microbial world that enable microorganisms to cope with the osmotic stress inherent to the presence of high salt concentrations: (i) cells maintain high intracellular salt concentrations (the 'salt-in' strategy) and (ii) cells may maintain low salt concentrations within their cytoplasm (the 'compatible-solute' strategy). In the latter case, the osmotic pressure of the cytoplasm is balanced by compatible solutes. Compatible solutes are defined as intracellular organic solutes which, at high concentrations, allow 'conventional' enzymes to function efficiently (Brown, 1990). In most halophilic and halotolerant microorganisms, the osmotic balance is provided by small organic molecules (compatible solutes) that are either synthesized by the cells or taken up from the medium when available (Oren, 1999; Welsh, 2000; O'Byrne and Booth, 2002). In contrast to the salt-in strategy, the compatible-solutes strategy does not involve the need for specially adapted proteins and intracellular systems (Oren, 1999). In addition to their role as

osmotic balancers, compatible solute function as effective stabilizers of enzyme function, providing protection against salinity, high temperature, freeze-thaw treatment and even drying (Welsh, 2000).

Many microorganisms possess transport systems for compatible solutes whose transcription and/or activity is directly regulated by osmotic pressure (Welsh, 2000). The uptake of solutes from the environment, when available, is expected to be advantageous in complex microbial communities in which different metabolic types of microorganisms coexist, as it diminishes the energy cost of life at high salt concentrations (Oren, 1999). Given that osmolyte uptake is often energetically more favorable than synthesis, accumulation of compatible solutes from exogenous sources generally inhibits endogenous synthesis (Whatmore and Reed, 1990; Dinnbier *et al.*, 1988). In natural ecosystems, the supply of compatible solutes is likely to be low and varying. Therefore, osmoprotectant transporters usually exhibit high affinity for their substrate with K_m values in the micromolar range, and their capacity is geared to permit high-level compatible solute accumulation (Kempf and Bremer, 1998).

Thus far, little is known concerning the use of compatible solutes as osmoprotectants in engineering applications. However, Yerkes *et al.* (1997) demonstrated that the addition of small concentrations (1 mM) of betaine, one of the most studied osmoprotectants, caused more rapid substrate uptake rates upon sudden changes in sodium concentration (0 to 500 mM of Na^+) in sucrose fed batch assays, CSTRs, fluidized bed reactors and UASB reactors operating at mesophilic (35ºC) conditions. To the best of our knowledge, there are no reports about the use of compatible solutes to alleviate sodium toxicity in thermophilic sulfate reducing systems. If effective, adopting a policy of adequate dosing of these compatible solutes could obviate the need for time consuming adaptation (Chapter 6) or biological augmentation of sulfate reducing treatment systems. The use of compatible solutes would enable the adoption of sulfate reducing bacteria (SRB)-based bioprocesses in closed water cycles, as the deliberate reduction of the bleed in bioreactors leads to salt accumulation (Lens and Kuenen, 2001). In this work, batch experiment were conducted to determine the effect of different compatible solutes in alleviating the toxic effect of sodium chloride on unadapted granular sludge cultivated in a thermophilic (55ºC) sulfate reducing UASB reactor.

MATERIAL AND METHODS

Activity tests were carried out in 117 mL vials as described in Chapter 3. The vials were inoculated with methanol as the sole substrate (2 g $COD.L^{-1}$) and sulfate, added as sodium sulfate, to provide a COD/SO_4^{2-} of 0.5 (excess of sulfate). All vials were inoculated with 25 g $NaCl.L^{-1}$, either in the presence or the absence of an antagonist of sodium toxicity (Table 1). The sulfidogenic granular sludges were harvested from two lab-scale (6.5 L)

thermophilic (55ºC) sulfate reducing UASB reactors operating at a low (2 $g.L^{-1}$) sodium concentration, so that the antagonists of sodium toxicity were evaluated in non-adapted sulfidogenic sludges. Both reactors operated at an organic loading rate (OLR) of 5 g $COD.L^{-1}.day^{-1}$, a hydraulic retention time (HRT) of 10 hours and a COD/SO_4^{2-} of 0.5. Thus, theoretically, all methanol, added as sole electron donor and carbon source, could be converted via sulfate reduction.

The first UASB reactor (UASB I, Chapter 6) was operating under overloading conditions, as evidenced by the 80 % COD removal (Chapter 6). Sulfide production accounted for about 71 % of the electron flow, whereas acetate was a secondary product, accounting for about 28 % of the electron flow (Chapter 6). Methanogenesis was rather insignificant, as it consumed less than 1 % of the electron flow (Chapter 6).

The second UASB reactor (UASB II; which is the UASB A described in Chapter 4 operating at 55°C) was inoculated with a different sludge in order to assess the influence of the inoculum on potential antagonists of sodium toxicity. In contrast to UASB I, full methanol removal was achieved in UASB II (Chapter 4). Sulfide production accounted for about 74 % of the electron flow in UASB II, whereas methane and acetate were a secondary product, accounting for, respectively, 17 % and 9% of the electron flow (Chapter 4).

Sulfide was determined photometrically as described by Trüper and Schlegel (1964). Methanol, VFA and methane were measured by gas chromatography (GC), as described by Weijma *et al.* (2000).

Table 1. Antagonists of sodium toxicity used in this study.

Reactor	Antagonist used	Formula	Conditions and concentrations
UASB I	Betaine	$C_5H_{11}NO_2$	10 mM
	Glutamate	$C_5H_9NO_4$	10 mM
	Ectoine	$C_6H_{10}N_2O_2$	10 mM
	Choline	$C_5H_{14}NOCl$	10 mM
	Mixture of cations (K^+ and Mg^{++})	KCl and $MgCl_2$	40 mM of K^+ and 26 mM of Mg^{++} (according to Muthumbi *et al.*, 2001)
UASB II	Potassium (in place of sodium as salt inhibitor)	KCl	9.8 $g.L^{-1}$ K^+ (250 mM), as also 9.8 g $Na^+.L^{1-}$ applied when 25 g NaCl. L^{-1}
	Mixture of compatible solutes (betaine, glutamate and choline)	$C_5H_{11}NO_2$ $C_5H_9NO_4$ $C_5H_{14}NOCl$	2.5 mM of each compatible solute
	Mixture of cations (K^+ and Mg^{++})	KCl and $MgCl_2$	40 mM of K^+ and 26 mM of Mg^{++} (according to Muthumbi *et al.*, 2001)

RESULTS AND DISCUSSION

High NaCl concentrations (25 $g.L^{-1}$) exerted a strong effect on the methanol depletion rate, as evidenced by the considerable decrease in the methanol depletion rate for the sludges cultivated for both UASB I and UASB II (Tables 2 and 3) and a lag phase of approximately 3 and 7 days for the sludges cultivated in, respectively, UASB I and UASB II (Figs. 1A and 1B). This confirms previous experiments (Chapter 5). The 7 days lag phase and the only 2.5 fold decrease in the methanol depletion rate by UASB A sludge in the presence of 25 g $NaCl.L^{-1}$ suggest that microorganisms able to degrade methanol at high salinity were present in the sludge bed. Thus, bioreactors aiming methanol removal could be successfully operated at high salinity, provided that enough time is given to the biomass to adapt to the high osmolarity environment. This was also suggested by various authors as a successful strategy to treat wastewaters with high salinity (Soto *et al.,* 1993; Omil *et al.,* 1996). However, results in Chapter 5 shows that no methanol removal was observed for more than 15 days when starting-up an UASB reactor with an influent NaCl concentration of 25 $g.L^{-1}$. Moreover, a stepwise exposure of the sludge to increasing salt concentrations did not favor the development of a halotolerant sulfate reducing sludge (Chapter 6). The purpose of screening possible compatible solutes able to counteract the deleterious effect of sodium was to find an alternative way to the time-consuming (and non-successful) adaptation of the biomass to high influent salinity.

In addition to the sharp decrease in the methanol degradation rates, the presence of 25 g $NaCl.L^{-1}$ strongly affected the methanol degradation pathway of the sludges cultivated in both reactors (Tables 2 and 3). NaCl clearly increased acetate production at the expense of sulfide (for UASB I) and methane (for UASB II) production. Thus, the methane producing archaea (MPA) and SRB appear to exhibit a greater sensitivity to the toxic effects of sodium than the acidogens. This is in agreement with previous work, where the greater sensitivity of MPA versus acid-forming bacteria to most other environmental conditions, including cation toxicity, was demonstrated (Kugelman and McCarty, 1965).

The 22 % decrease in the methanol depletion rate due to the addition of 18.6 g $KCl.L^{-1}$ (9.8 g $K^{+}.L^{-1}$) compared with the 58 % decrease due to the addition of 25 g $NaCl.L^{-1}$ (9.8 g $Na^{+}.L^{-1}$) suggests that sodium was more toxic than potassium to the microorganisms of UASB II sludge (Table 3). As such, the addition of potassium salts instead of sodium salts to bioreactors (e.g. as an alkalinizing agent) may diminish the deleterious effects of high salinity to the performance of bioreactors. However, this contrasts with results of Kugelman and McCarty (1965), who reported the following increasing order of salt toxicity towards acetate-utilizing methanogens (on a molar basis): sodium, ammonium, potassium, calcium and magnesium. In addition, potassium has been shown to inhibit acetoclastic methanogenesis at concentrations above 3.8 $g.L^{-1}$ (van den Berg *et al.*, 1976). Therefore, the lower addition of

potassium compared to sodium (250 mM of K^+ when adding 9.8 g $K^+.L^{-1}$ vs. 430 mM of Na^+ when adding 9.8 g $Na^+.L^{-1}$) is probably the reason for the decreased negative effect on the methanol depletion rate when replacing sodium by potassium (Table 3).

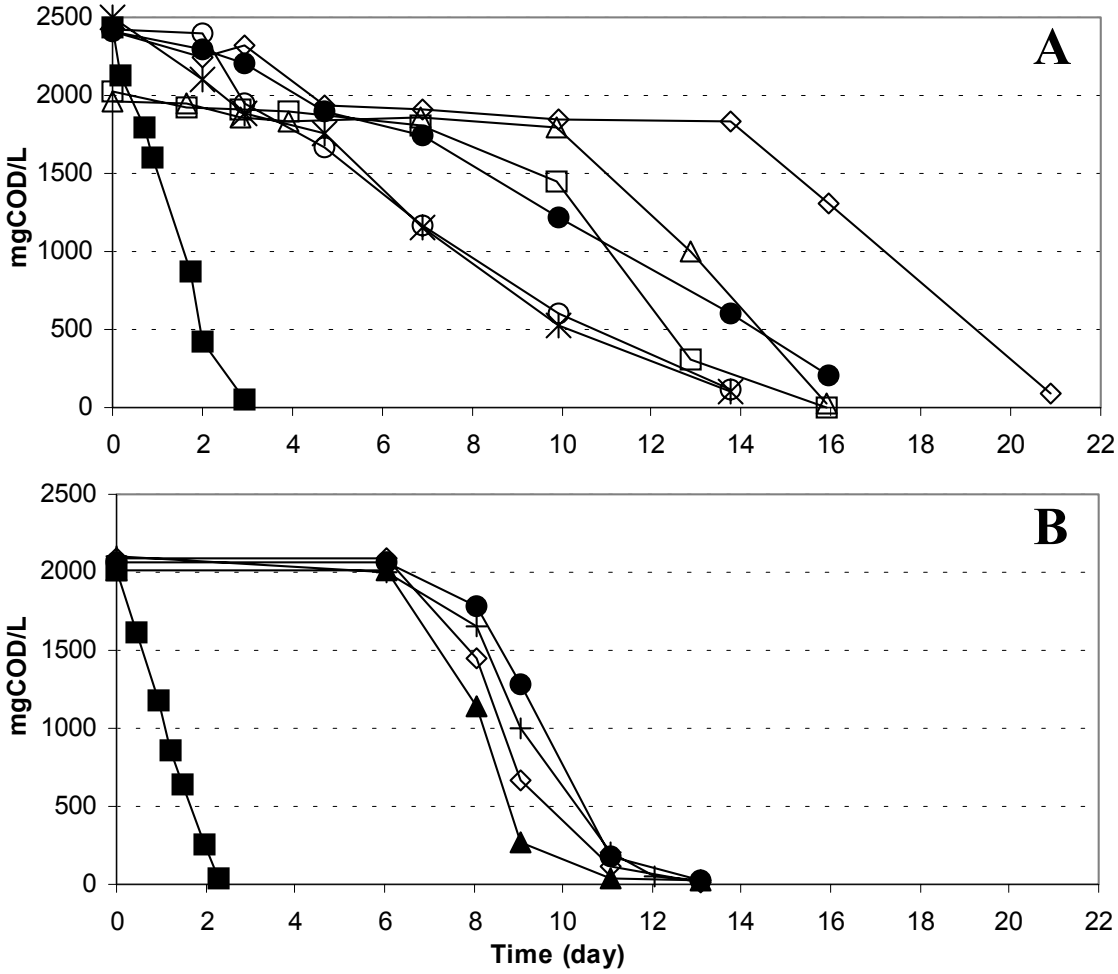

Figure 1. Evolution of the methanol depletion during activity assays with sludge harvested from UASB I (A) and UASB II (B). Vials inoculated without (■) and with 25 g $NaCl.L^{-1}$ and in the absence (•) and presence of potential antagonists of sodium toxicity: betaine (o), glutamate (∗), K^+ and Mg^{++} (◊), ectoine (□), choline (△), mixture of betaine, glutamate and choline (+) and K^+ as toxic agent (▲).

The addition of different osmoprotectants, viz. glutamate, betaine, ectoine, choline, a mixture of compatible solutes and K^+ and Mg^{2+}, slightly increased the methanol depletion rate for both sludges investigated (Tables 2 and 3). This was due to the increased activity of the homoacetogenic bacteria (AB), as the methanol breakdown was steered to the formation of acetate (Fig. 2 and Tables 2 and 3). In contrast, sulfide production was not stimulated by any of the osmoprotectants tested in this work (Fig. 2 and Tables 2 and 3). The addition of K^+ and Mg^{2+} yielded the highest sulfide production for the sludge harvested from UASB I (Fig. 2F and Table 2), although sulfide production was still lower compared to the control vials containing no added NaCl (Table 2). Moreover, K^+ and Mg^{2+} did not stimulate sulfate

reduction in UASB II sludge, where sulfide production accounted for less than 4 % of the electron flow (Table 3). Therefore, the compatible solutes used in this work were not effective as osmoprotectants to alleviate the acute NaCl toxicity on sulfate reducing granular sludges developed in thermophilic (55ºC) UASB reactors.

Table 2. Methanol depletion rates and electron flow share of the different methanol degradation products for the sludge harvested from UASB I.

Applied condition	Methanol depletion rate (mg COD.L^{-1}.day^{-1})	Electron flow share (%)		
		Methane	Sulfide	Acetate
No NaCl amended	710	22	40	38
25 g NaCl.L^{-1}	116	27	16	57
25 g NaCl.L^{-1} + Betaine	178	28	6	66
25 g NaCl.L^{-1} + Glutamate	148	32	10	58
25 g NaCl.L^{-1} + Ectoine	238	18	18	64
25 g NaCl.L^{-1} + Choline	278	11	11	78
25 g NaCl.L^{-1} + K^+ + Mg^{2+}	161	8	30	62

Table 3. Methanol depletion rates and electron flow share of the different methanol degradation products for the sludge harvested from UASB II.

Applied condition	Methanol depletion rate (mg COD.L^{-1}.day^{-1})	Electron flow share (%)		
		Methane	Sulfide	Acetate
No NaCl amended	610	37	53	10
25 g NaCl.L^{-1}	259	14	4	82
18.6 g KCl.L^{-1}	488	4	4	92
25 g NaCl.L^{-1} + K^+ + Mg^{2+}	292	10	4	83
25 g NaCl.L^{-1} + Betaine + Glutamate + Choline	291	27	2	71

The results found in this study contrast with the fact that sulfate reducers are reported to use compatible solutes as osmoprotectants. For instance, Welsh *et al.* (1996) demonstrated that the uptake and accumulation of K^+ and betaine from the growth medium alleviated the inhibition due to increasing NaCl concentration (up to 7%) for the halotolerant sulfate reducer *Desulfovibrio halophilus.* It may be that the organic compatible solutes used in this work were degraded by the anaerobic consortium prior to their accumulation in the cytoplasm. Indeed, the mass balance calculations did not fit for the bottles supplemented with betaine (Fig. 2B), glutamate (Fig. 2C) and choline (Fig. 2E), indicating that these organic compatible solutes were degraded via acetate as an intermediate. The anaerobic degradation of betaine (Thalasso *et al.*, 1999), glutamate (Plugge *et al.*, 2001) and choline (Sharma and Erdman, 1989) are well described.

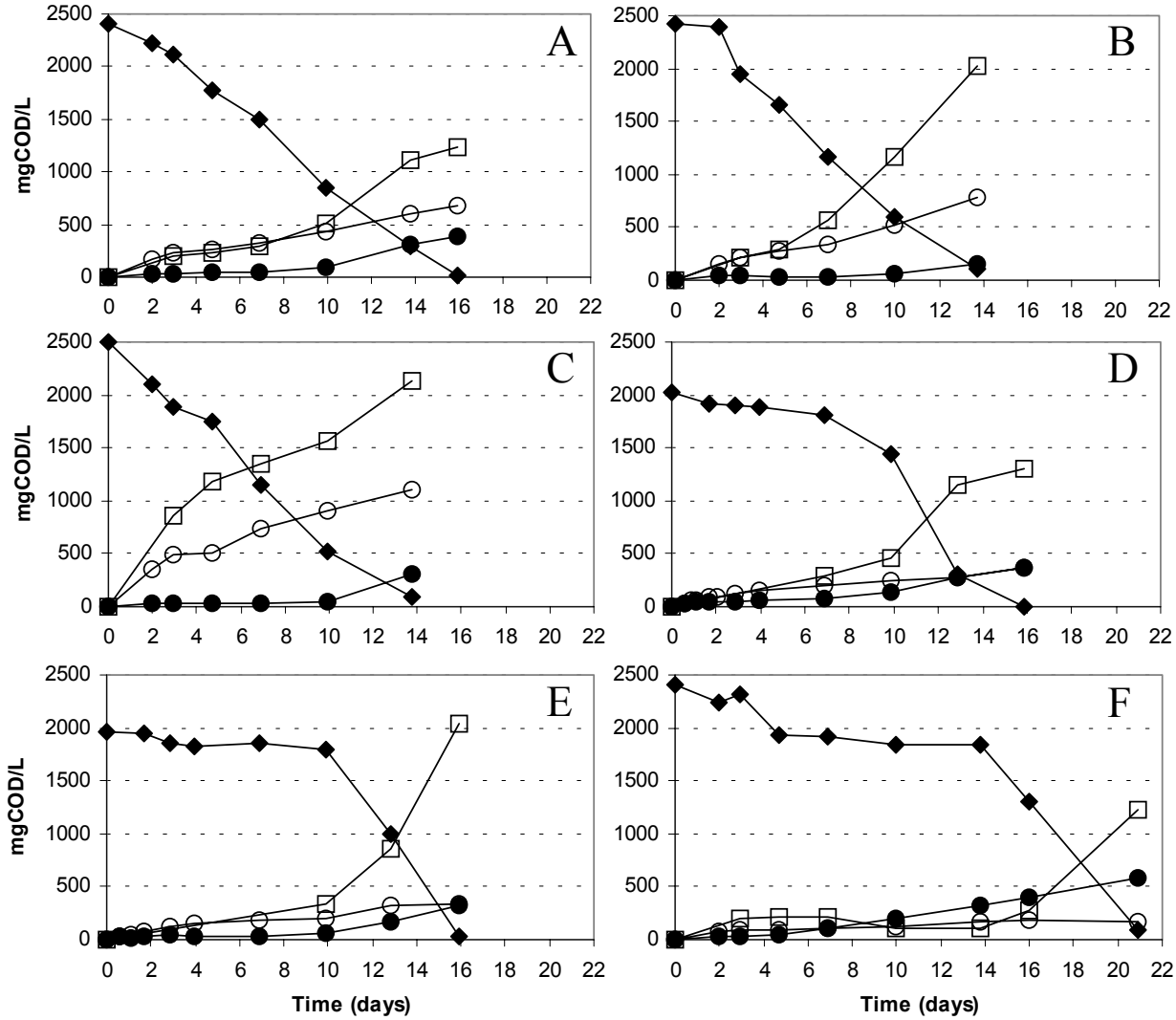

Figure 2. Evolution of methanol depletion (♦) and sulfide (•), acetate (□) and methane (o) formation during the activity test performed with the sludge harvested from UASB I with 25 g $NaCl.L^{-1}$ and in the absence (A) and presence of potential antagonists of sodium toxicity: betaine (B), glutamate (C), ectoine (D), choline (E) and K^+ and Mg^{++} (F).

The lack of osmoprotection observed in Fig. 1 implies that the tested compatible solutes are not the most prominent solutes accumulated by unadapted thermophilic sulfate reducing granular biomass used in these experiments. It might also be due to the fact that thermophilic microorganisms use different compatible solutes compared to their mesophilic counterparts. Indeed, the absence of glycine betaine and ectoine in extreme termophiles indicates that some common compatible solutes of mesophiles cannot be used by organisms that grow at high temperatures (Santos and Costa, 2001). The latter authors pondered that the compatible solutes of mesophiles are unstable at higher temperatures or do not meet the requirements of the organisms for osmotic adaptation and thermoprotection of macromolecules. As a matter of fact, there are reports pointing out that evolutionary pressures selecting for or against the accumulation of a specific compatible solute may not only depend

on its osmotic function, but also on secondary functions, such as heat or cold tolerance (Sleator and Hill, 2001; Ko *et al.*, 1994). However, Proctor *et al.* (1997) demonstrated that, upon an exposure to a mineral salt medium containing from 0.1 to 0.8 M NaCl, the thermophilic *Methanosarcina thermophila* TM-1 accumulated betaine within 10 minutes in concentrations up to 140 times of that encountered prior to salt exposure. Thus, it remains unclear if the osmoprotectants widely used by mesophiles are also used by thermophilic microorganisms.

Future research will also be oriented on the introduction of the so-called extremophiles (halophiles) in the sludge for the treatment of saline sulfate-rich waste streams. To the best of our knowledge, there are so far no reports on the successful immobilization of such halophilic SRB in bioreactor sludges. Such an approach will become essential, as it would push the current operation limits of bioreactors to very interesting working conditions, as when applying SRB based bioreactors in closed water cycles. Strategies to apply halophilic SRB in the treatment of saline sulfate-rich wastewaters (granular sludge bed engineering) are being developed.

CONCLUSIONS

The results obtained in this research allow to conclude that:

(1) High NaCl concentrations (25 $g.L^{-1}$) exerted a strong acute toxic effect on the methanol depletion rate, as evidenced by the considerable decrease in the methanol depletion rates for sludges harvested from lab-scale sulfate reducing UASB reactors.

(2) High NaCl concentrations (25 $g.L^{-1}$) strongly affected the fate of methanol degradation, with clear increase in acetate production at the expense of sulfide and methane production.

(3) The acceleration in the methanol uptake rate due to the addition of compatibles solutes in general favored the homoacetogenic bacteria, as the methanol (or the organic osmoprotectant) breakdown was steered to the formation of acetate.

(4) The compatible solutes used in this work were not effective as osmoprotectants to alleviate the acute NaCl toxicity on sulfate reducing granular sludges developed in thermophilic (55ºC) UASB reactors.

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Chapter 8

High rate sulfate reduction at high salinity (up to 90 mS.cm^{-1}) in mesophilic UASB reactors

Sulfate reduction in salt rich wastewaters using unadapted granular sludge was investigated in 0.9 L UASB reactors (pH 7.0 ± 0.2; hydraulic retention time from 8 to 14 hours) fed with acetate, propionate or ethanol at organic loading rates up to 10 g COD.L^{-1}.day^{-1} and in excess of sulfate (COD/SO_4^{2-} of 0.5). High sulfate reduction rates (up to 3.7 g SO_4^{2-}.L^{-1}.day^{-1}) were achieved at salt concentrations exceeding 50 g NaCl.L^{-1} and 1 g $MgCl_2$.L^{-1} (60-70 mS.cm^{-1}). Considerable sulfate reduction still proceeded at a rate of 1.40 g SO_4^{2-}.L^{-1}.day^{-1} at salt concentrations of up to 70 g NaCl.L^{-1} and 1 g $MgCl_2$.L^{-1} (corresponding to a conductivity of about 85 to 90 mS.cm^{-1}). Ethanol as well as propionate were suitable substrates for sulfate reduction with acetate and sulfide as the end products. The successful high rate treatment was due to the proliferation of a halotolerant incomplete oxidizing SRB population present in the unadapted inoculum sludge. Bioaugmentation of this sludge via the entrapment or immobilization of the acetate oxidizing halotolerant SRB *Desulfobacter halotolerans* was unsuccessful, as the strain washed out from the UASB reactor without colonizing the UASB granules.

A modified version of this chapter was submitted to Biotechnology & Bioengineering.

INTRODUCTION

The increasing adoption of water re-use strategies in industries leads to drastic changes in the wastewater strength and composition. Deliberate closing of water loops not only increases the pollutants concentration but also leads to a build up of sodium salts resulting in an increase in ionic strength. The latter cause osmotic stress to bacterial cells and/or inhibition of reaction pathways in the substrate degradation process (Pollice *et al.*, 2000). High salt concentrations are known to significantly reduce the treatment efficiency of conventional activated sludge (Kargi and Uygur, 1996), nitrification (Campos *et al.*, 2002; Panswad and Anan, 1999), denitrification (Dahl *et al.*, 1997; Glass and Silverstein, 1999), biological phosphorus removal (Intrasungkha *et al.*, 1999) and mesophilic (Guerrero *et al.*, 1997; Rinzema *et al.*, 1988) as well as thermophilic (Chapter 3, 4 and 5; Willets *et al.*, 2000) anaerobic processes. As a consequence, treatment systems that are tolerant to high salt concentrations must be developed in order to efficiently treat this type of (waste)waters to a quality allowing their final discharge or enabling their reuse in a given industrial process.

The desulfurization of flue gases is an example where a sulfur-rich saline (5-40 g $Cl.L^{-1}$) wastewater must be treated (Vredenbregt *et al.*, 1997). In addition, trends in the application of zero discharge concepts in flue gas desulfurization systems may result in chloride accumulation at concentrations as high as 80 $g.L^{-1}$ (Vredenbregt, personal communication). As a result of closed water loops, certain chemical factories generate sulfate rich (10 $g.L^{-1}$) flows with salinities as high as 105 $g.L^{-1}$ of total salts, which poses difficulties for the treatment of the water to a quality that can be returned to the process (Akzo-Nobel, personal communication). Tanning (Tadesse *et al.*, 2003) and seafood industries (Omil *et al.*, 1995) also generate large quantities of saline wastewater rich in sulfate and oxidized sulfur compounds.

The effect of sodium on the methanization of seafood wastewater has been extensively studied (Feijoo *et al.*, 1995; Gharsallah *et al.*, 2002; Guerrero *et al.*, 1997). These authors showed that mesophilic and thermophilic (up to 55ºC) high-rate methanogenic treatment of seafood wastewater proceeds successfully at NaCl concentrations ranging from 15 to 25 $g.L^{-1}$. In contrast to these methanogenic reactors, it has been found that sodium salts hinder thermophilic sulfate reducing processes with methanol as the substrate and a 50 % inhibition concentration (IC_{50}) was observed at about 9.3 g $NaCl.L^{-1}$ (Chapters 5 and 6). Addition of 25 g $NaCl.L^{-1}$ even completely inhibited methanol degradation in a methanol fed (COD/SO_4^{2-} of 0.5) thermophilic (55ºC) upflow anaerobic sludge bed (UASB) reactor (Chapter 5).

The easiest way to guarantee the activity of the sulfate reducing bacteria (SRB) present in granular sludges would be the dilution of the sulfate rich waste stream to a sufficiently low salinity. As this strategy is impossible in case of water shortage or high salinity, bioreactors using salt tolerant sludges/microorganisms need to be developed. Hence,

bioaugmentation of sludges via the entrapment and/or immobilization with specific halotolerant microorganisms may hold a promise in the treatment of saline wastewaters. This approach has already been successfully applied in the aerobic treatment of high saline wastewaters (Kargi and Uygur, 1996; Kargi and Dincer, 2000). As several halotolerant SRB have recently been isolated (Orus, 2002; Lens *et al.*, 2002), this approach would be possible for sulfate reducing reactors as well. The highest salinity at which complete oxidation via sulfate reduction occurs is around 13 % for the ethanol and acetate oxidizer *Desulfobacter halotolerans* (Brandt and Ingvorsen, 1997). The incorporation of halophilic SRB into granular sludges could extend the application of sulfate reducing systems to wastewaters that can currently not be treated biologically.

The aim of this work was to assess (1) whether VFA and ethanol halotolerant SRB were present in the granular sludge inoculum used in this work and (2) the possibility to entrap and/or immobilize (and grow) *Desulfobacter halotolerans* in this granular sludge. In addition, this work assessed the influence of the start up in flow through mode compared with the start up in batch mode (for 9 days) on the performance of sulfate reducing UASB reactors (without the inoculation of *D. halotolerans*) under saline conditions. The research was carried out under mesophilic (30°C) conditions in three sulfidogenic UASB reactors (COD/SO_4^{2-} of 0.5) fed with either acetate, propionate or ethanol as electron donors. The performance of the lab-scale UASB reactors was monitored as a function of the influent salinity and hydraulic retention time. The metabolic characteristics of the sludges were determined as well through activity tests.

MATERIAL AND METHODS

Continuous experiments

Experimental setup

Three lab-scale UASB reactors with a volume of 0.9 liter (30 cm high; internal diameter of 6.5 cm), described in detail in Chapter 3, were operated during 188 (UASB A), 103 (UASB B) and 50 (UASB C) days in order to study the performance of sulfate reduction at high salinity in UASB reactors.

The synthetic influent was provided by a peristaltic pump (Gilson Minipuls 2, Middleton, Wisconsin, USA). Recirculation of the effluent was applied using peristaltic pumps (Watson Marlow 505 S, Falthmouth, Cornwall, UK) to reach an upflow velocity of 1 $m.h^{-1}$. The pH was measured with a sulfide-resistant pH-electrode (Hamilton-Flushtrode, Hilkomij BV, Sliedrecht, the Netherlands) connected to a pH controller equipped with two changeable set points (Electronic workshop, Wageningen University, the Netherlands). The

pH of the reactors was controlled at pH 7.0 ± 0.2 by adding NaOH or HCl solution (0.05 M), except for the first 19 days in UASB A when the pH was only monitored, but not controlled. The biogas produced in all reactors was led through a 3 % NaOH solution and a column of soda lime pellets to remove the CO_2 and H_2S from the produced gas. The amount of biogas produced was measured using a wet test gas meter (Schlumberger Industries, Dordrecht, the Netherlands). To maintain the temperature at 30°C, the reactors were placed in a temperature-controlled waterbath (Julabo Labortechnik GmbH, Seelbach, Germany). Sampling ports were placed in the influent tube system, on top of the reactor and in the biogas collection system in order to obtain samples of the influent, effluent and biogas, respectively.

Inoculum

Granular anaerobic sludge was obtained from a full scale UASB reactor treating paper mill wastewater (Industriewater Eerbeek NV, Eerbeek, the Netherlands). Each of the three UASB reactors was supplemented with about 400 g wet granular sludge with a volatile suspended solids (VSS) content of about 8 % .

UASB A was inoculated 44 days after its start-up with the halotolerant bacterium *Desulfobacter halotolerans*. *Desulfobacter halotolerans* strain GSL-Ac1, kindly provided by Prof. Ingvorsen (Aarhus University, Denmark), was isolated from hypersaline sediments in the southern arm of Great Salt Lake (Utah, USA). Strain GSL-Ac1 uses acetate, ethanol and pyruvate as electron donor and carbon source. It is able to reduce sulfate, sulfite and thiosulfate at high salinity (up to 13% NaCl and 4.5% $MgCl{\cdot}6H_2O$), but grows optimally around 1-2% NaCl (Brandt and Ingvorsen, 1997).

Substrate and medium

Throughout the experiment, a mixture of acetate and propionate (UASB A, UASB B, UASB C) and ethanol (UASB B) was used as electron donor and carbon source, providing an influent COD concentration of about 2 $g.L^{-1}$ (Table I). All the reactors were fed with sulfate, added as sodium sulfate at a COD/sulfate ratio of 0.5 (g COD per g SO_4^{2-}). In addition, basal medium containing macro and micro nutrients was supplied to the influent at a ratio of 2.22 ml per g COD fed. Basal medium was prepared as described in Chapter 3 and a trace element (4.5 $mL.L^{-1}$) solution was prepared according to Zehnder *et al.* (1980). Both the basal medium and substrate stock solutions were prepared using demineralized water.

Table 1. Summary of the operational parameters applied to the UASB reactors.

UASB A	**Flow-through/ low salinity**	**Flow-through/ high salinity**	***D.halotolerans*** **/ batch mode**	**Flow-through/ high salinity**	**Flow-through/ high salinity**
Days	0 – 27	28 - 43	44 – 57	58 – 150	151-188
Substrate (in $gCOD.L^{-1}$)	Acet. (1) Prop. (1)	Acet. (1) Prop. (1)	Acet. (1) Prop. (1)	Acet. (1) Prop. (0.7-2.5)	Prop. (2)
NaCl ($g.L^{-1}$)*	0	25	50	50	50-70
Conductivity	7.6 ± 0.5	44.0 ± 13.4	67.0 ± 2.4	66.5 ± 9.4	80.4 ± 5.6
Influent flow ($L.day^{-1}$)	2.5 ± 0.3	3.9 ± 0.4	0	2.3 ± 0.3	2.1 ± 0.1
HRT (hour)	8.8 ± 0.9	5.6 ± 0.5	∝	9.7 ± 1.0	10.4 ± 0.4
OLR ($gCOD.L^{-1}.day^{-1}$)	5.2 ± 0.6	9.5 ± 1.0	/	7.1 ± 1.5	5.7 ± 1.6
SLR ($gSO_4^{2-}.L^{-1}.day^{-1}$)	11.6 ± 0.8	22.1 ± 1.2	/	11.0 ± 1.6	10.1 ± 1.6
COD/SO_4^{2-}	0.46 ± 0.1	0.44 ± .14	0.52	0.64 ± 0.1	0.57 ± 0.1
pH	7.5 ± 0.3	6.9 ± 0.1	7.0 ± 0.1	7.0 ± 0.2	7.1 ± 0.1

UASB B	**Batch mode/ high salinity**	**Flow-through/ high salinity**	***D.halotolerans*** **/ batch mode**	**Flow-through/ high salinity**	**UASB C**
Days	0 – 9	10 – 49	50 – 84	85 – 103	0 – 50
Substrate (in $gCOD.L^{-1}$)	Acet. (1) Prop. (2.5)	Acet. (1) Prop. (2.5)	Prop. (1) EOH (1)	EOH (2)	Acet. (1) Prop. (2)
NaCl ($g.L^{-1}$)*	50	50	50	50	50
Conductivity	61.9 ± 1.3	70.5 ± 1.1	67.4 ± 2.6	64.2 ± 3.0	72.6 ± 1.3
Influent flow ($L.day^{-1}$)	0	2.6 ± 0.2	2.5 ± 0.1	2.5 ± 0.1	1.8 ± 0.3
HRT (hour)	∝	8.6 ± 0.3	8.7 ± 0.2	8.9 ± 0.2	11.6 ± 1.8
OLR ($gCOD.L^{-1}.day^{-1}$)	/	10.0 ± 1.7	5.3 ± 1.7	4.8 ± 1.0	6.2 ± 1.9
SLR ($gSO_4^{2-}.L^{-1}.day^{-1}$)	/	21.2 ± 2.1	11.8 ± 1.2	9.9 ± 1.3	12.1 ± 2.4
COD/SO_4^{2-}	0.50	0.48 ± 0.1	0.45 ± 0.1	0.49 ± 0.1	0.49 ± 0.1
pH	7.4 ± 0.6	7.1 ± 0.1	7.1 ± 0.1	7.2 ± 0.1	7.0 ± 0.1

*1 g $MgCl_2.6H_2O.L^{-1}$ applied throughout the experiment (except between days 0 to 27 in UASB A).
/ = Not applicable; Acet = acetate; Prop. = propionate; HRT = hydraulic retention time; OLR = organic loading rate; COD = chemical organic demand; SRL = sulfate loading rate.

Experimental design

Table 1 summarizes the different operational conditions applied to the three UASB reactors. UASB A was operated for 188 days and fed with a mixture of acetate (1 g $COD.L^{-1}$), propionate (1 g $COD.L^{-1}$), sulfate and different influent salt concentrations, starting at low salinity (7.6 ± 0.5 $mS.cm^{-1}$) in the first 27 days of operation. On day 27, the influent salt concentration was increased to 25 g $NaCl.L^{-1}$ and 1 g $MgCl_2.6H_2O.L^{-1}$. On day 44, the influent salt concentration was further increased to 50 g $NaCl.L^{-1}$ and 1 g $MgCl_2.6H_2O.L^{-1}$

(conductivity between 65 to 70 $mS.cm^{-1}$). The reactor was operated in batch mode for 14 days (till day 57) after the addition of 50 mL of a pure culture of *D. halotolerans*. From day 58 onwards the continuous operation of UASB A was resumed. On day 70, the propionate concentration in the influent was further increased to 2 $gCOD.L^{-1}$. In order to confirm that propionate was the substrate of the SRB, the influent propionate concentration was decreased to about 0.7 g $COD.L^{-1}$ between days 97 and 106. Acetate was omitted from the influent from day 151 onwards. From day 151, the salinity was increased to a concentration exceeding 70 g $NaCl.L^{-1}$ on day 179, resulting in a conductivity of 90 $mS.cm^{-1}$. The pH was kept at values around 7 throughout the experimental run, except for the first 19 days when the pH varied between 7.4 and 7.8 and between days 100 to 110, when the pH increased to values of about 7.8.

UASB B and UASB C were operated without inoculation with *D. halotolerans* in order to investigate the presence of a halotolerant sulfate reducing population in the inoculum sludge and to determine whether running the reactor in batch mode stimulated the growth of indigenous halotolerant SRB or not, as observed when operating UASB A in batch mode. UASB B started up in batch mode for 9 days, whereas UASB C started up in flow through mode. UASB B and UASB C were operated for, respectively, 103 and 50 days with an influent salt concentration of 50 g $NaCl.L^{-1}$ and 1 g $MgCl_2.6H_2O.L^{-1}$, corresponding to a conductivity of about 70 to 75 $mS.cm^{-1}$ throughout the experiment.

UASB B was fed with 1 g $COD.L^{-1}$ acetate and 2.5 g $COD.L^{-1}$ propionate during the first 49 days. From day 50 onwards, ethanol (1 g $COD.L^{-1}$) replaced acetate as the substrate in the influent and the propionate concentration was decreased to 1 g $COD.L^{-1}$. On day 85, propionate was omitted from the influent and the ethanol concentration was increased to 2 g $COD.L^{-1}$. The OLR was about 10 g $COD.L^{-1}.day^{-1}$ in the first 51 days of the experiment and decreased to about 5 g $COD.L^{-1}.day^{-1}$ from day 52 onwards. The pH was kept at around 7 throughout the experimental run, except for two pH shocks, one of 7.6 on day 30 and another exceeding pH 8 on day 86.

UASB C was fed with a mixture of acetate (1 g $COD.L^{-1}$), propionate (2 g $COD.L^{-1}$) and started-up at a HRT of about 14 hours resulting in an OLR of about 5.0 g $COD.L^{-1}.day^{-1}$. In order to determine the maximal sulfate loading rate, the HRT was decreased till a minimum of 8 hours, resulting in an increase of the OLR up to 8.0 g $COD.L^{-1}.day^{-1}$. The pH was kept at around 7 throughout the experimental run.

Batch experiments

Complementary batch experiments were performed to assess the metabolic characteristics of the sludges with different electron donors and at low and high salinity. Batch experiments were performed, as described in Chapter 3, with Eerbeek sludge used as

inoculum, a pure culture of *Desulfobacter halotolerans* and sludge sampled from UASB A on days 43 and 80. The sludge used in the batch experiments was rinsed three times with basal medium prior to inoculation in order to remove substrate traces. Batch experiments were performed in duplicate at 30°C in 117 ml serum bottles containing 50 ml of basal medium, about 2 $g.L^{-1}$ volatile suspended solids (VSS) and 2 g $COD.L^{-1}$ of the selected substrate: acetate, propionate, ethanol or methanol. When 1.6 bar of H_2/CO_2 (80/20%) was supplemented, the amount of bicarbonate was recalculated to adjust the pH to 7. Experiments performed at high salinity contained 50 g $NaCl.L^{-1}$ and 1 g $MgCl_2.6H_2O.L^{-1}$. In order to assess the ability of indigenous SRB to use acetate as electron donor, methanogenic activity was suppressed in selected vials with 30 mM of sodium 2-bromoethanesulfonate (BES), a specific inhibitor of methanogenic archaea (Oremland and Capone, 1988).

Experiments using a pure culture of *Desulfobacter halotolerans* were performed in autoclaved (30 minutes at 121ºC) basal medium. The basal medium differed from the other batch experiments in that it was further supplemented with a 1 ml vitamin solution according to Stams *et al.* (1993) and buffered at pH 7.0 using 4 $g.L^{-1}$ $NaHCO_3$ and 1.6 bar of N_2/CO_2 (80/20%). An inoculum size of 5% (vol/vol) was used and tests were performed in duplicate.

Analysis and chemicals

The gas composition was measured on a gas chromatograph (Hewlett Packard HP 5890, Palo Alto, USA) according to Weijma *et al.* (2000). Volatile fatty acids (VFA) and alcohols were analyzed on a gas chromatograph (Hewlett Packard HP 5890A, Palo Alto, USA) according to Weijma *et al.* (2000). Sulfide was measured according to Trüper and Schlegel (1964). TSS and VSS were analyzed according to standard methods (APHA, 1995). The electrical conductivity (EC) was measured using a standard EC meter (WTW LF 196, Weilheim, Germany). Sulfate was measured on a DX-600 IC system (Dionex Corporation, Salt Lake City, USA). All chemicals used were of analytical grade and purchased from Merck (Darmstadt, Germany).

RESULTS

Effect of high NaCl concentrations on the performance of UASB A

When operated at fresh water salinity conditions (first 27 days; 7.6 $mS.cm^{-1}$), a complete acetate and propionate removal was achieved in UASB A (Fig 1B). The propionate removal efficiency dropped, however, to about 20% for a short period between day 11 and 15 (Fig. 1B). This sudden decrease was related to the decreased activity of the SRB, as evidenced by the drop in sulfide production during these days (Fig. 1C).

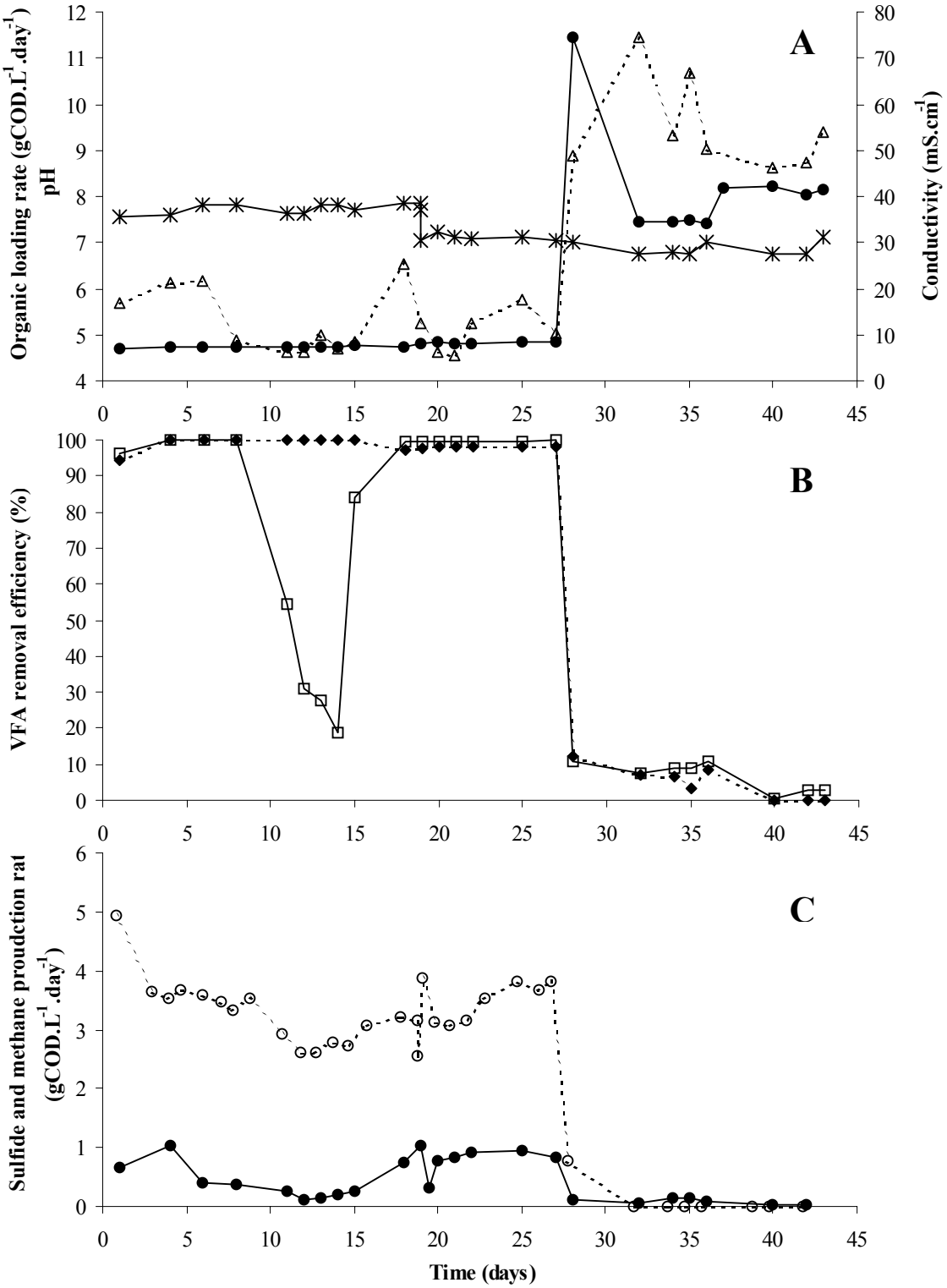

Figure 1. Process performance of UASB A in the first 43 days: (A) Evolution of the organic loading rate (•), pH (∗), and conductivity (△). (B) Evolution of the removals of acetate (♦) and propionate (□). (C) Evolution of the COD conversion rates to methane (o) and sulfide (•).

The slight drop in methane production during days 11 and 15 was probably the result of the decreased propionate conversion to acetate by the SRB, as the acetate consumption was

nearly complete (Fig. 2A). Table 2 shows that acetate was mainly converted by methanogens present in the sludge, as the amount of sulfide produced was similar to that in the controls without external substrate. Moreover, as acetate was not converted in the presence of 30 mM BES, it is obvious acetate was exclusively consumed by methanogens (Table 2). About 50 % of the propionate was converted via sulfate reduction (Table 2).

Table 2. Maximal specific activity of the inoculum sludge with acetate or propionate as the substrate and sulfate in a COD/SO_4^{2-} of 0.5. The experiments were performed at 30°C and pH 7 and at low salinity.

Substrate	Inhibitor	Substrate depletion rate (mgCOD.gVSS^{-1}.day^{-1})	Methane production rate (mgCOD.gVSS^{-1}.day^{-1})	Sulfide production rate (mgCOD.gVSS^{-1}.day^{-1})
-	-	nd	30 ± 5	nd
Acetate	-	440 ± 10	160 ± 12	11 ± 2
Acetate	30 mM BES	0	nd	nd
Propionate	-	360 ± 10	98 ± 2	95 ± 5

nd - not determined; BES - sodium 2-bromoethanesulfonate

Upon increasing the salinity in the influent on day 27 to conductivities of around 44.0 mS.cm^{-1} (Fig 1A), the acetate and propionate removal efficiency decreased sharply to values below 10 % (Fig. 1B). The methane production decreased sharply and was absent from day 31 onwards (Fig. 1C). Although the sulfide production dropped sharply upon increasing the salinity, a small sulfide production (< 15 mg.L^{-1}) could still be observed between day 27 and 44 (Fig. 2C). To test the reversibility of the effect of the increased salinity on the activity, a batch experiment with sludge sampled from UASB A on day 43 was performed at low salinity. The specific methanogenic activity with acetate as the substrate amounted to only 40 ± 5 mg COD.gVSS^{-1}.day^{-1} (data not shown), corresponding to 25 % of the activity of the inoculum sludge (Table 2). The propionate consumption and sulfide production rates were less affected by the salt exposure and had decreased to 80 % of the initial rates of the inoculum (data not shown). These results indicate that methanogens were most affected by the increased salinity.

Effect of the inoculation of UASB A with Desulfobacter halotolerans at high salinity

Batch mode: After the reactor was inoculated with *D. halotolerans*, both the acetate and propionate concentration decreased within a few days when operating UASB A in batch mode (Fig. 2A). Surprisingly, propionate removal proceeded faster than acetate removal (Fig. 2A), despite the fact that *D. halotolerans* does not consume propionate (Brandt and

Ingvorsen, 1997). Indeed, feeding the *D. halotolerans* culture with a mixture of acetate and propionate confirmed the complete consumption of acetate and the absence of propionate conversion (data not shown). The increasing acetate accumulation from day 47 onwards indicates the growth of incomplete propionate oxidizers (Fig. 2A). Nevertheless, a net acetate removal occurred only after propionate was fully converted on day 53 (Fig. 2A). The sulfide concentration in the reactor increased rapidly on day 44, but did not increase any further after day 51 (Fig. 2B), in contrast to the oxidation of propionate and acetate after day 51 (Fig. 2A). This may be partially caused by the dilution of the reactor bulk with the acid solution supplied by the pH controller, the precipitation of metal sulfides and formation of gaseous H_2S. Methane production was not observed at a salinity of 50 g $NaCl.L^{-1}$ and 1 g $MgCl_2.L^{-1}$.

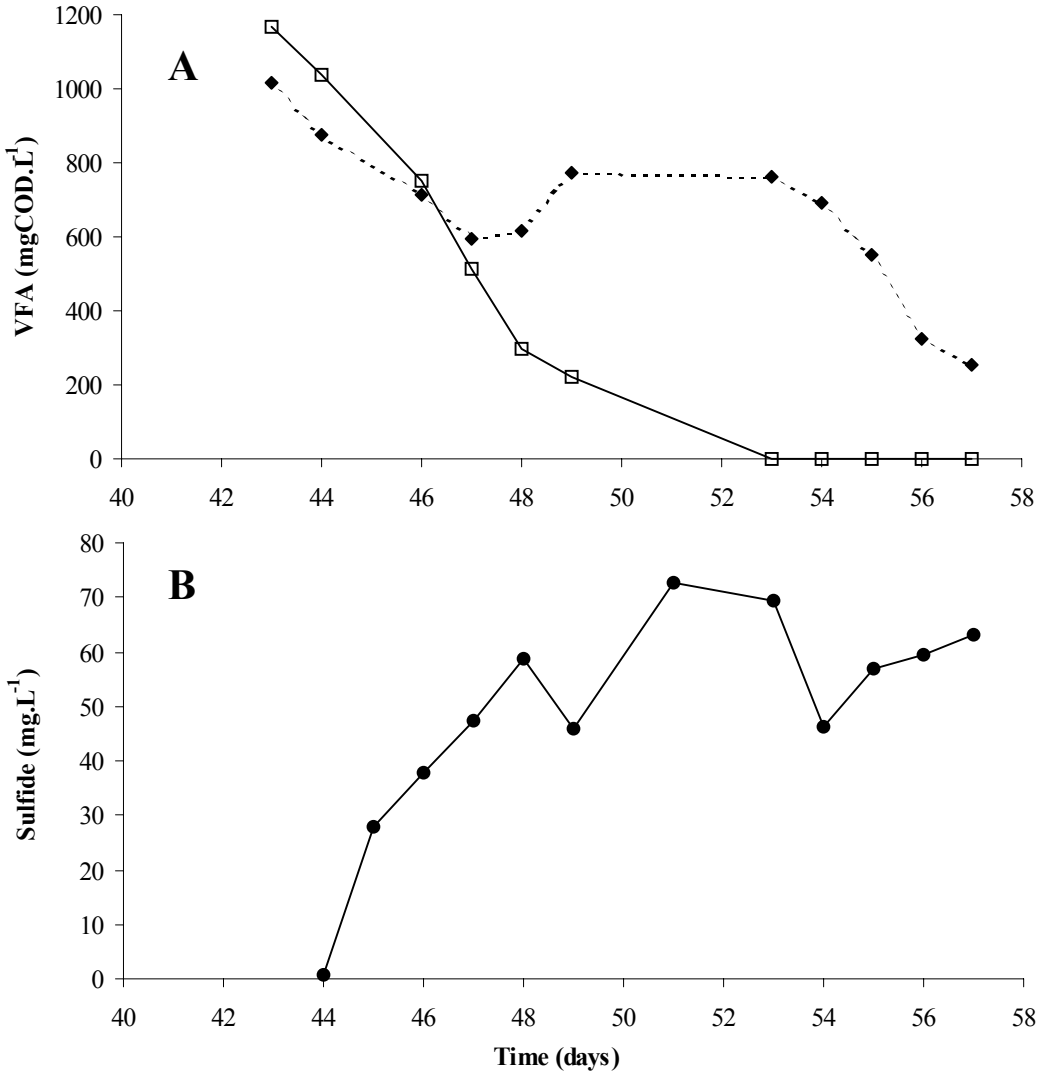

Figure 2. Process performance of UASB A between days 44 and 57 when operating in batch mode: (A) Evolution of the concentrations of propionate (□) and acetate (♦). (B) Evolution of the sulfide (●) concentration.

Flow through mode: The effluent acetate concentration was always higher than the influent acetate concentration (Fig. 3B) from day 58 onwards, when the continuous operation

of UASB A was resumed. On the other hand, a net propionate removal was observed from day 58 until the end of the experiment (Fig. 3C). The sulfide production steadily increased from day 58 onwards, reaching a concentration of about 200 $mg.L^{-1}$ on day 65 (Fig. 3D). After increasing the propionate concentration from 1200 $mgCOD.L^{-1}$ to about 2000 mg $COD.L^{-1}$ on day 70 (Fig. 3C), the sulfide production increased further to a maximum concentration of about 400 $mg.L^{-1}$ on day 96 (Fig. 3D), corresponding to a sulfate reduction rate exceeding 3.1 g $SO_4^{2-}.L^{-1}.day^{-1}$.

Effect of substrate limitation and pH shocks on sulfide production in UASB A operating at high salinity

The immediate drop in sulfide concentration (to about 250 $mg.L^{-1}$) after reducing the propionate concentration by 50 % indicates that acetate was not consumed by the SRB present in the reactor on day 97 (Fig. 3D). The decrease of the sulfide concentration clearly shows that propionate was the limiting substrate for sulfate reduction (Fig. 3D vs. Fig. 3C). The sulfide concentration further declined in the period following day 99 (Fig. 5C), which coincided with period of a elevated pH values (Fig. 3A). Apparently, the SRB population was rather sensitive to pH values higher than 7.6. The recovery of the sulfide production from day 109 onwards was rather slow. The sulfide production gradually increased to concentrations exceeding 400 $mg.L^{-1}$ on day 160 (Fig. 3D), corresponding to a sulfate reduction rate of 3.5 g $SO_4^{2-}.L^{-1}day^{-1}$ at a HRT of 10 hours. Omitting acetate from the influent on day 151 did not affect the sulfate reduction process (Fig. 3D). Results of batch experiments conducted with sludge harvested from UASB A on day 80 revealed the complete absence of acetate conversion (Table 3). Therefore, entrapment of *D. halotolerans* in the sludge appeared to be unsuccessful and an unknown halotolerant SRB present in the inoculum sludge proliferated in the reactor.

Assessing the upper salt concentration for sulfate reduction in UASB A

Sulfide production was not affected upon increasing the conductivity from 66 $mS.cm^{-1}$ (50 g $NaCl.L^{-1}$) to 75 $mS.cm^{-1}$ (60 g $NaCl.L^{-1}$), as shown in Fig. 3D. The sulfide concentration steadily declined upon changing the conductivity of the influent from 75 to 85 $mS.cm^{-1}$ on day 160 (Figs. 3A and 3D), corresponding to an influent NaCl concentration of 70 $g.L^{-1}$. The sulfide concentration gradually increased after decreasing the NaCl influent concentration back to 60 $g.L^{-1}$ (75 $mS.cm^{-1}$) on day 166 (Fig. 3D). Although the sulfide production could be maintained at about 200 $mg.L^{-1}$ for four days after increasing the NaCl back to concentrations exceeding 70 $g.L^{-1}$ (90 $mS.cm^{-1}$) on day 179, the sulfide production decreased slowly again. These results indicate that at 90 $mS.cm^{-1}$ (70 $g.l^{-1}$ NaCl) sulfate reduction is still possible, even though at lower rates.

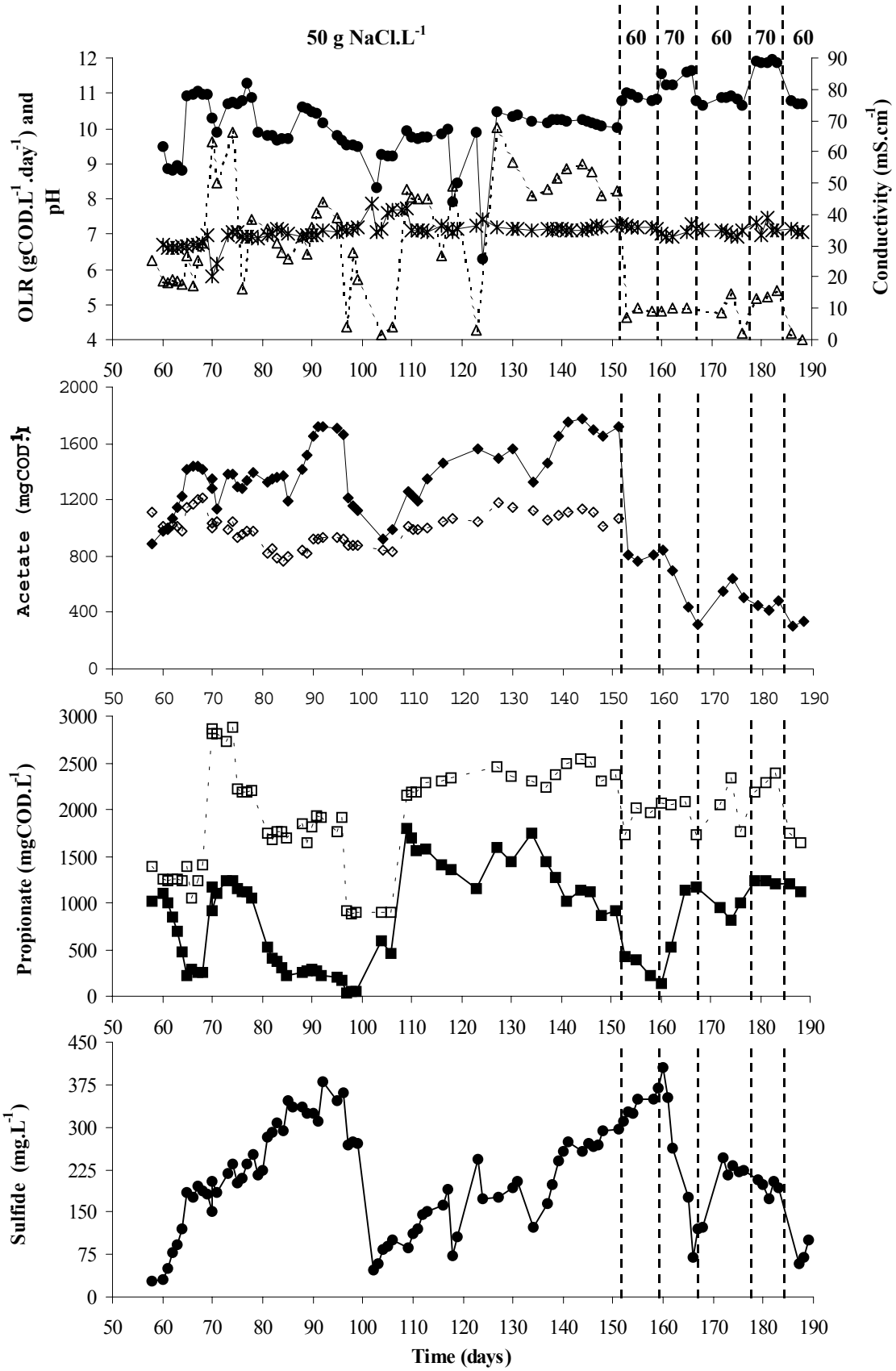

Figure 3. Process performance of UASB A between days 58 to 188: (A) Evolution of the organic loading rate (•), pH (∗), and conductivity (△). (B) Evolution of the concentrations of acetate in the influent (◊) and effluent (♦). (C) Evolution of the concentrations of propionate in the influent (□) and effluent (■). (D) Evolution of the sulfide (•) concentration.

Table 3. Sulfide production with alternative electron donors at high salinity (50 $g.L^{-1}$ NaCl and 1 $g.L^{-1}$ $MgCl_2.6H_2O$) at 30°C and pH 7, the COD/SO_4^{2-} was 0.5 and the sludge was sampled from UASB A on day 80.

Substrate	% substrate consumed	Sulfide production (mg $COD.L^{-1}$)
Propionate	100	790 ± 40
Ethanol	100	750 ± 250
Methanol	0	160 ± 30
H_2 / CO_2	0	150 ± 20

Performance of UASB B at high salinity: start up in batch mode and effect of high pH

The start-up of UASB B in batch-mode resulted in a low sulfide production (20 $mg.L^{-1}$) after 9 days (Fig. 4D). After switching to flow through mode on day 10, the sulfide production gradually increased to around 200 $mg.L^{-1}$ on day 15 (Fig. 4D). However, a sharp decrease of the sulfide concentration to 55 $mg.L^{-1}$ manifested on day 32, which was apparently caused by the elevated reactor pH (≥ 7.6) on day 30 (Fig. 4D). The reactor required two weeks to recover from this disturbance (Figs. 4A, 4B and 4D).

Performance of UASB C at high salinity: start up in flow through mode

The start-up of UASB C in continuous mode also resulted in a low sulfide production (20 $mg.L^{-1}$) after 9 days. The sulfide reached a concentration of 60 $mg.L^{-1}$ on day 15 (Fig. 5A) and the concentration gradually increased to values exceeding 450 $mg.L^{-1}$ on day 37, corresponding to a sulfate reduction rate of 2.5 g $SO_4^{2-}.L^{-1}d^{-1}$ (Fig. 5B). The HRT decrease to 8 hours on day 47 (Fig. 5B) did not affect the sulfide concentration in the reactor (Fig. 5A), which resulted in a maximal sulfate reduction rate of 3.8 g $SO_4^{2-}.L^{-1}.day^{-1}$ (Fig. 5B). As observed in UASB A and UASB B, acetate accumulated in the effluent as a result of incomplete propionate oxidation and the absence of acetate consumption (data not shown). Methane was not produced during this experiment.

Alternative electron donors for sulfate reduction at high salinity

The results of batch experiments conducted with the inoculum sludge revealed that ethanol, next to propionate, represents a suitable electron donor for sulfate reducing systems operating at high salinity (Table 3). However, this is not the case for hydrogen or methanol (Table 3). These substrates were not degraded in batch experiments at the high salinity tested, even though some sulfide production was observed (Table 3), apparently as a result of endogenous electron donor present in the sludge (Table 2).

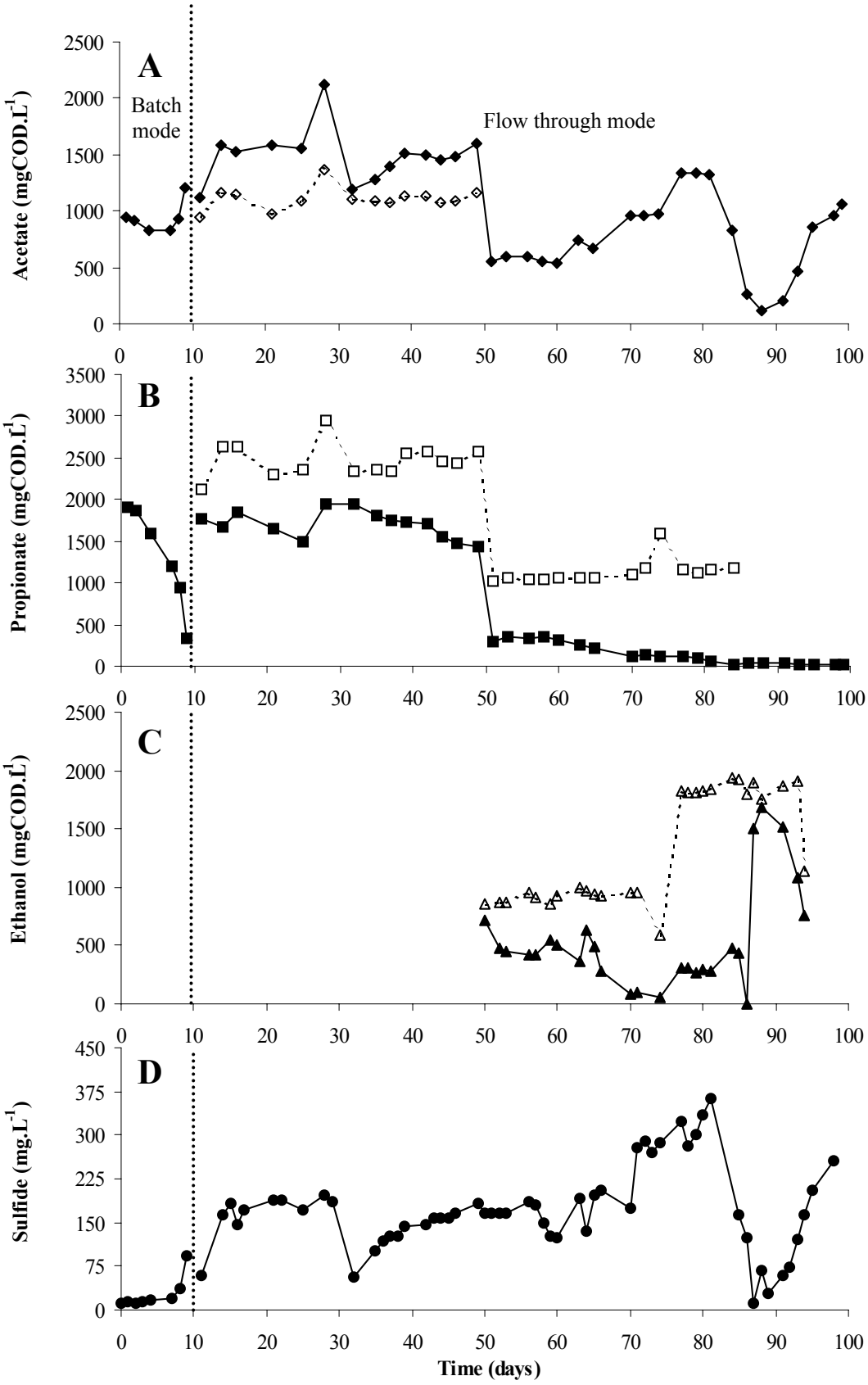

Figure 4. Process performance of UASB B: (A) Evolution of the concentrations of acetate in the influent (◊) and effluent (♦). (B) Evolution of the concentrations of propionate in the influent (□) and effluent (■). (C) Evolution of the concentrations of ethanol in the influent (△) and effluent (▲). (D) Evolution of the sulfide (•) concentration.

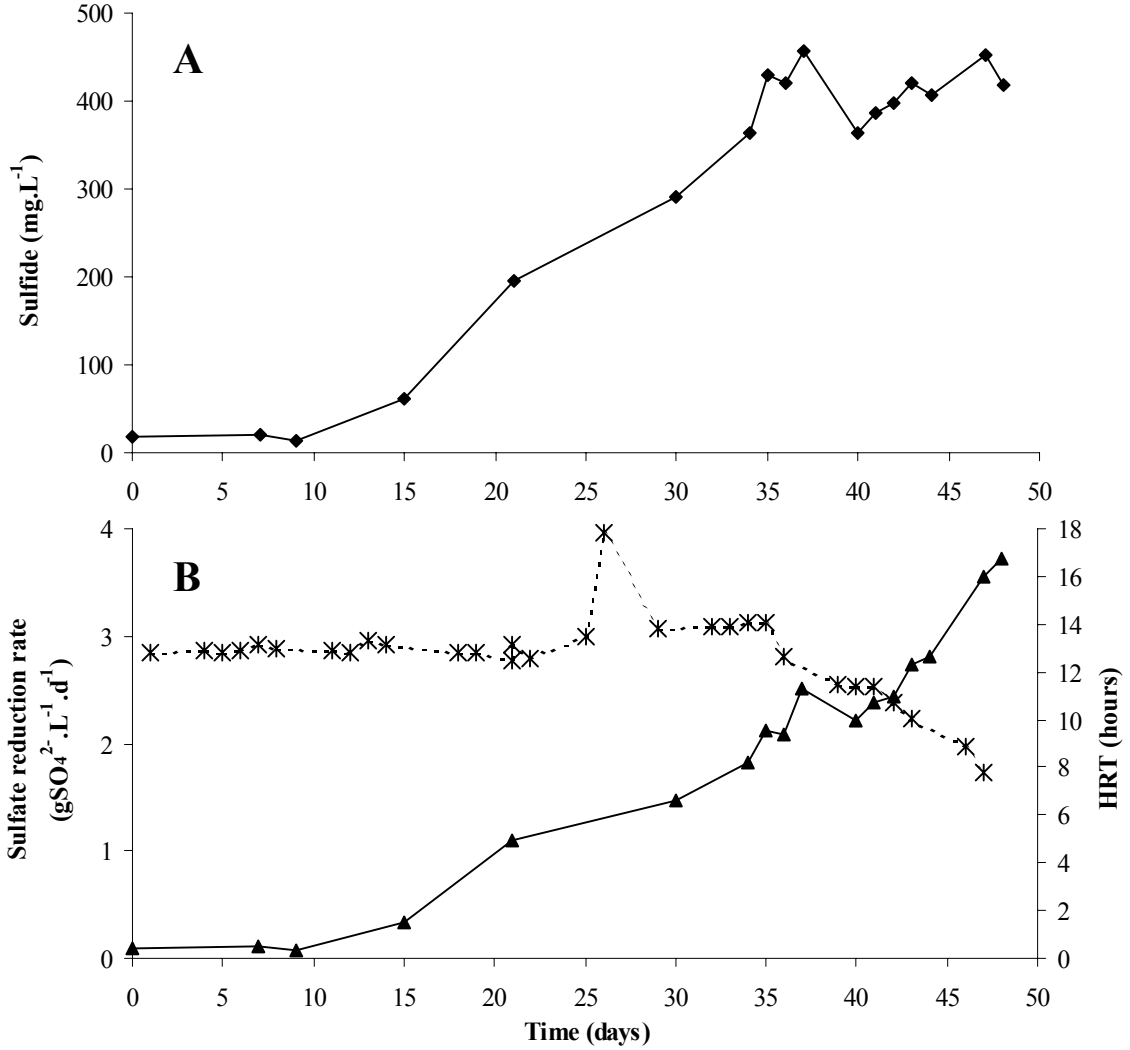

Figure 5. Process performance of UASB B: (A) Evolution of the sulfide (•) concentration. (B) Evolution of the hydraulic retention time (∗) and the sulfate elimination rate (▲).

Fig. 4 reveals that a partial replacement of the substrate propionate by ethanol in the influent of UASB B on day 52 did not affect the sulfide production. Moreover, even a full ethanol conversion was obtained 20 days after ethanol was supplied to the influent (Fig. 4C) and the sulfide concentration increased to values exceeding 300 $mg.L^{-1}$ (Fig. 4D) from day 70 onwards. The maximal sulfate reduction rate (2.8 g $SO_4^{2-}.L^{-1}day^{-1}$) was found on day 81 (data not shown), when UASB B was fed with 2 g $COD.L^{-1}$ ethanol and 1 g $COD.L^{-1}$ propionate (OLR of about 5g $COD.L^{-1}.day^{-1}$). The high acetate production from ethanol oxidation (Fig. 4A and 4C) reveals that ethanol was incompletely oxidized, as also found for propionate. A reactor pH shock (> 7.6) on day 82 caused a rapid drop in the sulfide production of UASB B. However, the reactor recovered from this shock within a few days (Fig. 4).

DISCUSSION

Development of high rate sulfate reducing reactors at high salinity

This work demonstrates, for the first time, that high rate sulfate reduction (up to 3.7 g $SO_4^{2-}.L^{-1}.day^{-1}$) can be achieved in UASB reactors at salinities exceeding 50 g $NaCl.L^{-1}$ and 1 g $MgCl_2.L^{-1}$ (65 to 70 $mS.cm^{-1}$) using unadapted granular sludge as inoculum. Sulfate reduction was found to proceed even at a salinity of up to 70 g $NaCl.L^{-1}$ and 1 g $MgCl_2.L^{-1}$ (conductivity of about 85 - 90 $mS.cm^{-1}$; Fig. 4A), although at considerably lower (about 65 % lower) rates than at 60 - 70 $mS.cm^{-1}$ (Figs. 3, 4 and 5). Moreover, this work clearly shows that a distinct halotolerant SRB population, other than the inoculated *D. halotolerans* (in UASB A), proliferated in all three reactors, as evidenced by (1) the high sulfide production rates in the UASB B (Fig. 4D) and UASB C (Fig. 5A), which were not inoculated with *D. halotolerans*, and (2) the observation that propionate oxidation was coupled to sulfate reduction in UASB A (Figs. 3C and 3D). According to Brandt and Ingvorsen (1997), *D. halotolerans* grows on acetate, ethanol and pyruvate as carbon sources, but is unable to use propionate. Thus, the methanogenic granular sludge used as inoculum in this experiment is apparently a potential source for halotolerant microorganisms. Currently, attempts are being made to isolate this sulfate reducing bacterium present in the granular sludge cultivated in these bioreactors. The physiological characteristics of this isolated strain will provide useful information that will enable the further optimization of the operational window of bioreactors operating under halophilic conditions. The high salinity tolerance found has already significant practical implications, as it enables the direct treatment of sulfate rich brines without prior dilution, thus making the application of SRB based bioreactors in closed cycles possible.

The relatively fast (less than 50 days) adaptation of a sulfate reducing granular sludge to high salinity observed in this work (Fig. 5A) is so far only reported for the operation of a methanogenic semi-continuous flow-through fixed film reactor fed with ethanol and acetate (no sulfate) in a saline solution (65 $gNaCl.L^{-1}$) and inoculated with digested sewage sludge from a conventional digester (de Baere *et al.*, 1984). They observed an initial inhibition at 65 g $NaCl.L^{-1}$, while a 50 % inhibition was found only at 95 $g.L^{-1}$ (de Baere *et al.*, 1984). This suggests that methanogenesis could also proceed in the sulfate fed UASB reactors, although the comparison with a methanogenic fixed film reactor (de Baere *et al.*, 1984) is difficult. It appears that the source (and selection) of inoculum is very important if one whishes to develop either methanogenic or sulfate reducing sludges in anaerobic reactors.

The relatively lower sulfide production found with ethanol (Fig. 4D) relative to propionate (Fig. 5A) is explained on the basis of the stoichiometry of the incomplete substrate degradation:

$CH_3CH_2COOH^- + 0.75\ SO_4^{2-} \rightarrow CH_3COO^- + HCO_3^- + 0.75\ HS^- + 0.25\ H^+$

$CH_3CH_2OH + 0.5\ SO_4^{2-} \rightarrow CH_3COO^- + 0.5\ HS^- + 0.5\ H^+ + H_2O$

At first sight, propionate looks the more attractive electron donor, in view of the amount of sulfate reduced per mol of substrate added. However, as the price of the electron donor represents a major selection criteria in the biodesulfurization of inorganic types of wastewaters and ethanol is much cheaper than propionate, ethanol represents a more attractive substrate than propionate. Opposed to propionate, ethanol is already used as electron donor in full-scale plants (Janssen *et al.*, 2000) and to the best of our knowledge there are so far no reports on the use of propionate as the sole electron donor for sulfate reducing processes. Another reason to select ethanol is the observations from both batch (data not shown) and continuous experiments (Fig. 4) that ethanol oxidation proceeds faster than propionate oxidation. Further research must be directed to the enhancement of sulfate reduction processes with ethanol as electron donor as well as to understand the reasons why acetate is an end product rather than a metabolic intermediate in sulfate reducing reactors.

The proliferation of the new SRB population is independent of the start up in continuous or batch mode. In addition, the apparent proliferation of an exclusively sulfate reducing population in the reactors does not suffer from the competition with MPA at high salinity, as is the case in sulfidogenic bioreactors operating at low salinity, where competition for substrates such as acetate (Fig. 1C) and ethanol (O'Flaherty *et al.*, 1998) occurs. The absence of methanogenesis at high salinity found in this work is highly advantageous for sulfate reducing processes, as there is no loss of electron donor as a result of methane production.

The procedure of immediate exposure of the sludges to high salinity as a strategy to select a halotolerant population contrasts the procedure adopted by Omil *et al.* (1995), who suggested to stepwise increase the salt concentration when using inoculum adapted to non-saline environments. In previous works (Chapter 6), it was found that it is unlikely that the adaptation of thermophilic (55°C) sulfidogenic methanol degrading biomass to high salinity would occur, due to the absence of a population of thermophilic halophilic methanol consuming SRB in the inoculum used. This implies that the success for the application of anaerobic treatment of saline wastewaters greatly depends on the sludge characteristics, viz. the source of biomass and the types of substrate present in the wastewater. In order to assess the presence of the required targeted microorganisms that will ultimately determine the feasibility of the process at extreme conditions, a screening of different types of sludges, environmental conditions and substrates is required. For instance, the 39 days lag phase found for propionate oxidation in batch vials amended with high salt (50 $gNaCl.L^{-1}$ and 1 $gMgCl_2.6H_2O.L^{-1}$) concentrations (data not shown) indicates that sufficient time must be

invested to allow the growth of halotolerant microorganisms in the potential inoculum sludge under investigation.

pH and salt sensitivity of unadapted granular sludges

This study clearly highlights the high sensitivity of the SRB present in the reactor sludges to high pH shocks, because in all cases a sharp drop in sulfide concentrations occurred at pH > 7.6 in UASB A (Fig. 3D) and UASB B (Fig. 4D). Moreover, the recovery of the SRB from such an exposure to high pH proceeded rather slowly (Figs. 3 and 4), and consequently it can be concluded that the operational pH must not exceed 7.5.

Interestingly, results of batch tests shows that the toxicity caused by a 15 day exposure of the granular sludge to a relatively high salinity (25 $g.L^{-1}$ NaCl and 1 $g.L^{-1}$ $MgCl_2$) is partially reversible (data not shown). The only 25 % recovery of the initial activity for the MPA compared to the 80 % recovery for the SRB activity indicates that the methanogens cultivated at low salinity are more sensitive to sudden salt concentration increases.

Inoculation with *Desulfobacter halotolerans*

The attempt to incorporate the acetate oxidizing halotolerant SRB *Desulfobacter halotolerans* the reactor sludge via the entrapment/immobilization was unsuccessful, as indicated by the net accumulation of acetate after switching back the reactor operation to flow through mode (Figs. 2A and 3B) and the dependence of the sulfate removal on propionate removal (Figs. 2A and 2C). Apparently, *D. halotolerans* was not entrapped in the sludge granules and was ultimately washed out from UASB A after returning to the continuous operation on day 58. Although some of the *D. halotolerans* might have been sorbed on the surface of the granules (Tawfiki-Hajji *et al.*, 2000), they certainly were not incorporated into the micro-ecosystem and consequently the reactor removal efficiency remained unaffected after resuming continuous operation. This is in agreement with other studies which have highlighted the difficulties of introducing a new strain into UASB reactors (Nagpal *et al.*, 2000; O'Flaherty *et al.*, 1999; Omil *et al.*, 1997). Thus, anaerobic granules seem to be an inadequate matrix for efficient attachment of exogenous microorganism.

Interestingly, successful colonization of anaerobic granules by new microorganisms is only reported for dechlorinating microorganisms (Ahring *et al.*, 1992; Christiansen and Ahring, 1996; Horber *et al.*, 1998; Lanthier *et al.*, 2002; Tartakovsky *et al.*, 1999; Tawfiki Hajji *et al.*, 1999). According to Lanthier *et al.* (2002), entrapment and colonization of anaerobic granules by new microorganisms are most successful when the newly introduced strain establishes commensal or mutualistic relationships within a natural consortium. For instance, the retention and proliferation of strain *Desulfitobacterium frappieri* PCP-1

(dechlorinating microorganism) in the granules of a UASB reactor was explained by its complementary role within the anaerobic consortium exposed to pentachlorophenol (PCP; Tartakovsky *et al.*, 1999). It was suggested that the dechlorinating activity of strain PCP-1 protected other members of the anaerobic consortium, in particular methanogens, from PCP toxicity (Tartakovsky *et al.*, 1999). Fluorescent in situ hybridization (FISH) showed that the strain PCP-1 was present in the outer layer of the UASB granules three weeks after reactor inoculation and by the end of the experiment (nine weeks) a dense outer layer of strain PCP-1 manifested (Lathier *et al.,* 2002). The observed deep penetration and more uniform distribution of entrapped *Dehalospirillum multivorans* (Horber *et al.*, 1998) and *Desulfobacterium hafniense* (Christiansen and Ahring, 1996) in autoclaved granules (thus heat treated dead granules) contrasts the hypothesis of the importance of need for commensal or mutualistic relationships for successful colonization of anaerobic granules by new microorganisms (Lanthier *et al.*, 2002). This warrants further research towards inoculation techniques that guarantee a successful retention and proliferation of newly added strains into anaerobic granules or biofilms.

Effect of sudden salt shock (25 gNaCl.L^{-1}) on reactor performance and perspectives

At low salinity (7.6 mS.cm^{-1}), acetate and propionate are easily removed in mesophilic UASB reactors operated at an OLR of 11.5 g COD.L^{-1}.day^{-1} and a HRT of 5 hours (Fig. 2A). Under these conditions, propionate is the preferred substrate for sulfate reducers and acetate is mainly degraded by methanogens (Oude Elferink *et al.*, 1998). Fig. 1 shows the high sensitivity of sludge cultivated at low salinity (7.6 mS.cm^{-1}) to a sudden increase in the influent salinity imposed on day 27, as similarly observed for methanol-fed thermophilic (55°C) sulfate reducing UASB reactors (Chapter 5). Indeed, high osmolarity environments trigger rapid fluxes of cell water, causing a reduction in turgor and dehydration of the cytoplasm (Kempf and Bremer, 1998). This would suggest, at a first sight, that high salt concentrations are highly detrimental to the operation of mesophilic sulfidogenic reactors operating originally at low salinity (Fig. 1).

The maximal sulfate reduction rates obtained with propionate (3.7 g SO_4^{2-}.L^{-1}.day^{-1} in UASB C at a HRT of 7.8 hours) and ethanol (2.8 g SO_4^{2-}.L^{-1}.day^{-1} in UASB B at a HRT of 9 hours) can probably be further increased by decreasing the HRT, e.g. to values usually applied in UASB reactors (i.e. 4 hours). This was already achieved for non-saline conditions by, for instance, Dries *et al.* (1988) who reported a sulfate removal rate as high as 10 g SO_4^{2-}.L^{-1}.day^{-1} in an acetate fed EGSB reactor operated at low salinity and a HRT of about 1.9 hours. On the other hand, de Smul *et al.* (1997) reported that in an ethanol fed EGSB reactor operated at low salinity a sulfate reduction rates of only 1 g SO_4^{2-}.L^{-1}.day^{-1} was achieved after 40 days of

operation and more than 150 days were needed to obtain sulfate elimination rates as high as 8 g $SO_4^{2-}.L^{-1}.day^{-1}$.

CONCLUSIONS

The results of this work lead to the following conclusions:

(1) Sulfidogenic UASB reactors inoculated with unadapted granular sludge can be operated satisfactorily at salinities as high as 70 g $NaCl.L^{-1}$and 1 g $MgCl_2.L^{-1}$ (90 $mS.cm^{-1}$) when using propionate or ethanol as electron donor.

(2) The granular inoculum sludge used in this work can be adapted to high salinity in less than 50 days, reaching maximal sulfate reduction rate of 3.7 g $SO_4^{2-}.L^{-1}.day^{-1}$ at a HRT of 7.8 hours.

(3) The successful start up and operation of UASB reactor systems used in this investigation can be attributed to the proliferation of a halotolerant incomplete oxidizing SRB population present in the inoculum sludge. Incorporation of the acetate oxidizing halotolerant SRB *Desulfobacter halotolerans* in the inoculum sludge via entrapment and/or immobilization was unsuccessful.

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Chapter 9

High rate sulfate reduction in a submerged anaerobic membrane bioreactor (SAMBaR) at high salinity

Sulfate reduction in salt rich wastewaters (50 g $NaCl.L^{-1}$ and 1 g $MgCl_2.6H_2O.L^{-1}$; conductivity 60-70 $mS.cm^{-1}$) was investigated in a 6 L submerged anaerobic membrane bioreactor (SAMBaR) and inoculated solely with the halotolerant sulfate reducing bacterium *Desulfobacter halotolerans*. The SAMBaR was fed with acetate and ethanol at organic loading rates up to 14 $gCOD.L^{-1}.day^{-1}$ in excess of sulfate (COD/SO_4^{2-} of 0.5) and operated at pH 7.2 ± 0.2 and a hydraulic retention time (HRT) from 8 to 36 hours. A sulfate reduction rate up to 6.6 $gSO_4^{2-}.L^{-1}.day^{-1}$ was achieved in the SAMBaR operating at a flux of 17.1 $L.m^{-2}.h^{-1}$, which resulted in a HRT of 9 hours. The fairly constant very high specific sulfate reduction rate of 5.5 $gSO_4^{2-}.gVSS^{-1}.day^{-1}$ showed that the performance of the reactor was limited by the low amount of biomass (0.85 $gVSS.L^{-1}$) present in the reactor at the end of the experiment. It was shown that sulfate reducing submerged anaerobic membrane bioreactors can be operated over extended periods of time without chemical cleaning of the membranes at a certain fixed flux if this flux is substantially below the nominal critical flux determined experimentally (18-21 $L.m^{-2}.h^{-1}$). Intermittent operation as well as backflush of the membranes were shown to slow the fouling in the membranes. Frequent backflush (e.g. 1 minute each 10 minutes) is the suggested operational strategy to minimize fouling in anaerobic MBRs.

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INTRODUCTION

Biomass retention is one of the most important aspects of modern anaerobic technology. Uncoupling of the hydraulic retention time (HRT) and cell retention time by self-aggregation (e.g. granular sludges) or biofilm formation, is essential for the successful operation of conventional high rate anaerobic bioreactors (Young and McCarty, 1969; Lettinga *et al.,* 1980). Conventional anaerobic reactors, however, are less suited for the introduction of a particular metabolic capacity via the addition and retention of specialized microorganisms to granular sludge based reactor systems, since the added microorganisms mostly do not entrap or immobilize the granules and are washed out from biofilm systems. The unsuccessful immobilization of specific strains into reactor biomass has been reported in fluidized bed (Nagpal *et al*., 2000), upflow anaerobic granular sludge bed (UASB; Omil *et al.*, 1997; Chapter 8) and hybrid (UASB + packed bed; O'Flaherty *et al.*, 1999) reactor systems. A complete retention of all microorganisms in the bioreactor, including newly added bacterial species with a specific metabolic capacity, can be achieved in anaerobic membrane bioreactors. In addition, membrane bioreactors (MBR) are not dependent on granulation or biofilm formation, so that MBRs can also be operated with cell suspensions or flocs with poor settling characteristics. Thus, inoculation of the MBRs with a pure culture or a combination of known bacterial species can be performed without any risk of washout of the inoculated biomass.

Anaerobic membrane bioreactors might offer advantages in terms of volumetric loading rates (resulting in a small footprint reactor), effluent quality and process stability (Fuchs *et al.,* 2002). Another problem sometimes manifesting in practice is that anaerobic biomass can be sensitive to high salinity environments. High salt concentrations are known to significantly reduce the treatment efficiency of methanogenic and sulfidogenic conventional mesophilic (Guerrero *et al.*, 1997; Rinzema *et al.*, 1988) and thermophilic (Willets *et al.*, 2000; Chapters 4, 5 and 6) anaerobic bioreactors. Indeed, high osmolarity environments trigger rapid fluxes of cell water, causing a reduction in turgor and dehydration of the cytoplasm (Kempf and Bremer, 1998). Thus, the successful operation of sulfate reducing bacteria (SRB)-based bioreactors operating at high salinity requires the retention of halophilic SRB in anaerobic reactors.

The ability of halophilic anaerobic microorganisms to degrade different organic substrates has been reviewed and appears that only a few easily degradable substrates such as simple sugars and amino acids can be fermented via dissimilatory sulfate reduction (Oren, 2002a; Oren, 2002b). The upper limit of salinity at which dissimilatory sulfate reduction has been observed is 240 g $NaCl.L^{-1}$, for the incomplete lactate, ethanol and pyruvate oxidizer *Desulfohalobium retbaense* (Ollivier *et al.*, 1991). The highest salinity for the complete oxidation via sulfate reduction reported so far is around 130 g $NaCl.L^{-1}$ for the acetate

oxidizer *Desulfobacter halotolerans* (Brandt and Ingvorsen, 1997). The incorporation of such a halophilic SRB in a membrane bioreactor would greatly extend the application of desulfurization to wastewater treatment systems that can presently not be treated biologically.

The aim of this work was to assess the performance of a sulfate reducing submerged anaerobic membrane bioreactor (SAMBaR) fed with acetate and ethanol as the sole electron donors operated at high salinity (50 g $NaCl.L^{-1}$ and 1 g $MgCl_2.6H_2O.L^{-1}$; conductivity 60-70 $mS.cm^{-1}$) and inoculated with the pure strain *Desulfobacter halotolerans*. The major limitation to the use of membranes is the continuous reduction in permeate flux by membrane fouling and the operational costs associated with it (Chang *et al.*, 2002). The reduction in permeate flow is known to be the main factor in determining the economic feasibility of membrane processes (Chang *et al.*, 2002). Therefore, different operational procedures for the minimization of fouling were studied, including the determination of the critical flux and the assessment of the influence of flux stoppage and membrane backflush on the increase in transmembrane pressure (TMP).

MATERIAL AND METHODS

Continuous experiments

Experimental setup

A submerged anaerobic membrane bioreactor (SAMBaR) of 6 L (1 m high, internal diameter 10 cm) was operated during 92 days in order to study the feasibility of high rate sulfate reducing processes at high salinity (50 g $NaCl.L^{-1}$ and 1 g $MgCl_2.6H_2O$ L^{-1} in the influent; 60-70 $mS.cm^{-1}$). The SAMBaR (Fig. 1) was equipped with a set of 5 cylindrical polysulfone membranes (Triqua bv, Wageningen, the Netherlands) with a total effective surface of 0.07 m^2 (Fig. 1). The mean pore size of 0.2 μm guaranteed the uncoupling of the hydraulic retention time (HRT) and the cell retention time. The SAMBaR was equipped with a double wall, through which water, heated in a thermostatic waterbath (Julabo, Seelbach, Germany), was recirculated to maintain the reactor temperature at 33 ± 1ºC. This temperature was selected because it is the optimum temperature for the growth of *Desulfobacter halotolerans* (Brandt and Ingvorsen, 1997), used as reactor inoculum.

The pH in the reactor was maintained at 7.25 ± 0.2 (within the optima pH range for growth of *D. halotolerans*, Brandt and Ingvorsen, 1997) by means of an automatic pH control, adding HCl when necessary (Fig. 2B). The pH was measured with sulfide resistant Flushtrode pH-electrodes (Hamilton Flushtrode, Hilkomij bv, Rijswijk, the Netherlands) connected to an automatic pH controller with two changeable set points to adjust the pH (Elektronika Wageningen, the Netherlands). The pH electrodes were checked and calibrated three times per week.

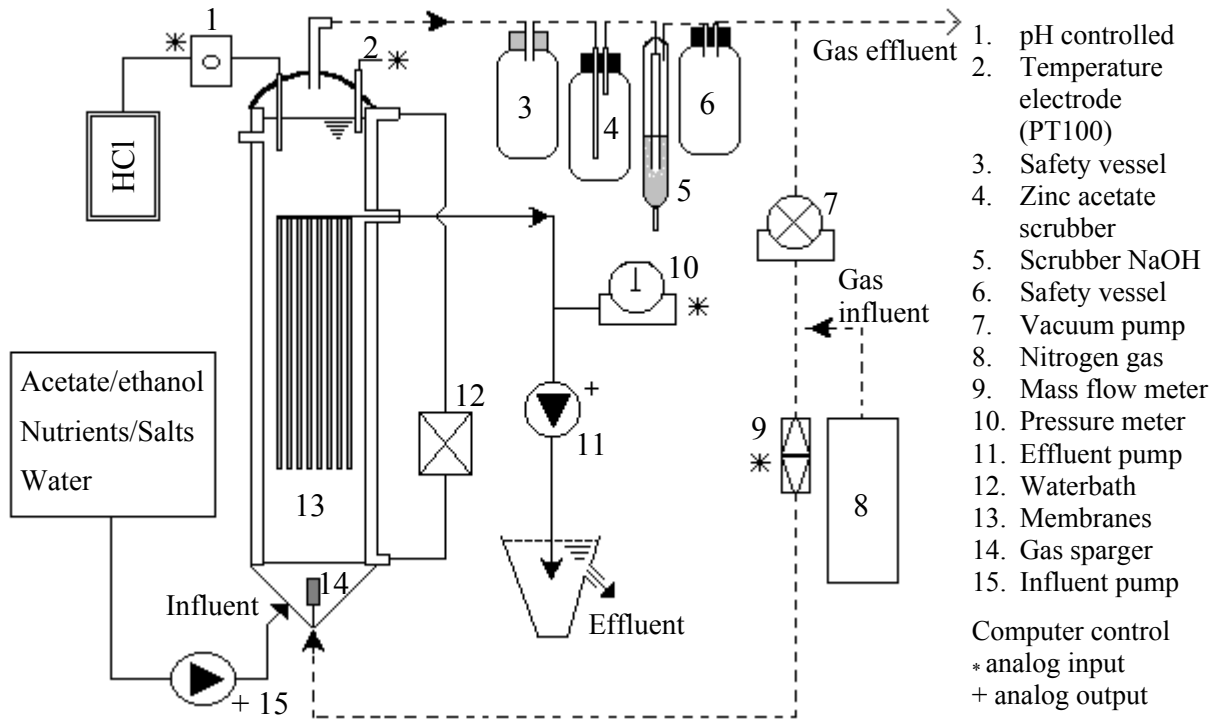

Figure 1. Schematic representation of the submerged anaerobic membrane bioreactor (SAMBaR).

Nitrogen gas was sparkled in the bottom of the SAMBaR (at a gas loading rate of 14 $L.L_{reactor}.h^{-1}$) in order to promote reactor mixing, to strip off the sulfide and to prevent the fast accumulation of foulants onto the membrane surface (Chang *et al.*, 2002). A vacuum pump was installed for the recirculation of the (bio)gas. Four bottles were mounted in the recirculation gas line. The first bottle was used for the collection of the reactor bulk that was eventually transported with the gas out of the SAMBaR. The second bottle was filled with a zinc acetate solution to selectively retain the gaseous H_2S. The third bottle was filled with a 1M NaOH solution where the carbon dioxide (CO_2) was removed from the gas prior to its recirculation into the reactor. The fourth bottle was used to avoid the alkaline solution to flow into the vacuum pump. The effluent gas was led to a waterlock placed between the vacuum pump and the fourth bottle. The scrubbed (H_2S and CO_2-free) recirculation gas was finally combined with the influent N_2 gas and led into the reactor through a gas sparkler (Fig. 1). A mass flow meter was placed before the reactor inlet in order to determine the gas sparkling rate (Fig. 1).

The influent flow, consisting of substrate, micro and macro nutrients (diluted with demineralized water), was provided by means of a computer controlled peristaltic pump (Watson-Marlow 501 U, Falthmouth, Cornwall, UK). Effluent was generated by operating a computer controlled peristaltic pump (Watson Marlow 501 U) after the membrane module, thus regulating the flux over the membranes. The latter was measured by weighing the amount of permeate on an electrical balance. A pressure transducer (Fig. 1-10, Farnell,

BTE6000 series 0-10V output, Germany) was placed in line between the membranes and the effluent peristaltic pump so that the transmembrane pressure (TMP) applied to the membranes was recorded. Sampling ports were placed in the influent and effluent tube systems in order to collect samples. Temperature, pH, TMP and gas flow signals were sent to a computer, where the data were recorded.

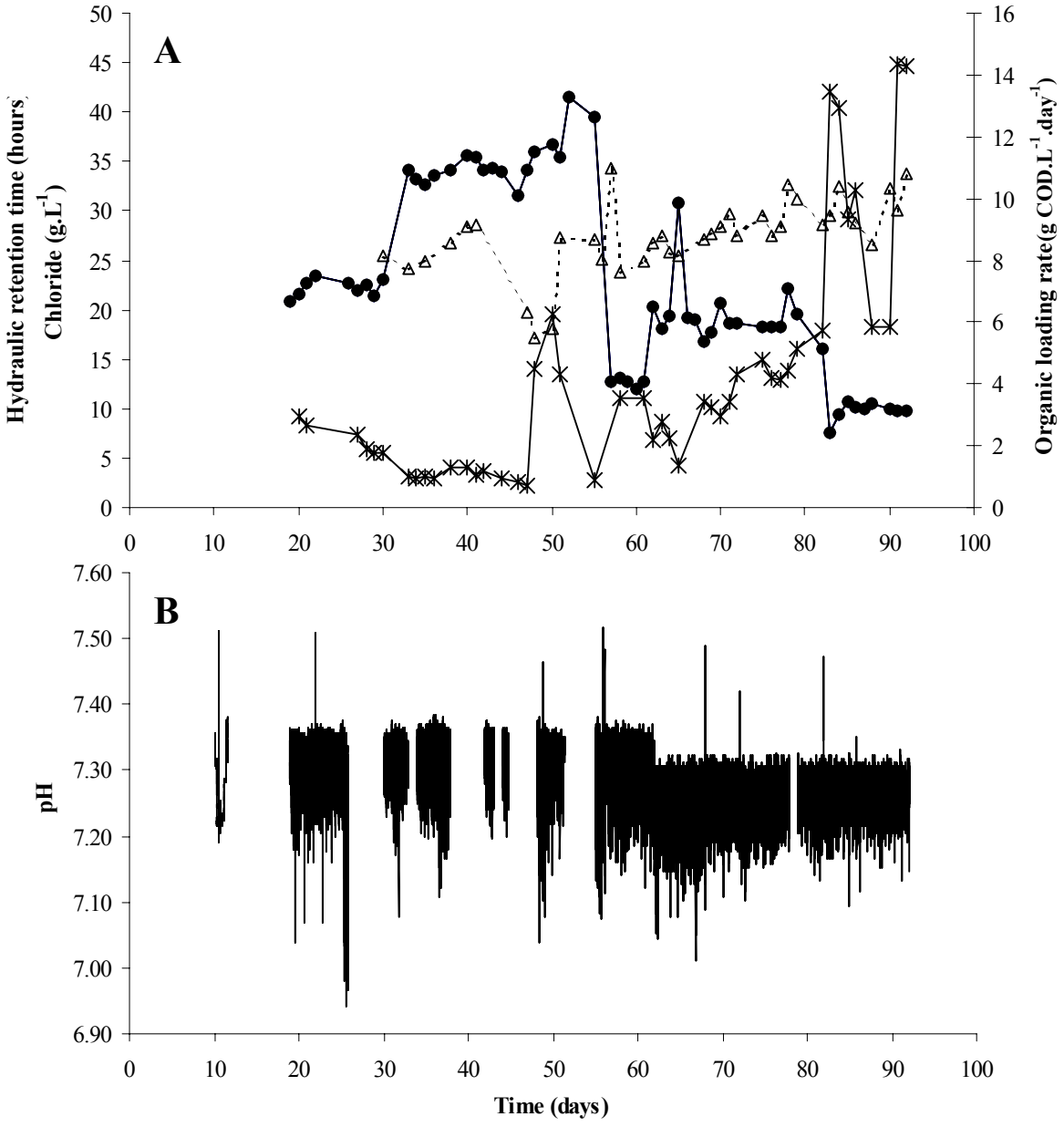

Figure 2. Evolution of the organic loading rate (∗), hydraulic retention time (●) and chloride concentration (△) applied to the SAMBaR.

Membrane operational modes

In order to minimize membrane fouling0, two distinct operational procedures were applied in the SAMBaR, viz. production/relaxation mode or backflush mode (Table 1). The operational mode was selected depending on the TMP registered. If the TMP was higher than

0.15 bar, the membranes were backflushed with the permeate at a flow two times higher than that normally applied. Otherwise (TMP < 0.15) the reactor operated in the production-relaxation mode (Table 1). Fig. 3 shows a typical 3 hours representation of the TMP in relation with the two operational procedures adopted to minimize fouling. Fig. 3 also illustrates the mathematical procedure (linear regression) to calculate the TMP increase rate (defined as dP/dt and proportional to the membrane fouling rate) in the membranes during the experiment.

Table 1 Operational procedures applied to the membranes in order to minimize fouling during the operation of the SAMBaR.

Operational Modes	
Production/relaxation mode	**Backflush mode**
6 minutes production (pumps on - flux)	1 minute backflush ($Q_{Bf} = 2 \times Q_P$)
2 minutes relaxation (pumps off - no flux)	2 minutes production (to compensate the flow backflushed to the SAMBaR)
2 minutes production (pumps on - flux)	1 minute relaxation (pumps off - no flux)
2 minutes relaxation (pumps off - no flux)	Go to production/relaxation mode sequence
Verification of transmembrane pressure (TMP)	
If TMP < 0.15 bar → production/relaxation mode	
If TMP > 0.15 bar → backflush mode	

Q_{Bf} – flow rate of backflush; Q_P – flow rate of production

Whenever a TMP of 0.4 bar was reached, an ex-situ chemical cleaning of the membranes was carried out. The membrane set was removed from the SAMBaR and immersed in a 1 $g.L^{-1}$ hypochlorite (NaOCl) solution for one hour, followed by another one hour immersion in 3 $g.L^{-1}$ of citric acid ($C_6H_8O_7$) solution. During these immersions, the membranes were backflushed with the solutions at a flux of 5 $L.m^{-2}.h^{-1}$. Before placing the membrane back inside the reactor, the membranes were backflushed (at a flux of 5 $L.m^{-2}.h^{-1}$) with water for one hour in order to remove any residual chemical solution.

Critical flux determination

In this work, the flux-step method was used to determine the critical flux value, as described in Le-Clech *et al.* (2002). The flux was stepwise increased for a fixed duration (10 minutes) for each increment (3 $L.m^{-2}.h^{-1}$), giving a relatively stable TMP at low flux but an ever-increasing rate of TMP increase at higher fluxes. This flux-step method yielded the highest flux for which TMP increase remains stable as the critical flux. The linear regression of the recorded TMP for each flux applied determined the rate of TMP increase. The TMP

value was recorded in the computer each 30 seconds. The critical flux determination was carried out with a suspension (1.5 g VSS.L^{-1}) of crushed anaerobic sludge.

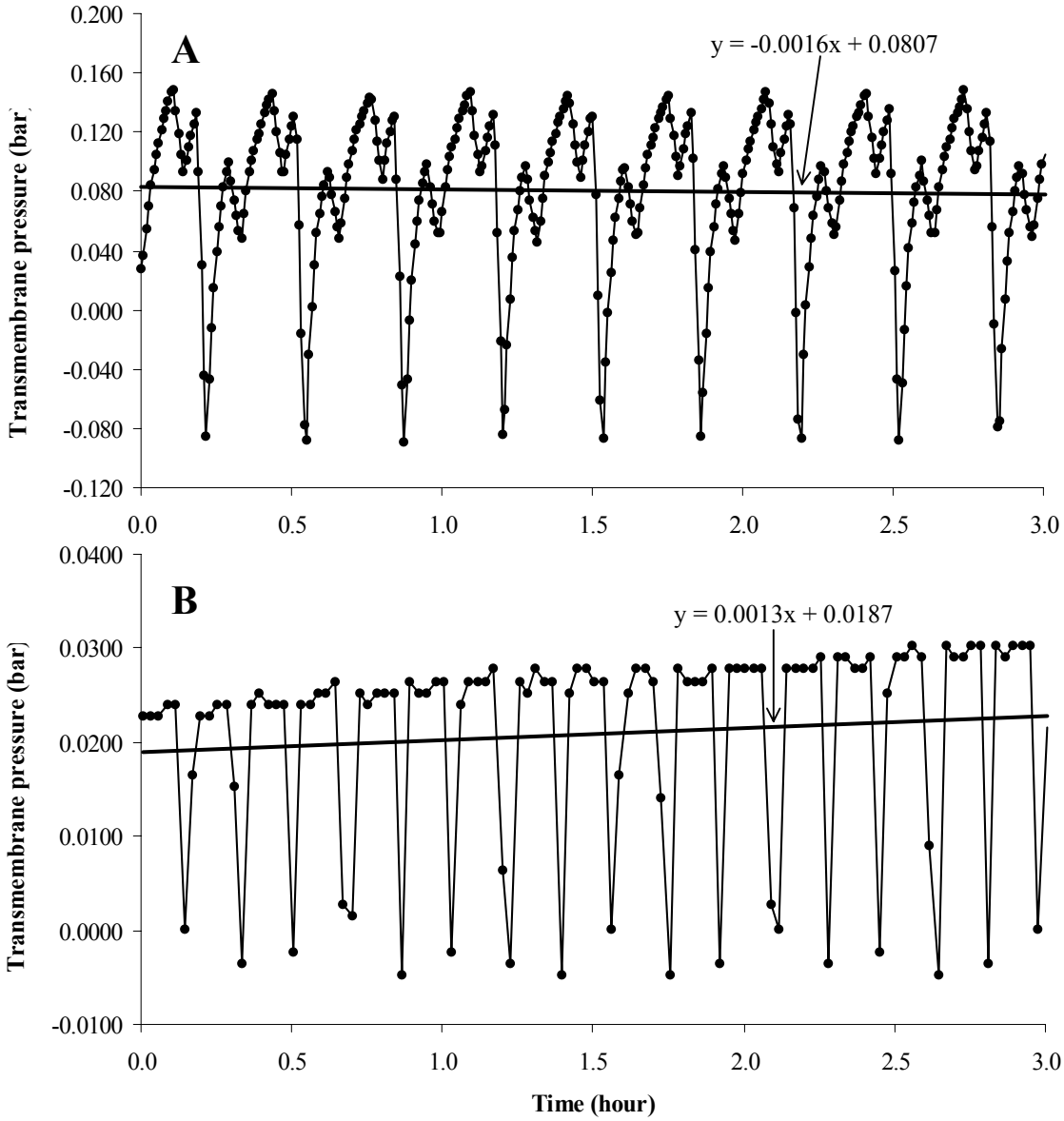

Figure 3. Typical transmembrane pressure (TMP) variation in function of the SAMBaR operational mode. Note that linear regression (trend line) allowed to calculate the TMP increase rate.

Inoculum

A culture of the mesophilic acetate oxidizing SRB *Desulfobacter halotolerans*, initially cultured in a defined medium (Brandt and Ingvorsen, 1997) and subsequently subcultured in the medium described below, was used as the inoculum in this study. *Desulfobacter halotolerans* strain GSL-Ac1, kindly provided by Prof. Ingvorsen (Aarhus University, Denmark), was enriched from moderate hypersaline sediments in the southern arm

of Great Salt Lake (Utah, USA) and isolated in a synthetic medium containing 10 % NaCl and 1 % $MgSO_4.7H_2O$ (Brandt & Ingvorsen, 1997). Strain GSL-Ac1 uses acetate, ethanol and pyruvate as electron donor and carbon source. It is able to reduce sulfate, sulfite and thiosulfate at high salinity (up to 13 % NaCl and 4.5 % $MgCl{\cdot}6H_2O$), but grows optimally around 1-2 % NaCl (Brandt and Ingvorsen, 1997). *Desulfobacter halotolerans* grows at a pH ranging from 6.2 to 8.1 (pH optimum, 6.2-7.4) and the maximum growth temperature is 37^0C (optimum between 32-34^0C).

Substrate and medium

Acetate (day 0 to 79) and ethanol (day 68 to 92) were supplied as the electron donor and carbon sources, providing an influent COD concentration between 1 and 5.9 $g.L^{-1}$. Sulfate was added to the reactor as sodium sulfate at a COD/SO_4^{2-} of 0.5 (g COD per g SO_4^{2-}), so theoretically all substrate could be degraded via sulfate reduction. Sodium chloride (50 g $NaCl.L^{-1}$) and magnesium chloride (1 g $MgCl_2.6H_2O.L^{-1}$) were used as model compounds to increase the salinity of the wastewater. In addition, basal medium containing macro and micro nutrients was supplied to the influent at a ratio of 2.22 ml per g COD fed. Basal medium was prepared as described in Chapter 3 and a trace element (4.5 $mL.L^{-1}$) solution was prepared according to Zehnder *et al.* (1980). From day 68 onwards the basal medium was further supplied with a vitamin solution (50 $mg.L^{-1}$ biotin and 50 $mg.L^{-1}$ 4-aminobenzoate). Both the basal medium and substrate stock solutions were prepared using demineralized water.

Desulfobacter halotolerans was cultivated in autoclaved (30 minutes at 121ºC) mineral medium. This mineral medium differed from the basal medium supplied in the reactor in that it was further supplemented with a 1 ml vitamin solution according to Stams *et al.* (1993) and buffered at pH 7.0 using 4 $g.L^{-1}$ $NaHCO_3$ and 1.6 bar of N_2/CO_2 (80/20%). An inoculum size of 5% (vol/vol) was used.

Experimental design

The SAMBaR was operated for 92 days at a high salinity of 50 g $NaCl.L^{-1}$ and 1 g $MgCl_2.6H_2O.L^{-1}$ (about 60-70 $mS.cm^{-1}$). The SAMBaR was inoculated with 1000 mL (17 % reactor volume) of a pure culture of *D. halotolerans* growing in the exponential phase. The organic loading rate and the HRT of the SAMBaR varied as a function of the flux and the strategy applied for the maintenance of the membrane, viz. relaxation production of backflush mode (Table 1 and Fig. 2A). The reactor was operated in batch mode (no effluent production) for 19 days till a drop in the redox potential to values around –240 mV and a significant sulfide production were observed. On day 19, the set of membranes was installed in the reactor (Fig. 1) and a flux (J) of 4.7 $L.m^{-2}.h^{-1}$ was applied, corresponding to a HRT of about 24 hours when operating in production/relaxation mode. Between days 25 to 26, the flux

occasionally increased to 32 $L.m^{-2}.h^{-1}$, which caused the mechanical collapse of the membranes (due to the acute increase in the transmembrane pressure). New membranes were placed in the SAMBaR and the same flux of 4.7 $L.m^{-2}.h^{-1}$ was applied till day 54 (when the membranes were chemically cleaned). On day 55, the flux was increased to 9.4 $L.m^{-2}.hour^{-1}$, resulting in a HRT of about 12 or 18 hours when operating in, respectively, production/relaxation or backflush mode. The membranes were chemically cleaned on day 82, before further increasing the flux to 17.1 $L.m^{-2}.hour^{-1}$, resulting in a HRT of about 10 hours (in backflush mode). On days 85 and 89, the membranes were mechanically cleaned by gentle displacement of the cake layer deposited on the membrane with a brush.

Analysis and chemicals

The gas composition was measured on a gas chromatograph (Hewlett Packard HP 5890, Palo Alto, USA) according to Weijma *et al.* (2000). Volatile fatty acids (VFA) and alcohols were analyzed on a gas chromatograph (Hewlett Packard HP 5890A, Palo Alto, USA) according to Weijma *et al.* (2000). Sulfide was measured according to Trüper and Schlegel (1964). Occasional samples were taken from the reactor bulk in order to determine the amount of volatile suspended solids (VSS) and total suspended solids (TSS) inside the SAMBaR, analyzed according to standard methods (APHA, 1995). The electrical conductivity (EC) or the reactor mixed liquor was measured using a standard EC meter (WTW LF 196, Weilheim, Germany). Sulfate was measured on a DX-600 ion chromatograph (IC) system (Dionex Corporation, Salt Lake City, USA). The specific sulfate elimination rate was calculated from the total amount of sulfate reduced divided by the concentration of VSS in the SAMBaR. The particle size distribution (PSD) was measured by laser diffraction analysis (Coulter LS230, Beckman Coulter, USA). A reactor sample was harvested on day 64 for microscope observations (Olympus BH-2). All chemicals used were of analytical grade and purchased from Merck (Darmstadt, Germany).

RESULTS

Reactor performance

Reactor performance in batch mode (day 0 to 19)

During the start-up of the SAMBaR in batch mode (no membranes and no gas sparkling), the reactor acetate concentration decreased from 1000 to 270 $mgCOD.L^{-1}$ in 12 days (Fig. 4A). The sulfate concentration decreased from 3200 to 1880 $mgSO_4^{2-}.L^{-1}$ in the same period (Fig. 5A), resulting in an increase of the sulfide concentration up to a maximum of 105 $mg.L^{-1}$ on day 17 (Fig. 5C).

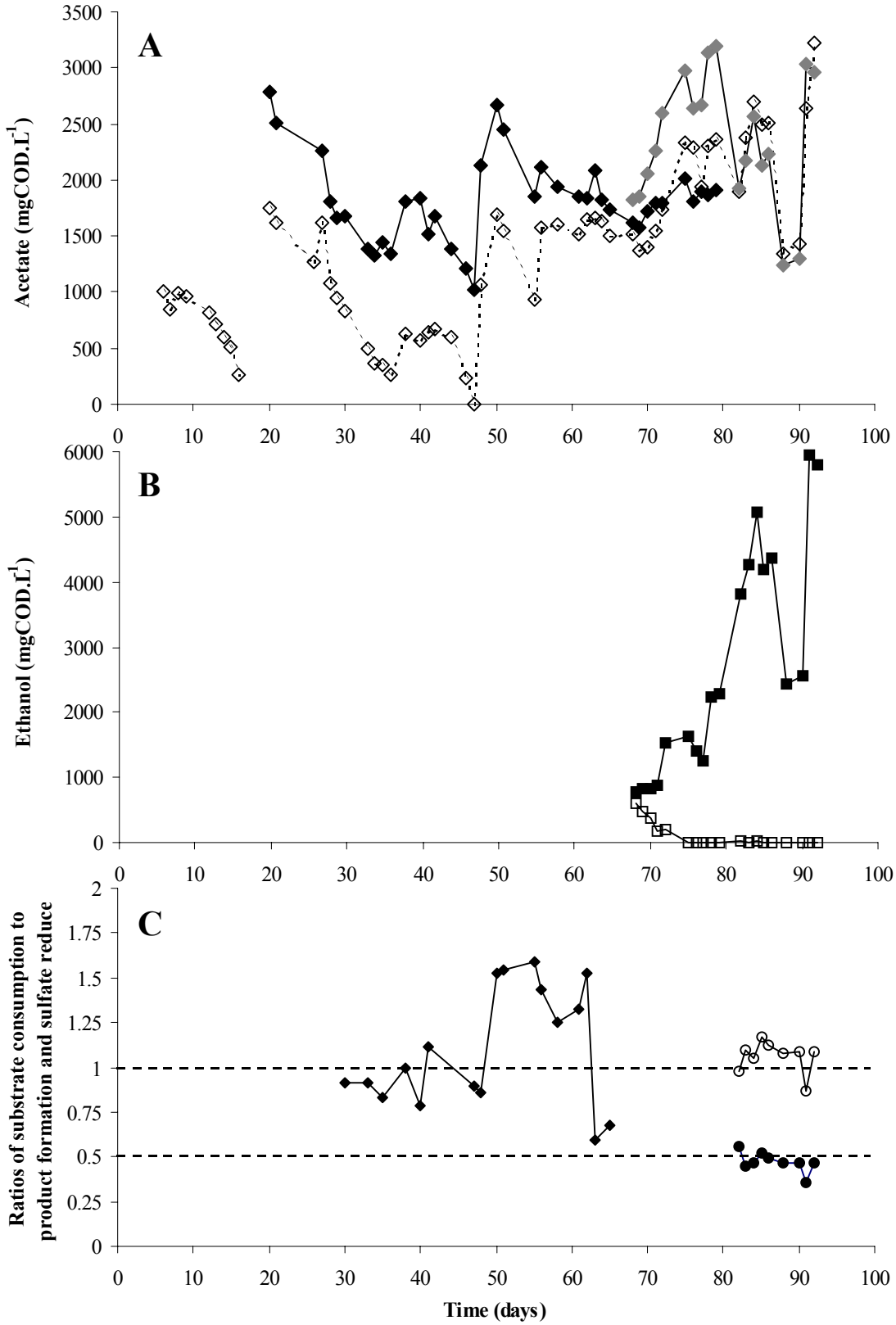

Figure 4. Process performance of the SAMBaR. (A) Evolution of the acetate concentration in the influent (♦), effluent (◇) and calculated reactor acetate concentration based on the stoichiometry of incomplete ethanol oxidation (◆). (B) Evolution of the ethanol concentration in the influent (■)and effluent (□). (C) Evolution of the stoichiometrical molar ratio of acetate consumption to sulfate reduced (♦), acetate produced to ethanol consumed (o) and sulfate reduced to ethanol consumed (●).

Reactor performance when operating at flow through conditions (day 20 to 92)

After switching to flow through mode, an acetate removal efficiency of 80 % was obtained on day 36 (Fig. 4A). During this period, the SAMBaR was operated in backflush mode at a flux of 4.7 $L.m^{2-}.h^{-1}$, corresponding to a HRT of about 34 hours (Fig. 2A). The acetate removal efficiency dropped to about 60 % between days 36 and 44 due to a lack of micro-nutrients in the feed (Fig. 4A). After the micro-nutrient supply was resumed on day 44, full acetate removal was achieved on day 47 (Fig. 4A). From day 47 to 50, the effluent acetate concentration increased due to an unintentional increase in the influent acetate concentration (Fig. 4A).

The sulfate removal efficiency increased continuously till day 55, reaching a maximum sulfate removal efficiency of 85% on day 55 (Fig. 5A). Upon increasing the flux to 9.4 $L.m^{-2}.h^{-1}$ (resulting in a HRT decrease from 39 to 12 hours) on day 55, the sulfate removal efficiency dropped to around 20 % (Fig 5A). Note that this was an effect of decreasing the HRT, as the reactor kept working at a fairly constant sulfate elimination rate of around 1.5 g $SO_4^{-2}.L^{-1}.d^{-1}$ till day 61 (Fig. 5B). After the flux was increased to 9.4 $L.m^{-2}.h^{-1}$ on day 55, the acetate removal efficiency also decreased to 15 % (Fig. 4A). In addition, neither biotin nor 4-aminobenzoate, essential vitamins required for the growth of *D. halotolerans* (Brandt and Ingvorsen, 1997), were added to the SAMBaR till day 68, which may have contributed to the performance deterioration of the reactor. Indeed, a remarkable increase in the sulfate elimination rate of the SAMBaR was observed after the addition of vitamins (biotin and 4-aminobenzoate), and the replacement of the substrate acetate by ethanol on day 68 (Fig. 5B), resulting in an increase in the sulfate removal efficiency from 7 to 68 % on, respectively, days 65 and 82 (Fig. 4A).

The addition of ethanol and vitamins to the system not only boosted the sulfate removal efficiency, but also the acetate removal efficiency. Although apparently there was no acetate removal from day 72 onwards (Fig. 4A), the calculated amount of acetate present in the reactor mixed liquor, based on the stoichiometry of the ethanol oxidation to acetate (Equation 1) shows that in fact there was a net removal of acetate till day 79 (Fig. 4A).

$$2\ C_2H_5OH + SO_4^{2-} \rightarrow 2\ CH_3COO^- + HS^- + H^+ + 2\ H_2O \qquad (1)$$

Complete ethanol removal was observed 10 days after its addition into the SAMBaR (Fig. 4B). The full removal of ethanol at a maximal concentration of 5950 $mgCOD.L^{-1}$ on day 91 (Fig. 4B) at a flux of 17.1 $L.m^{2-}.h^{-1}$ (HRT of 9.7 hours; Fig. 2A) indicates that the reactor was operated at underloaded conditions. In addition, it shows that the biomass had a higher affinity for ethanol than for acetate (Fig. 4A vs. Fig. 4B). Under these operational conditions, a maximal sulfate elimination rate of 6.60 g $SO_4^{-2}.L^{-1}.d^{-1}$ proceeded on day 92 (Fig. 4B). The sulfate reduction is correlated to ethanol oxidation, as evidenced by the sharp drop in the sulfate elimination rate between days 86 and 91 (Fig. 4B), when the influent ethanol

concentrations were much lower (Fig. 4B). This is confirmed by the results in Fig. 4C, which shows that the stoichiometry of ethanol utilization closely followed that of Equation 1, with ~ 0.5 mole sulfate reduced, and ~ 1.0 mole acetate produced per mole of ethanol utilized.

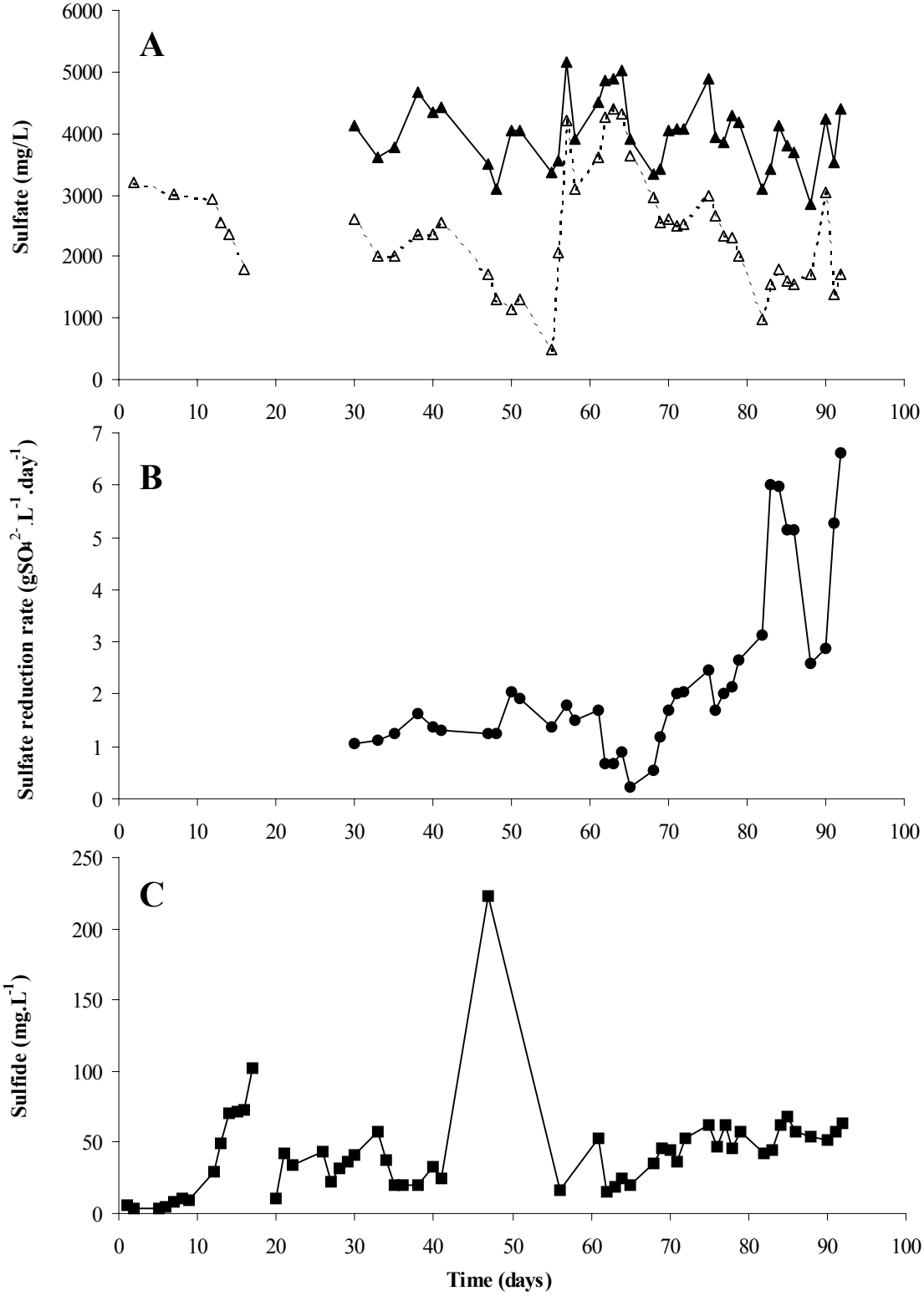

Figure 5. Process performance of the SAMBaR. (A) Evolution of the sulfate concentration in the influent (▲) and effluent (△). (B) Evolution of the sulfate reduction rate (●). (C) Evolution of the sulfide concentration in the effluent (■).

Due to the high gas loading rate (to clean up the membranes), the sulfide concentration remained rather constant at concentrations around 80-100 $mg.L^{-1}$ during the whole experimental run (Fig. 5C). An exceptional sulfide peak manifested around day 47 (Fig. 4C), when the gas load in the SAMBaR was unintentionally very low.

Reactor biomass characteristics

Solids concentration and specific biomass activity

The TSS and VSS concentration in the mixed liquor present in the reactor could not be measured during the first days of SAMBaR operation, as this required a too big reactor liquid sample (due to the dilute nature of the freshly inoculated reactor mixed liquor at the beginning of the experiment) for the solids determination. The TSS and VSS concentrations increased from day 55 onwards till a maximal concentration of around 0.85 g $VSS.L^{-1}$ and 1.75 g $TSS.L^{-1}$ on day 91 (Fig. 6A). The VSS/TSS ratio remained fairly constant at around 0.4 (± 0.09), except at the beginning of the experiment and on day 68, when the VSS/TSS ratio was equal to 0.10 and 0.27, respectively. The specific activity of the sludge was very high with values of 5.5 (± 1.0) g $SO_4^{2-}.gVSS^{-1}.day^{-1}$ between days 55 and 92 experiment (Fig 6B), irrespective of the sulfate removal efficiency found (Fig. 5A).

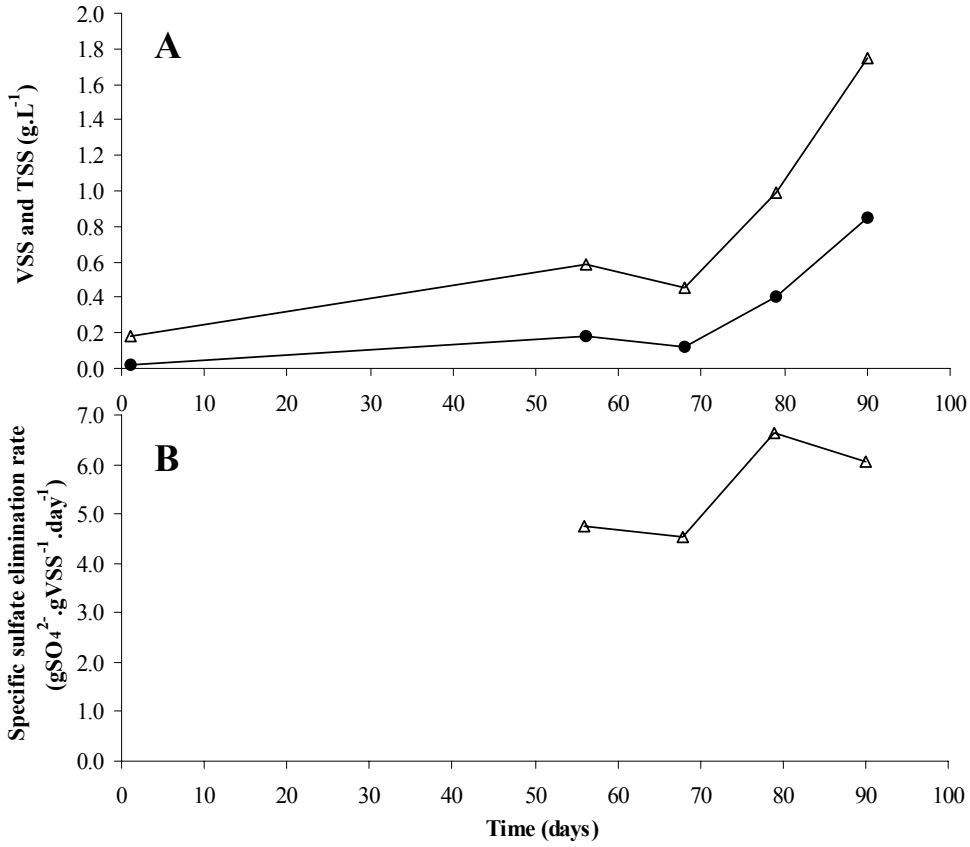

Figure 6. Process performance of the SAMBaR. (A) Evolution of the total (△) and volatile (●) suspended solids in the reactor mixed liquor. (B) Evolution of the specific sulfate reduction rate (△).

Particle size distribution

The results of the particle size distribution measurements of the inoculum show that 90 % of the particles were bigger than 38 μm and particles smaller than 0.2 μm, the size of the membrane pore, were absent (Fig. 7A). After 50 days of operation, 90 % of the particles were bigger 70 μm, whereas only 0.31% of the particles were smaller than 1 μm (Fig. 7B) and only 0.0043 % of the particles were smaller than 0.2 μm (Fig. 7B). The mean particle size of the inoculum and the SAMBaR mixed liquor on day 50 was, respectively, 370.8 and 463.2 μm, with no particles bigger than 2000 μm (the upper detection limit of the equipment). The SAMBaR sludge flocs contained many blackish spots, most probably metal precipitates. Surprisingly, the particle size distribution could not be measured anymore by laser difraction on day 56, as a small fraction of the particles surpassed the upper detection limit of the equipment (2000 μm).

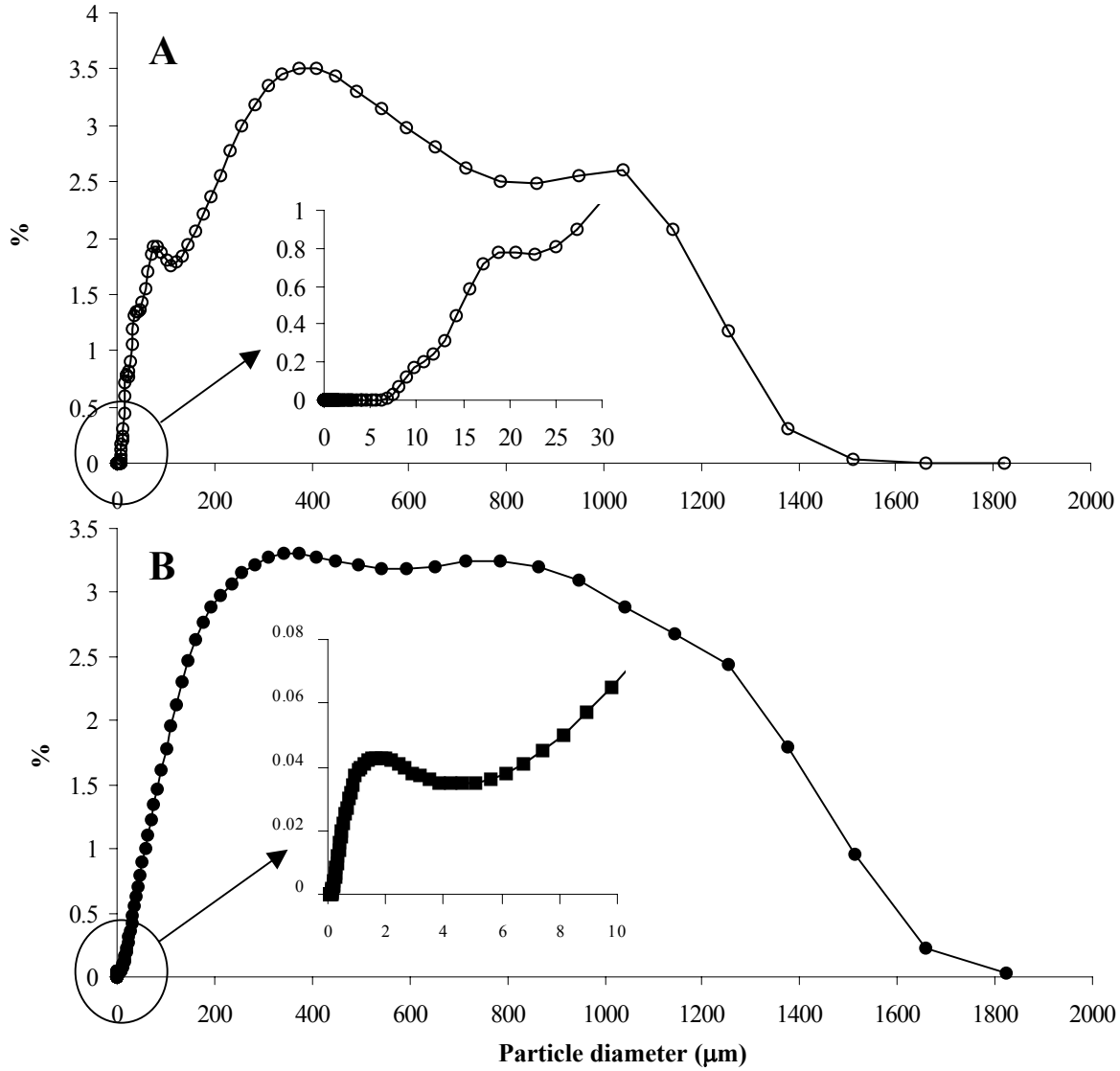

Figure 7. Particle size distribution. (A) Reactor inoculum consisting of a pure culture of *Desulfobacter halotolerans*. (B) Reactor mixed liquor sampled on day 56.

Microscopic observations

Fig. 8 shows the morphology of a pure culture of *Desulfobacter halotolerans* and the biomass that grew in the SAMBaR. Although the SAMBaR sludge contained bacteria other than *D. halotolerans* (Fig. 8B), *D. halotolerans* still accounted for most of the microorganisms present. In addition, many crystals, presumably metal sulfides, were present in the SAMBaR sludge.

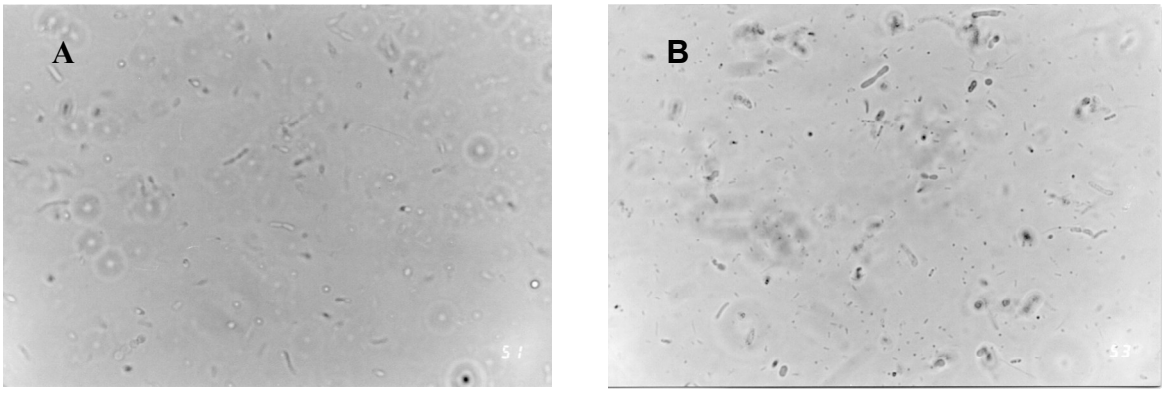

Figure 8. Microscopic pictures of the biomass. (A) Reactor inoculum consisting of a pure culture of *Desulfobacter halotolerans*. (B) Microorganisms present in the reactor mixed liquor sampled on day 64.

Membrane operation and fouling experiments

Critical flux and TMP increase rate dependence on flux

No severe increase in the TMP was observed as a function of the stepwise increase of the flux (up to 80 $L.m^{-2}.h^{-1}$) when only basal medium was present in the SAMBaR (data not shown). It was also observed that each increase in the flux produced an equal increase in the TMP. In addition, a relatively low TMP (0.15 bar) was observed for the maximal applied flux of 80 $L.m^{-2}.hour^{-1}$ when only basal medium was added to the SAMBaR.

According to Chen *et al.* (1997), the critical flux is defined as the last flux step at which the TMP remains constant. A closer examination of the initial flux steps, however, reveals that the TMP never remains absolutely constant at any point during the test (Fig. 9A). Even a flux as low as 3 $L.m^{-2}.h^{-1}$ produced a TMP increase rate (dP/dt) of 3.7 $mbar.day^{-1}$ (Fig. 9B). Fluxes higher than 18 to 21 $L.m^{-2}.hour^{-1}$ caused a rapid increase in the TMP (Fig. 9A), resulting in very high dP/dts (Fig. 9B). As such, the value of the critical flux for the crushed sludge was determined to be between 18 to 21 $L.m^{-2}.hour^{-1}$, corresponding to a TMP of about 0.16 to 0.18 bar (Fig. 9A). An overall dP/dt of 27.6 $mbar.day^{-1}$ was obtained for fluxes below 15 $L.m^{-2}.h^{-1}$, whereas a very high dP/dt of 692.4 $mbar.day^{-1}$ was obtained when operating at fluxes higher than 18 $L.m^{-2}.h^{-1}$ (Fig. 9B). The maximal TMP of 1 bar was reached at a flux of 30 $L.m^{-2}.hour^{-1}$ and the TMP started to decrease only when the flux was diminished to 15 $L.m^{-2}.hour^{-1}$ (Fig. 9A).

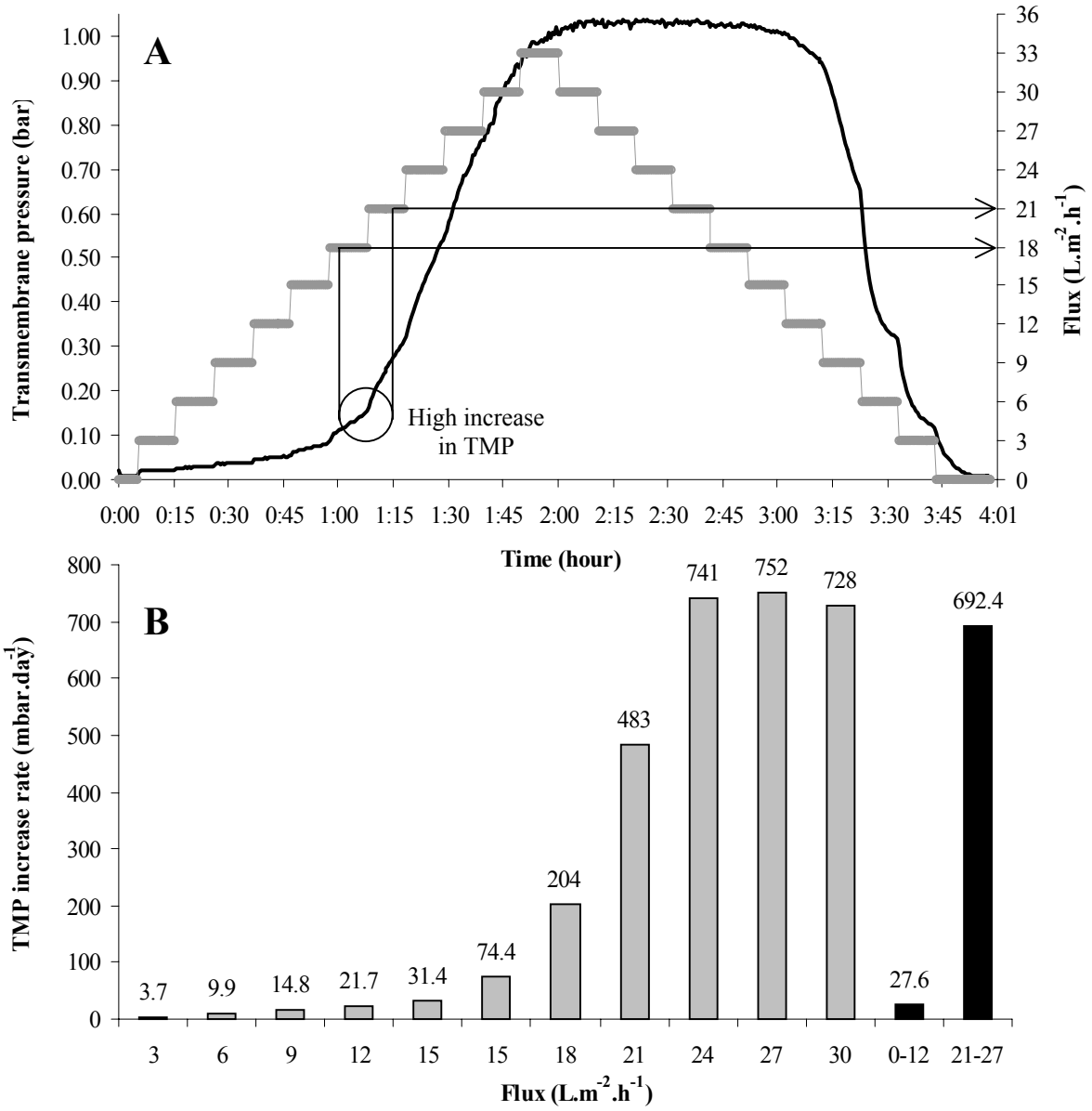

Figure 9. Critical flux experiments. (A) Evolution of the transmembrane pressure in function of the applied flux. (B) Calculated TMP increase rate in function of the applied flux.

Occurrence of membrane fouling in the SAMBaR

Flux of 4.7 $L.m^{-2}.h^{-1}$: Fig. 10A shows the full set of TMP values obtained from the operation of the SAMBaR. From day 19 till day 22 a constant flux of 4.7 $L.m^{-2}.h^{-1}$ was applied to the reactor (Fig. 10B), resulting in a TMP increase rate (dP/dt) of about 13 $mbar.day^{-1}$ (Table 2). On day 22, however, a constant permeate flux (no relaxation or backflush) was imposed to the reactor, resulting in an immediate increase of the dP/dt to 137 $mbar.day^{-1}$ (Table 2). The relaxation/production operational mode was resumed on day 23. On this day, however, a low influent gas load (3 $L.L^{-1}.h^{-1}$) was imposed to the reactor, resulting in a dP/dt of around 92 $mbar.day^{-1}$ (Table 2).

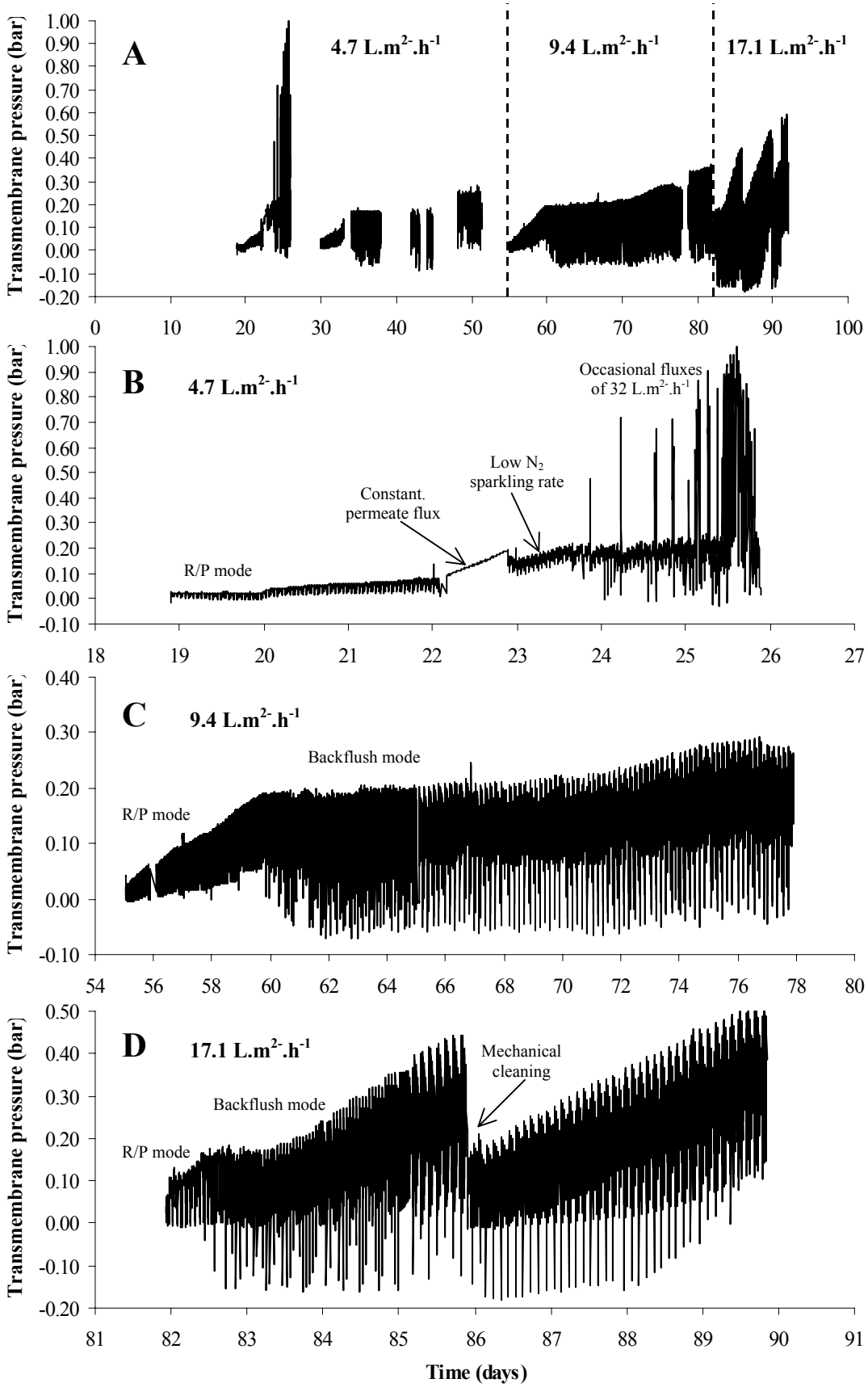

Figure 10. Evolution of the transmembrane pressure. (A) TMP values during the whole experimental run. (B) TMP values between days 19 and 26 (flux of 4.7 $L.m^{-2}.h^{-1}$). (C) TMP values between days 54 and 78 (flux of 9.4 $L.m^{-2}.h^{-1}$). (D) TMP values between days 82 and 90 (flux of 17.1 $L.m^{-2}.h^{-1}$). Note the differences in the TMP and time scales.

Table 2. TMP increase rates (dP/dt; mbar.day^{-1}) according to the flux and the different operational procedures to minimise fouling.

J = 4.7 L.m^{2-}.h^{-1}				**J = 9.4 L.m^{2-}.h^{-1}**				**J = 17.1 L.m^{2-}.h^{-1}**					
Production/ relaxation mode		**Backflush mode/ chemical cleaning** (3)		**Production/ relaxation mode**		**Backflush mode/ chemical cleaning** (3)		**Production/ relaxation mode**		**Backflush mode/ chemical cleaning**		**Backflush mode/ mechanical cleaning** (4)	
Day	**dP/dt**	**Day**	**dP/dt**	**Day**	**dP/dt**	**Day**	**dP/dt**	**Day**	**dP/dt**	**Day**	**dP/dt**	**Day**	**dP/dt**
10	13	33-38	-15.3	55-60	27.8	61	-50.5	82	129	83	-41.2	86-89	70.2
22	137 (1)	48-54	4.5			62-70	5.1			84	39	90-92	104.3
23	92 (2)					71-81	10.3			85	75.4		
26-32	18.5												

(1) Constant permeate flux

(2) Low N_2 gas sparkled in the reactor (20 L.h^{-1})

(3) Membranes chemically cleaned on days 54 and 81

(4) Membranes mechanically cleaned on days 85 and 89

J – Flux

R/P – relaxation production

The SAMBaR started to operate in backflush mode on day 25 (Fig. 10B). Due to improper input of information in the computer control, occasional fluxes of 32 $L.m^{2-}.h^{-1}$ were imposed to the membranes during the 2 minutes reserved for the compensation of flow after backflushing the membranes (see material and methods section). These occasional high fluxes caused an immediate increase in the TMP to values around 1 bar, which caused an irreversible mechanical collapse of the membrane (Fig. 10B). Note that this occasional flux of 32 $L.m^{-2}.h^{-1}$ is higher than the critical flux of 18 to 21 $L.m^{2-}.h^{-1}$ determined for crushed anaerobic sludge (Fig. 9A).

The SAMBaR operated in relaxation-production mode from day 26 to 32 (after replacing the membrane on day 26), resulting in a dP/dt of around 18.5 $mbar.day^{-1}$ (Table 2). The SAMBaR operated in backflush mode between days 33 to 54 (Fig. 10B). Surprisingly, the TMP diminished in the first days after switching to backflush mode, as indicated by the negative dP/dt of -15 $mbar.day^{-1}$ (Table 2). On day 48, however, the dP/dt started to increase again to values around 4.5 $mbar.day^{-1}$ (Table 2).

Flux of 9.4 $L.m^{-2}.h^{-1}$: The reactor operated in relaxation-production mode from day 55 to 60, resulting in a dP/dt of around 27.8 $mbar.day^{-1}$ (Table 2). As for when operating at a flux of 4.3 $L.m^{-2}.h^{-1}$, the TMP diminished in the first days after the operation of the SAMBaR switched to backflush mode on day 61 (Fig. 10C), as indicated by the negative values of the dP/dt of -50 $mbar.day^{-1}$ (Table 2). On day 62, however, the dP/dt started to increase again, to values around 5.1 and 10.3 $mbar.day^{-1}$ between days 62-70 and between days 71-81, respectively (Table 2).

Flux of 17.1 $L.m^{-2}.h^{-1}$: The reactor operated in relaxation-production for only one day and experienced a high dP/dt of 129 $mbar.day^{-1}$ (Table 2). Again, the TMP dropped after the operation of the SAMBaR switched to backflush mode on day 83 (Fig. 10D), as indicated by the negative values of the dP/dt (–41.2 $mbar.day^{-1}$; Table 2). The dP/dt started to increase again on day 84 up to 75.4 $mbar.day^{-1}$ (Table 2). On days 86 and 90, the membranes were mechanically cleaned by gentle displacement of the membrane cake with a brush (Fig. 10D). The dP/dts measured on day 86 and 90 were equal to, respectively, 70.2 and 104.3 $mbar.day^{-1}$ (Table 2).

DISCUSSION

Reactor Performance

This paper clearly shows that high rate sulfate reduction at salinities of 50 g $NaCl.L^{-1}$ and 1 g $MgCl_2.6H_2O.L^{-1}$ (60-70 $mS.cm^{-1}$) can be achieved by using a submerged anaerobic membrane bioreactor (SAMBaR) inoculated with a pure culture of the halophilic SRB *Desulfobacter halotolerans* using acetate or ethanol as electron donors. The high salt

tolerance reported in this paper has significant practical implications as it enables the direct treatment of sulfate rich brines without prior dilution, thus enabling the direct application of SRB based bioreactors in closed cycles. It is worth mentioning that the substrate spectrum of *D. halotolerans* is broader than merely sulfate, and also includes sulfite and thiosulfate. Thus, it can also be adopted in processes where these compounds are dominant, e.g. in scrubbed waters from flue gas desulfurization systems and in photographic effluents, respectively.

The maximal sulfate reduction rate of 6.6 g $SO_4^{2-}.L^{-1}.day^{-1}$ (at a flux of 17.1 $L.m^{-2}.h^{-1}$ and a HRT of 9.7 hours) found in this work (Fig. 5B) is comparable to sulfate reduction rates reported for ethanol-fed immobilized biomass reactors operated at low salinity. The highest sulfate reduction rate reported so far in ethanol-fed sulfidogenic reactors is 9.9 g $SO_4^{2-}.L^{-1}.day^{-1}$ for an ethanol-fed mesophilic expanded granular sludge bed (EGSB) reactor (HRT = 5 to 6 hours) operated at low salinity (de Smul *et al.* 1997). Nagpal *et al.* (2000) obtained sulfate elimination rates up to 6.33 g $SO_4^{2-}.L^{-1}.day^{-1}$ in an ethanol-fed recirculating CSTR vessel and fluidized bed reactor operated at a HRT of 5.1 hours and inoculated with a mixed culture of SRB (*Desulfovibrio desulfuricans* and *Desulfobacter postgatei*) immobilized on porous glass beads. According to a mathematical model the low volume of the bed relative to the total liquid volume of the system (V_{bed}/V_{total} = 0.074) was the limiting factor in the sulfate elimination rate of the fluidized bed reactor (Nagpal *et al.*, 2000).

Kalyuzhnyi *et al.* (1997) achieved a sulfate reduction rate of 6 g $SO_4^{2-}.L^{-1}.day^{-1}$ in an ethanol-fed UASB reactor operated at a HRT of 20 hours and the system was found to be limited by sulfide toxicity (180 $mg.L^{-1}$ undissociated H_2S) of acetotrophic SRB. Such concentrations of undissociated H_2S are known to inhibit acetotrophic SRB (O'Flaherty and Colleran, 2000). In the present work, sulfide toxicity hardly could occur as the sparkling of the reactor mixed liquor with N_2 to minimize membrane fouling provided an excellent H_2S stripping, thus avoiding the build up of sulfide in the reactor mixed liquor (Fig. 4C). Results obtained by de Smul *et al.* (1997) show that a remarkable increase in the sulfate removal rate can be achieved in a ethanol-fed EGSB reactor after stripping sulfide with N_2 and by controlling the reactor pH above 7.75. Prevention of H_2S toxicity is particularly important in bioreactors using cell suspensions as H_2S can cause acute toxicity to SRB without any recovery (Lens *et al.*, 2003). The stripping effect of the gas sparkling is thus of paramount importance and circumvents the need of other H_2S removal methods, as e.g. extractive membranes (de Smul and Verstraete, 1999) or the formation of iron sulfide precipitates (McFarland and Jewell, 1989).

The maximal specific sulfate elimination rate of 6.64 g $SO_4^{2-}.gVSS.day^{-1}$ found in this work (Fig. 6B) is significantly higher than those obtained in any previous investigations in sulfidogenic bioreactor configurations, either using ethanol or different electron donors (Table 3). A possible explanation for this difference is that only part of the biomass in the granules or (thick) biofilms participate in the sulfate reduction process when the reactor configuration relies on granules or (thick) biofilms, as in UASB, EGSB, fixed or fluidized bed reactors.

Table 3 Maximal specific sulfate reduction rate from biomass of anaerobic sulfidogenic bioreactors

Reactor Concept	Temperature (°C)	pH	HRT (hours)	Substrate	Sulfate reduction rate ($gSO_4^{2-}.gVS^{-1}.day^{-1}$)	Reference
CSTR vessel + fluidized bed reactor	rt	6.9	5.1	ethanol	6.0 – 18.9 (1)	Nagpal *et al.* 2000
EGSB	30 to 35	7.8 to 8.3	5 to 6	ethanol	0.95 (2)	de Smul *et al.* 1997
EGSB	65	7.5	3	methanol	1.22 (3)	Weijma *et al.*, 2000b
UASB	32 ± 1	8.3	2	acetate	0.64 (4)	Muthumbi *et al.*, 2001
Gas lift (with pumice stones)	55	7.0	4.5	hydrogen	3.75 (5)	van Houten *et al.*, 1997
Gas lift (with pumice stones)	35	7.0	2.25 to 4.5	hydrogen	4.2 (6)	van Houten *et al.*, 1997
SAMBaR	33 ± 1	7.2 ± 0.1	9.5	ethanol	6.64	this work

rt – room temperature

(1) The specific rate was calculated from the reported 0.07 g-0.22 $gSO_4^{2-}.g\ protein^{-1}.h^{-1}$ and the reported ratio of 0.278 g protein/g biomas dry weight.

(2) The specific rate was calculated from the final concentration of 10.5 $gVS.L^{-1}$ and the maximal sulfate reduction rate (9.9 $gSO_4^{2-}.L^{-1}.day^{-1}$) reported by the authors

(3) The specific rate was calculated from the final concentration of 9 to 10 $gVSS.L^{-1}$ in the reactor and the maximal sulfate reduction rate (11 $gSO_4^{2-}.L^{-1}.day^{-1}$) reported by the authors

(4) The specific rate was calculated from the initial concentration of 21.7 $gVSS.L^{-1}$ (assuming no growth or loss of biomass) and the maximal sulfate reduction rate (14 $gSO_4^{2-}.L^{-1}.day^{-1}$) reported by the authors.

(5) The specific rate was calculated from the reactor mixed liquor concentration of 1.2 $gVSS.L^{-1}$ and the maximal sulfate reduction rate (7.5 $gSO_4^{2-}.L^{-1}.day^{-1}$) reported by the authors.

(6) The specific sulfate elimination rate was calculated from the reported 1.4 g $S.gbiomass^{-1}.day^{-1}$ in van Houten *et al.*, 1997 (in reference to van Houten *et al.*, 1995). We assume 1 g biomass equal to 1 g VSS.

The results in Table 3 show that high specific sulfate reduction rates only were achieved for hydrogen fed gas lift reactors. The poor aggregation of SRB on pumice stones, used as inorganic carrier, in these gas lift reactor, resulted in the formation of thinner biofilms and therefore, an overall more active biomass (van Houten *et al.*, 1997). Nagpal *et al.* (2000) also noticed the substrate diffusion limitations in biofilms and the presence of dead/inactive (inert) biomass in the sludge of an ethanol-fed fluidized bed reactor. This was based on the differences between the specific sulfate reduction rates found in batch growth experiments (0.15-1.34 g SO_4^{2-}.g protein^{-1}.day^{-1}) compared to those found in the reactor (0.07 g - 0.22 g SO_4^{2-}.g protein^{-1}.day^{-1}). In the present work, the absence of inorganic carrier induced the growth of small bioparticles (Fig. 7B), conceivably diminishing the substrate limitation transport phenomena as reported for aggregates bigger than 0.5 mm (van Houten *et al.*, 1995). Thus, bioreactors systems which apply the concept of suspended growth offer the advantage that they cultivate biomass with very high specific sulfate reduction rates. Remains to be answered, however, what will be the type of biomass (and specific sulfate reduction activity) that develops in long reactor runs.

The observed fairly constant specific sulfate reduction rate of 5.5 g SO_4^{2-}.gVSS^{-1}.day^{-1} shows that the performance of the reactor was limited by the low amount of biomass (0.85 gVSS.L^{-1}; Fig. 5A) present in the reactor. It is well known that membrane bioreactors can be operated at much higher solid concentrations and mixed liquor suspended solids (MLSS) for aerobic membrane bioreactors typically range from 3 to 31 g.L^{-1} (Stephenson *et al.*, 2000). The low biomass concentration in the SAMBaR, that was never bled during the experiment, is due to the very low growth rate of *D. halotolerans*, equal to about 36 hours at 5 % salinity (Ingvorsen, personal communication). As such, it can be expected that the capacity of the reactor to be increased further by allowing the biomass to grow to higher VSS concentrations in the reactor. Paulo *et al.* (2003) developed a system that allows SRB to grow continuously at their near-maximum μ_{max} based on a limiting substrate dosing regime. With the adoption of this substrate dosing regime to a *D. halotolerans* inoculated SAMBaR would enable to develop and maintain much higher biomass concentrations in a short period of time.

The sulfate elimination rate in principle can also be increased by the reduction of the HRT, but this investigation shows that increasing the flux to values close to or beyond the nominal critical flux is highly detrimental to the operation of the membranes (Fig 10B). However, taking into account that a membrane surface area to reactor volume of only 0.011 m^{-2}.L^{-1} was available in the experimental rig, this surely can be improved by adjusting the reactor design. This can be achieved by constructing a new experimental rig equipped with a high membrane surface area to reactor volume, thus enabling a decrease of the HRT while operating the SAMBaR at very low fluxes. Another alternative would be the adoption of membranes with a bigger pore size than that adopted in this work, thus decreasing the pressure drop without compromising the biomass separation.

Metabolical characteristics of the sludge

The results showed that ethanol was incompletely oxidized by *D. halotolerans*, and the stoichiometry of ethanol utilization followed closely that of Equation 1, with about 0.5 mol sulfate reduced and 1 mol of acetate produced per mol of ethanol utilized (Fig. 4C). The higher affinity of the biomass for ethanol found in the present work contrasts with the findings of Brandt and Ingvorsen (1997) who found that, rather than ethanol, acetate is the preferential substrate for *D. halotolerans*. When grown on ethanol, cell yields were only 30 % of acetate grown cultures, but intense sulfide production is reported when using ethanol as the substrate (Brandt and Ingvorsen, 1997). As such, in case a full COD removal is also required in the sulfate reducing ethanol fed SAMBaR reactor, it must be taken into account that the acetate oxidation is the rate limiting step and therefore the rate of acetate degradation will define the design of the ethanol-fed sulfate reducing reactor. A similar observation with respect to the big importance of the acetate degradation rate on the reactor performance has been reported for methanol fed thermophilic (Weijma *et al.*, 2000; Chapters 4, 5 and 6) and VFA fed mesophilic (Omil *et al.*, 1998) reactors.

Operational strategies

The results of the present investigation show that anaerobic membrane bioreactors can be operated over extended periods of time at a fixed flux provided that this flux is substantially below the nominal critical flux determined experimentally (18-21 $L.m^{-2}.h^{-1}$). However, it must be taken into account that even below the nominal critical flux the transmembrane pressure tends to rise slowly (Fig. 10B and 10C). Operating membrane bioreactor at fluxes higher than the critical flux must be avoided at any price, otherwise the TMP will than raise dramatically, resulting in a collapse of the membrane (Fig. 10B). Turbulence induced by the sparkling of nitrogen gas is beneficial for the operation of the membranes for extended periods of time. According to Chang *et al.* (2002), the injection of coarse gas in membrane bioreactors keeps the solids in suspension and scours the membrane surface, suppressing fouling. Indeed, the results of this work show that a 4 to 5 times increase in the TMP increase rate when the SAMBaR was not mixed with nitrogen gas (Table 2 and Fig. 10B). The constant permeate flux on day 22 resulted in the TMP increase rate within 6 to 11 times compared to operating the SAMBaR in relaxation/production or backflush mode, respectively (Table 4.2 and Fig. 10B). The intermittent operation mode (Cho and Fane, 2002) as well as the backflush operation mode of the membranes (Lee *et al.*, 2001) have been reported to slow the fouling rate in membrane filtration of biomass. Based on the results of this work, it looks attractive to operate anaerobic membrane bioreactor with the occasional backflush of the membranes. If backflush is adopted as the operational strategy to minimize

fouling at a flux of 4.7 $L.m^{-2}.h^{-1}$, chemical cleaning of the membranes will be required only at about 106 days (adopting a TMP increase rate of 4.5 $mbar.day^{-1}$; Table 2).

Future research is required to further optimize the system both with respect to the required time as well as the frequency of the backflush operation. In addition, the optimization of the gas loading rate as well as the improvement of reactor design are required. This will improve the contact of the coarse bubble gas (which cause the scour of the membrane) with the set of membranes, thus further reducing the membrane fouling. The dP/dt values measured for each flux applied during the critical flux experiments are not equivalent to the values found the long term operation in the SAMBaR (Fig. 9B vs Table 2). However, the dP/dt values obtained in the critical flux test indicates at what flux in the SAMBaR fouling starts to become severe. As such, the flux-step method is a valuable tool to determine the operational conditions for the operation of membrane bioreactors.

CONCLUSIONS

The results of this work allow to conclude that:

(1) High rate sulfate reduction (6.6 g $SO_4^{2-}.L^{-1}.day^{-1}$ at a HRT of 9 hours) at salinities of 50 g $NaCl.L^{-1}$ and 1 g $MgCl_2.L^{-1}$ (60-70 $mS.cm^{-1}$) can be achieved in a submerged anaerobic membrane bioreactor (SAMBaR) inoculated with a pure of the halophilic SRB *Desulfobacter halotolerans* using either acetate or ethanol as electron donor.

(2) The rather constant very high specific sulfate reduction rate of 5.5 g $SO_4^{2-}.gVSS^{-1}.day^{-1}$ found indicate that the performance of the reactor was limited by the low amount of biomass (0.85 g $VSS.L^{-1}$) present in the SAMBaR.

(3) Sulfate reducing submerged anaerobic membrane bioreactors can be operated over extended periods of time without chemical cleaning of the membranes at a certain fixed flux provided that this flux remains well below the nominal critical flux determined experimentally (18-21 $L.m^{-2}.h^{-1}$).

(4) Intermittent operation as well as backflush of the membranes slow down the fouling of the membranes. Frequent backflush (e.g. 1 minute each 10 minutes) is the suggested operational strategy to minimize fouling in anaerobic MBRs.

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Chapter/Hoofdstuk 10

Summary and general discussion

Samevatting en discussie

MAIN FINDINGS

Chapter 3: Methanol was fully converted (organic loading rate (OLR) up to 20 $gCOD.L^{-1}.day^{-1}$; pH 7.0 ± 0.2; hydraulic retention time (HRT) of 7.5 hours) both via sulfate reduction (up to 13 % when operating at a COD/SO_4^{2-} of 5) and methanogenesis (85 %) in a thermophilic UASB reactor fed with increasing sulfate concentrations. Surprisingly, the sulfate reduction efficiency of the system strongly deteriorated when it was operated in excess of sulfate (COD/SO_4^{2-} of 0.5), which likely can be due to the poor immobilization of SRB in the sludge bed and the presence of relatively high sodium concentrations (about 6 g $Na^+.L^{-1}$) originating from the supply of sulfate as its sodium salt. Activity tests showed that methanol was converted syntrophically via H_2/CO_2 by homoacetogenic bacteria, in combination with either SRB or MPA.

Chapter 4: A full methanol and formate degradation was achieved at temperatures up to, respectively, 70 and 75°C, when operating UASB reactors (pH 7.5 ± 0.2) with sulfate rich (COD/SO_4^{2-} = 0.5) synthetic wastewater. MPA outcompeted SRB in the formate-fed UASB reactor at all temperatures tested (65 - 75°C). In contrast, SRB outcompeted MPA in methanol-fed UASB reactors at temperatures equal or exceeding 65°C, whereas a strong competition between SRB and MPA was observed in the reactors at a temperature of 55°C. Based on the very high sulfate reduction rate found, i.e. 14.5 g $SO_4^{2-}.L^{-1}day^{-1}$, and the complete absence of methane and acetate production, it looks that 70°C is the most attractive operational temperature for methanol-fed sulfate reducing reactors. A short term (2 days) temperature increase from 55 to 65-70°C was found to be an effective strategy to suppress methanogenesis in methanol-fed sulfidogenic UASB reactors operated at 55°C. However, the process of acetogenesis resumed after resetting the temperature to 55°C. Inhibition of the sulfidogenesis by NaCl manifested in a methanol-fed sulfidogenic UASB reactor at concentrations as low as 10 $g.L^{-1}$. Batch experiments demonstrated that SRB (mainly hydrogenotrophic) are the most NaCl (25 $g.L^{-1}$) sensitive microorganisms in the sludge cultivated at 70°C, and that acetogenesis was observed after a 10 days period of lag-phase.

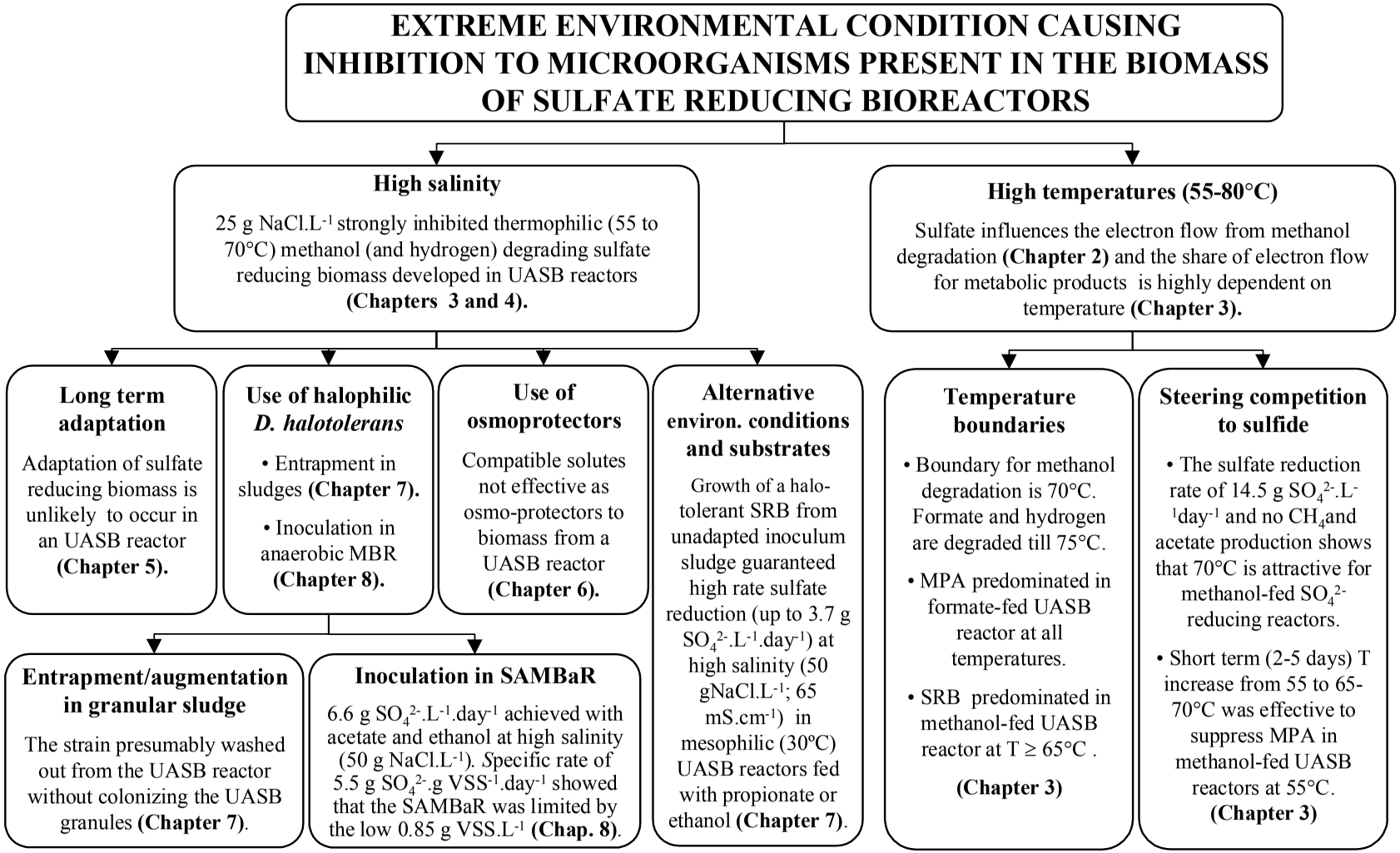


Figure 1. Overview of the structure of this thesis and main results obtained.

Chapter 5: Methanol was almost completely used for sulfate reduction in UASB reactors (COD/SO_4^{2-} of 0.5; pH 7.5 ± 0.2; OLR of 5 g $COD.L^{-1}.day^{-1}$, HRT of 10 hours) in the case the feed did not contain NaCl. In this work, the SRB outcompeted MPA for methanol at 55°C, which contrasts with previous experiments conducted at 55°C (**Chapter 4**) where the strong competition between SRB and MPA was found. Acetate was produced as a side-product, accounting for maximal 25 % of the total COD converted. The accumulation of acetate resulted from the fact that either MPA or SRB were unable to use acetate as substrate in activity tests. A high NaCl concentration of 25 $g.L^{-1}$ completely inhibited the methanol degradation, whereas a low NaCl concentration 2.5 $g.L^{-1}$ provoked considerable changes in the metabolic fate of methanol. The MPA were most sensitive for the 25 g $NaCl.L^{-1}$ shock, while the addition of only 2.5 $g.L^{-1}$ of NaCl apparently stimulated MPA and homoacetogenic bacteria (AB).

Chapter 6: The adaptation of thermophilic (55°C) sulfidogenic methanol degrading biomass to a high osmolarity environment apparently did not proceed in an UASB reactor (pH 7.5 ± 0.2; HRT of 10 hours) fed solely with methanol and in excess of sulfate (COD/SO_4^{2-} of 0.5). Even though sulfide was the main mineralization product from methanol degradation, regardless of the NaCl concentration present in the influent, the sulfide production in the system steadily decreased following the addition of 7.5 g $NaCl.L^{-1}$, whereas the production of acetate was stimulated. Batch activity tests performed with sludge samples harvested from the UASB reactor when the system was operated at different influent salinities indeed confirmed that acetate was the main metabolic product at NaCl concentrations higher than 12.5 $g.L^{-1}$. The apparent order of NaCl toxicity towards the different trophic groups is: SRB > MPA > AB.

Chapter 7: The addition of different osmoprotectants, viz. glutamate, betaine, ectoine, choline, a mixture of compatible solutes and K^+ and Mg^{2+}, slightly increased methanol depletion rates for sludge samples harvested from a thermophilic (55°C) sulfate reducing (COD/SO_4^{2-} of 0.5) UASB reactor. However, this higher methanol depletion rate mainly favored the homoacetogenic bacteria, as the methanol breakdown was steered to the formation of acetate without increasing sulfate reduction and methane production rates. Thus, none of these compounds were effective as osmoprotectants to alleviate the acute NaCl toxicity on sulfate reducing granular sludges samples harvested from a methanol degrading UASB reactor.

Chapter 8: In contrast to the unsuccessful adaptation of thermophilic (55-70°C) methanol oxidizing sulfidogenic biomass to NaCl observed in **Chapters 4, 5 and 6,** the proliferation of a halotolerant mesophilic SRB population present in the unadapted inoculum sludge (the same used in **Chapter 4**) guaranteed fairly high rate sulfate reduction (up to 3.7 g $SO_4^{2-}.L^{-1}.day^{-1}$) at a high salinity of 50 g $NaCl.L^{-1}$ and 1 g $MgCl_2.L^{-1}$ in mesophilic UASB

reactors (pH 7.0 ± 0.2; HRT from 8 to 14 hours) fed with acetate, propionate or ethanol in excess of sulfate (COD/SO_4^{2-} of 0.5). A considerable sulfate reduction even proceeded at a rate of 1.40 g $SO_4^{2-}.L^{-1}.day^{-1}$ at salinities of up to 70 g $NaCl.L^{-1}$ and 1 g $MgCl_2.L^{-1}$ (corresponding to a conductivity of about 85 to 90 $mS.cm^{-1}$). Ethanol as well as propionate were suitable substrates for sulfate reduction, although acetate accounted as one of the end products. Attempts to entrap and/or immobilize the acetate oxidizing halotolerant SRB *Desulfobacter halotolerans* in this sludge was unsuccessful, because the strain washed out from the UASB reactor without any clear colonization of the UASB granules.

Chapter 9: Sulfate reduction rates up to 6.6 g $SO_4^{2-}.L^{-1}.day^{-1}$ were achieved in a submerged anaerobic membrane bioreactor (SAMBaR; pH 7.2 ± 0.2; HRT from 8 to 36 hours) inoculated solely with the halotolerant sulfate reducing bacterium *Desulfobacter halotolerans* and fed with acetate and ethanol in saline medium (50 g $NaCl.L^{-1}$ and 1 g $MgCl_2.6H_2O.L^{-1}$; conductivity 60-70 $mS.cm^{-1}$). Based on the assessed fairly constant high specific sulfate reduction rate of 5.5 g $SO_4^{2-}.gVSS^{-1}.day^{-1}$, it can be concluded that the performance of the reactor was limited by the low amount of viable biomass (0.85 $gVSS.L^{-1}$) was present at the termination of the experiment. It was also found that sulfate reducing submerged anaerobic membrane bioreactors can be operated over extended periods of time (around 100 days) without chemical cleaning of the membranes at a certain fixed flux, provided that this flux is substantially below the nominal critical flux determined experimentally (18-21 $L.m^{-2}.h^{-1}$). Intermittent operation as well as backflush of the membranes were shown to slow down the fouling in the membranes. Frequent backflush (e.g. 1 minute each 10 minutes) can be recommended as an operational strategy to minimize fouling in sulfate reducing MBRs.

GENERAL DISCUSSION

Operation of sulfate reducing UASB reactors under extreme conditions (high salinity and high temperature)

Start-up of UASB reactors (direct exposure vs. stepwise exposure)

The efficiency of the strategy of a stepwise increase of the salt concentration to acclimatize the sludge to high salinity, resulting presumably in gradual selection for salt tolerant microorganisms in an initially non-adapted inoculum sludge, as proposed by Omil *et al.* (1995) and Feijoo *et al.* (1995), looks questionable. The results obtained in our investigations **(Chapters 4, 5, 6 vs. Chapter 8)** indicate that the appearance of a targeted metabolic property (sulfate reduction at high salinity) is independent of the strategy for biomass acclimation. The occurrence of the stepwise adaptation of thermophilic sulfidogenic methanol degrading biomass to a high osmolarity environment, both at 55°C **(Chapters 6)** or

at 70°C **(Chapter 4)**, likely does not occur in UASB reactors, as probably no methanol halotolerant thermophilic SRB were present in the thermophilic inoculum sludge used in these investigations (Table 1). On the other hand, **Chapter 8** shows that exposing the sludge (the same one used in **Chapter 4**) directly to a very high salinity (50 g $NaCl.L^{-1}$) stimulated the growth of a mesophilic (propionate- and ethanol-utilizing) halotolerant SRB population, which supported the high rate sulfate reduction (up to 3.6 $gSO_4^{2-}.L^{-1}.day^{-1}$) in a UASB reactor. Therefore, the key for the successful treatment of high salinity wastewater is to invest enough time for the growth of the targeted microorganism in the biomass. As such, the above mentioned strategy for salt adaptation using a stepwise increase in salt concentration is absolutely irrelevant. The targeted extremophile (in this case salt tolerant) does not benefit from the strategy of a stepwise increase of the salt concentration, because the microorganism probably does not alter its physiology (and boundaries) in function of the salt concentration. It can be therefore concluded that the adaptation of the SRB population to high salinity should be done immediately at the targeted salinity, because this will result in a fast growth of a salt tolerant population in the biomass.

Table 1. Description of the sludges used as inoculum in the reactor systems used in the investigations of this thesis.

Chapter	pH	T (°C)	Feed	Morphology	Remark	Reference
3	7	55	Methanol (no sulfate added)	Whitish well-shaped settleable granules	Methanol converted to methane syntrophically via H_2/CO_2. Sludge not exposed to sulfate during 130 days in previous reactor run.	Paulo *et al.*, (2001)
5	6	30	Starch, sucrose, lactate, propionate acetate (COD/SO_4^{2-} of 10)	Black well-shaped granules and also of dispersed flocs	Sludge harvested from the UASB reactors described in **Chapter 5** was used in the experiments presented in **Chapters 6 and 7.**	Lens *et al.*, (2003)
4, 8	6.9	30 - 37	Starch, acetate, propionate, butyrate and formate (COD/SO_4^{2-} of 9.5)	Black, well-shaped granules and also of dispersed flocs	COD and sulfate removal efficiencies were approx. 70 % and 95 %, respectively. One of the UASB reactors from **Chapter 8** was also inoculated with the halotolerant SRB *D halotolerans*.	Oude Elferink *et al.*, (1998)

The start-up of thermophilic (55 to 65°C) and extreme thermophilic (70°C or higher) reactors at the targeted temperature using mesophilic sludges as inoculum is another example

where a stepwise increase of the inhibitory factor is not adopted for achieving a successful start-up and operation of the bioreactor. The start-up of (extreme) thermophilic anaerobic bioreactors inoculated with mesophilic sludges at the targeted temperature even proceeds generally fast and stable **(Chapters 3, 4, 5 and 6)**. The start-up of the bioreactor at the targeted temperature provokes the rapid selection of (extreme) thermophiles. The organisms readily colonize on the outer surface and in the interstices and/or cavities of mesophilic granules, as demonstrated by van Lier (1995). However, further research is required to assess whether a direct exposure procedure is effective as a strategy to stimulate the growth of targeted microorganisms when two or three inhibitory factors are involved. Such a situation, for instance, prevails in the treatment of strongly buffered (bicarbonate) wastewaters, where a polluted stream with high pH and high salinity (in some cases also hot) must be treated.

Competition for substrate in sulfate reducing reactors

The results of this investigation show that the competition between SRB, MPA and AB for substrate is highly dependent of the substrate and operational conditions imposed to each experiment. In this thesis, the suppression of methane and acetate production was pursued. It was found that the production of methane can be easily suppressed in thermophilic methanol fed reactors, either by running the reactor at temperatures equal or higher than 65°C or by exposing 55°C operated reactors to a short (2 days) temperature (65 – 70°C) shocks **(Chapter 4)**. Methanogenesis can also be easily suppressed in mesophilic propionate- and ethanol-fed reactors, provided high salinity conditions prevail **(Chapter 8).** It seems, however, that the production of acetate, with exception of methanol fed reactors operated at 70°C, is unavoidable either in thermophilic **(Chapters 4, 5 and 6)** or in mesophilic **(Chapters 8 and 9)** reactors. Even though hydrogen was suggested to play a key role as an intermediate for sulfate reducers in thermophilic methanol fed reactors, acetogenic bacteria are capable to compete with hydrogen producers for methanol at temperatures between 55°C and 65°C **(Chapters 3 and 4)**. In mesophilic reactors this problem is even more evident, as the SRB growing on either propionate or ethanol are incomplete oxidizers, making acetate an unavoidable end-product of the process **(Chapters 8 and 9)**.

This thesis describes a situation where the production of acetate and methane was completely suppressed in methanol-fed sulfate reducing UASB reactors operated at 70°C **(Chapter 4)**, which is the desired situation, i.e. no loss of the supplied methanol for other unwanted conversions. As a matter of fact, this is the first time where a fully sulfate reducing granular sludge has been cultivated in a thermophilic sulfate reducing reactor. According to Weijma (2000), formation of acetate cannot be suppressed in thermophilic (65°C) methanol-fed sulfate reducing reactors without diminishing the formation of sulfide as well. Weijma (2000) suggested that the microbial community responsible for sulfate reduction might be

involved directly or indirectly in the formation of acetate. The optimal flow of electrons to sulfate reduction with methanol at 70°C, as found in the present work, indicates that there are practical ways to suppress acetate production without compromising sulfide production in bioreactors. In addition, it is suggested that distinct microbial communities are involved in the production of either acetate or sulfide in bioreactors operated at 70°C.

Recalcitrance of acetate in sulfate reducing bioreactors: addressing the problem

The results obtained in this work indicates that acetate (when produced) is an end product rather than an metabolic intermediate in sulfate reducing bioreactors, regardless the source of the inoculum sludge (Table 1), the type of substrate used, temperature, salinity, pH or imposed operational conditions, such as OLR and HRT. The accumulation of acetate is highly undesired in sulfate reducing bioreactors, because it induces the need for further treatment steps, either when the treated water is meant for reuse or when the sulfide is to be biologically converted to elemental sulfur (Janssen, 1997). Moreover, when an exogenous electron donor is supplied in a SRB based bioprocesses for the removal of sulfur oxyanions, acetate production obviously represents a loss of electron donor.

The absence of acetate degradation via sulfate reduction has been reported in thermophilic (e.g. Weijma, 2000) and mesophilic (e.g. Omil *et al.*, 1997) systems, with only few reports of this metabolism in thermophilic (Rintala, 1997) and mesophilic (Visser, 1995) systems. The unsuccessful acetate oxidation in sulfate reducing reactors from this thesis and in many other reports can be attributed to the very slow growth rates of acetate oxidizing SRB (Omil *et al.*, 1998) and MPA (reviewed by Paulo, 2002). According to Omil *et al.* (1998), basing their view on theoretical calculations with the growth kinetics of acetotrophic SRB, a long time (1000 days) is needed for the development of a substantial population of acetate oxidizing SRB. Besides this well known slow growth of acetate oxidizing SRB, the activity of this trophic group might also be negatively affected by the high sulfide concentrations prevailing in sulfate reducing reactors (O'Flaherty and Colleran, 2000). It has been shown that mesophilic acetotrophic SRB are much more sensitive to sulfide toxicity than the hydrogenotrophic SRB (O'Flaherty and Colleran, 2000). It can be concluded, therefore, that the acetate oxidizing SRB are the most sensitive group of microorganisms in sulfate reducing bioreactors. The recalcitrance for acetate conversion has also observed for methanol-fed thermophilic (55°C) methanogenic UASB reactors, where acetoclastic methanogens were easily suppressed and washed out during adverse conditions (Paulo, 2002). Three alternatives to resolve the problem of acetate accumulation and recalcitrance in sulfate reducing reactors can be adopted:

(1) Adoption of bioreactor designs (e.g. by compartmentalization of the sludge bed) which would result in (a) the protection the acetotrophic SRB from harsh (and inhibitory) environmental conditions (b) the stimulation of their growth.

(2) Stimulation of acetate oxidation via an alternative metabolical route, as for instance via denitrification after the addition of the alternative electron acceptor nitrate (Lens *et al.*, 2000).

(3) Selection of electron donors (e.g. formate or hydrogen, **Chapter 4**) and operational conditions (e.g. operating methanol-fed UASB reactors at 70°C, **Chapter 4**) that suppress (or at least does not stimulate) the acetate production in sulfate reducing bioreactors.

Sulfate reduction under extreme conditions: technological solutions

Key role of inoculum sludge for achieving successful treatment of "extreme" wastewaters

The successful operation of bioreactors inoculated with granular sludges not adapted to high salinity and temperatures at very high temperatures (up to 75°C, **Chapter 4**) and at very high salinities (up to 70 g $NaC.L^{-1}$, **Chapter 8**) as demonstrated in the investigations of this thesis, shows that granular sludges can represent a quite attractive inoculum for the treatment of wastewaters at extreme temperature and salinity conditions. No salt tolerance, however, was found in thermophilic UASB reactors, regardless the type of the inoculum: mesophilic **(Chapter 4)** or thermophilic **(Chapters 5, 6 and 7)** sludges; irrespective of the type of substrates: methanol, acetate, hydrogen or ethanol **(Chapter 4)**; and the presence of different antagonistic salts or osmoprotectants **(Chapter 7)**. However, in contrast to what was found in thermophilic conditions, a fairly high rate sulfate reduction was achieved in bioreactors operated at mesophilic conditions **(Chapter 8)**, even though the same inoculum sludge was used for the start-up of thermophilic UASB reactors **(Chapter 4)**. In addition, a satisfactory sulfate reduction at mesophilic conditions at high salinity (> 50 g $NaCl.L^{-1}$) only could be obtained when using ethanol and propionate as the electron donors, but not with hydrogen, acetate or methanol **(Chapter 8)**.

Obviously, the diversity (and number) of extremophiles present in granular sludges in (many) presently available types of granular sludges can be presumed to be very low, as extreme conditions generally do not prevail in the full scale reactors from which these granular sludges presently can be harvested. As such, the versatility of the available granular sludges in terms of environmental boundaries, viz. salinity, temperature and pH, and ability to oxidize a broad range of substrates is likely rather limited, as in fact observed in **Chapter 8** in terms of pH and substrates. The results of this thesis therefore indicate that the successful use of granular sludges for the anaerobic treatment of highly saline and very hot wastewaters greatly depends on the sludge inoculum characteristics, viz. the origin of the biomass and the types of substrates on which they were cultivated. In order to assess the presence of the required targeted microorganisms that will ultimately determine the feasibility of the process under specific extreme conditions, experiments need to be conducted with different types of sludges, environmental conditions and substrates. Batch activity tests represent a powerful

tool to screen all these variables collectively, and these tests frequently provide relevant information regarding metabolical capacity of the sludge as well as the environmental boundaries which the sludges can be applied, and so a proper pre-selection of the inoculum sludge can be made. Nevertheless, long term continuous experiments in lab-scale and pilot reactors inoculated with the pre-selected sludge sources(s) are ultimately needed, as quite different experimental conditions prevail in bioreactors to those in batch activity tests (i.e. hydraulic regime, mixing degree, and substrate and nutrients concentration and availability). Furthermore, batch activity tests are conducted under conditions of infinite cell retention time, neglecting therefore the possibility of cell washout as generally is the case in continuously operating bioreactors, so the batch activity tests do not provide information about the immobilization of microorganisms.

Use of specialized cells in granular sludge reactors

Even though the results obtained in our investigations demonstrated that the metabolical flexibility and the microbial diversity of granular sludges should not be underestimated for the treatment of sulfate rich wastewaters at extreme conditions, still important question remains to be answered. A relevant question for instance is whether these indigenous SRB have better physiological properties (in terms of metabolical boundaries and substrate turnover) than the bacterial SRB strains that so far isolated from specific native environments in which these extreme conditions prevails (reviewed by Lens *et al.*, 2002).

Using such a selected specialized microorganisms harvested from an extreme environment and cultivating them in a bioreactor **(Chapter 9)** may ultimately lead to a much better performance of a bioreactor than when using indigenous organisms present in available granular sludge, by stimulating their growth by exposing the inoculum sludge to an extreme condition **(Chapter 8)**. As explained before (see previous section), the diversity (and number) and versatility of extremophiles present in granular sludges is likely rather low, and consequently the treatment of extreme wastewaters which require the association of different strains that exercise multiple functions (e.g. substrate fermentation and intermediates mineralization) might be impeded by the lack of a specific extremophile in a mutualistic tie of microorganisms (which is normally observed in the anaerobic degradation of complex substrates). As a result, irrespective of the potentials of granular sludges, research on fundamental microbial physiology and ecology of extremophiles (isolated from specific extreme environments and cultivated in laboratories) as well as on their application in bioreactors are essential to broaden the operational window of bioreactor systems for the treatment of a broad range of wastewaters under extreme conditions. Especially the mechanisms in which externally added specific bacteria interact with granules still needs to be carefully investigated. It is, for instance, not clear if these bacteria merely grow in suspension

or they agglomerate via reversible adhesion, immobilize (irreversible adhesion) or colonize (irreversible adhesion + growth) in granular micro-environments. Moreover, the extent in which they might become incorporated (integrated) in existing granules during their multiplication (growth of sludge bed) is unknown. The role of these factors needs to be elucidated in order to enable the engineering of UASB granules by the incorporation of the microorganisms which hold specific desired metabolical and physiological properties, needed for the successful treatment of wastewater under extreme conditions.

Retention of specialized organisms in bioreactors for the treatment of extreme wastewaters

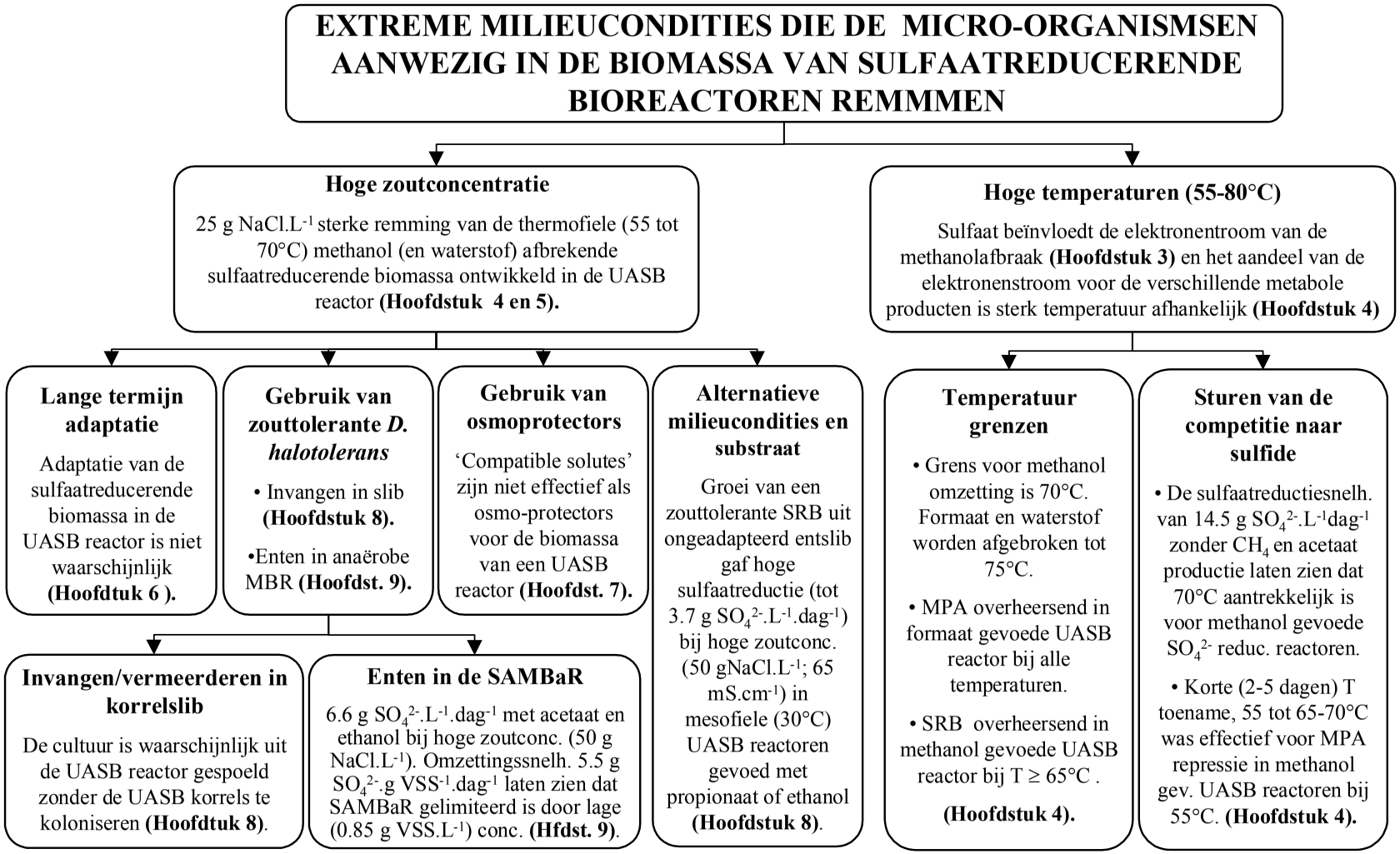
Along with the discussion about using granular sludges or specialized cells for the treatment of wastewaters under extreme conditions, the choice of the reactor configuration to treat these wastewaters is equally important. Obviously, after the selection of a proper granular sludge (see section 'Key role of inoculum sludge…) with the required metabolical characteristics to treat a specific wastewater, conventional high rate anaerobic bioreactors (UASB, expanded granular sludge bed - EGSB, internal circulation – IC or packed bed reactors) are the logical choice as treatment system. Alternatively, hybrid configurations (e.g. UASB + packed bed) can be adopted to provide additional niches for the colonization of specialized microorganisms into the bioreactor environment (which was not tried in this thesis). However, in cases where the retention of the microorganisms remains problematic, the adoption of alternative reactor configurations, such as membrane bioreactors **(Chapter 9)** might be considered.

The concept of submerged anaerobic membrane bioreactors (SAMBaR) as described in **Chapter 9** looks attractive. As demonstrated in this thesis, indeed very high specific sulfate reduction rates (up to 6.6 g SO_4^{2-}.$gVSS^{-1}$.day^{-1}) can be obtained in this completely mixed tank reactors where the biomass (the halophilic *D. halotolerans*) grows in suspension and can be efficiently retained in the system. Conversely, it appears that a big fraction of the microorganisms present in granular sludges developing in conventional high rate anaerobic reactors are not active. Consequently, relatively low specific sulfate reduction rates are attained in these bioreactors, as discussed in **Chapter 9**. As a conclusion, in view of the results obtained in this work, which showed that anaerobic granules seem to be an inadequate matrix for efficient attachment or entrapment of exogenous microorganisms **(Chapter 8),** reactors based on suspended growth (such as the SAMBaR) must be adopted (and further developed) for the use of extremophiles in environmental biotechnological applications. For instance, growth of high cell density cultures using, e.g. reactor systems based on pH induced continuous growth at the apparent maximal growth rate - μ_{max} (Paulo *et al.*, 2003) coupled to a very high biomass retention via membranes are likely to further increase the specific sulfate reduction conversion rates that were obtained in **Chapter 9**, thus leading to reactors with enhanced substrate turnovers resulting in small footprint units.

BELANGRIJKSTE RESULTATEN

Hoofdstuk 3: Methanol werd volledig omgezet (OLR tot 20 g CZV.L^{-1}.dag^{-1}; pH 7.0 ± 0.2; hydraulische verblijftijd (HVT) of 7.5 uur zowel via sulfaatreductie (tot 13 % bij een CZV/SO_4^{2-} van 5) en methanogenese (85 %) in een thermofiele UASB reactor gevoed met toenemende sulfaat concentraties. Verrassend genoeg verminderde de efficiëntie van de sulfaatreductie sterk wanneer de reactor werd bedreven met een overmaat sulfaat in het influent (CZV/SO_4^{2-} of 0.5), vermoedelijk veroorzaakt door slechte immobilisatie van SRB in het slibbed en de aanwezigheid van relatief hoge zout concentraties (ongeveer 6 g Na^+.L^{-1}), dit als gevolg van het feit dat sulfaat wordt gedoseerd als natriumzout. Activiteitstests toonden aan dat methanol syntroof werd omgezet via H_2/CO_2 door homoacetogene bacteriën, in combinatie met SRB of MPA.

Hoofdstuk 4: Wanneer een UASB reactor (pH 7.5 ± 0.2) wordt bedreven met sulfaatrijk (CZV/SO_4^{2-} = 0.5) synthetisch afvalwater werd volledige omzetting van methanol en acetaat bereikt bij temperaturen tot respectievelijk, 70 en 75°C. MPA winnen de competitie met SRB in de formaatgevoede UASB reactor bij elke geteste temperatuur (65 - 75°C). Daarentegen winnen de SRB de competitie van MPA in methanol gevoede UASB reactoren bij temperaturen gelijk aan of hoger dan 65°C, terwijl in de reactor een sterke competitie tussen SRB en MPA werd gevonden bij een temperatuur van 55°C. Gebaseerd op de zeer hoge, d.w.z. 14.5 g SO_4^{2-}.$L^{-1}dag^{-1}$ sulfaatreductiesnelheid, en de volledige afwezigheid van methaan en acetaat productie, lijkt 70°C de aantrekkelijkste temperatuur voor methanol gevoede sulfaatreducerende reactoren. Een korte (2 dagen) stijging van de temperatuur van 55 naar 65-70°C blijkt een effectieve manier om methanogense te onderdrukken in methanol gevoede sulfidogene UASB reactoren bedreven bij 55°C. Echter, de acetogenese begint opnieuw zodra de temperatuur werd teruggebracht tot 55°C. Remming van de sulfidogenese door NaCl in een methanol gevoede sulfidogene UASB reactor ontstond bij lage concentraties van 10 g.L^{-1}. Uit de batch experimenten blijkt dat SRB (met name hydrogenotrofe) de meest NaCl (25 g.L^{-1}) gevoelige micro-organismen zijn in het slib ontwikkeld bij 70°C. De acetogenese begon na een lagfase van 10 dagen.



Figuur 1. Overzicht van de structuur van dit proefschrift en de belangrijkste resultaten.

Hoofdstuk 5: Wanneer het influent geen NaCl bevat werd de methanol in de UASB reactoren bijna volledig gebruikt voor sulfaatreductie (CZV/SO_4^{2-} of 0.5; pH 7.5 ± 0.2; OLR of 5 g $CZV.L^{-1}.dag^{-1}$, HVT van 10 uur). In dit onderzoek winnen de SRB de competitie voor methanol bij 55°C van MPA, in tegenstelling tot eerdere experimenten bij 55°C (**Hoofdstuk 4**) waar een sterke competitie tussen SRB en MPA werd gevonden. Acetaat werd gevormd als bijproduct, maximaal 25 % van de totaal omgezette hoeveelheid CZV. Dit werd veroorzaakt door het feit dat zowel de MPA en SRB in de activiteitstest niet in staat zijn acetaat als substraat te gebruiken. Een hoge concentratie van 25 $g.L^{-1}$ NaCl remde de methanolomzetting volledig, terwijl lage concentraties NaCl (2.5 $g.L^{-1}$) behoorlijke veranderingen in de metabole omzettingsroute van methanol veroorzaakten. De MPA waren het meest gevoelig voor de 25 g $NaCl.L^{-1}$ schok, terwijl het toevoegen van een NaCl concentratie van slechts 2.5 $g.L^{-1}$ de MPA en homoacetogene bacteriën (AB) kennelijk stimuleerden.

Hoofdstuk 6: Adaptatie van thermofiel (55°C) sulfidogeen methanol omzettende biomassa aan een hoog osmolair milieu deed zich klaarblijkelijk niet voor in een UASB reactor (pH 7.5 ± 0.2; HVT van 10 uur), wanneer alleen gevoed met methanol en een overmaat sulfaat (CZV/SO_4^{2-} of 0.5). Hoewel sulfide het belangrijkste mineralisatieproduct van de methanol omzetting was, ongeacht de NaCl concentratie in het influent, nam de sulfide productie gestaag af na het toevoegen van 7.5 g $NaCl.L^{-1}$, terwijl de productie van acetaat werd gestimuleerd. Activiteitstesten met het slib bemonsterd uit de UASB reactor, terwijl het systeem werd bedreven bij verschillende zoutconcentraties in het influent, bevestigden dat acetaat daadwerkelijk het belangrijkste metabole product was bij NaCl concentraties hoger dan 12.5 $g.L^{-1}$. De volgorde van NaCl toxiciteit voor de verschillende trofische groepen is: SRB > MPA > AB.

Hoofdstuk 7: Het toevoegen van verschillende osmoprotectantia, te weten glutamaat, betaine, ectoine, choline, een mengsel van 'compatible solutes' en K^+ and Mg^{2+}, verhoogde de methanol omzettingssnelheid van een slibmonster uit een thermofiele (55°C) sulfaat reducerende (CZV/SO_4^{2-} of 0.5) UASB reactor lichtelijk. Echter, de hoger omzettingssnelheid begunstigt voornamelijk de homoacetogene bacteriën, de methanolomzetting werd in de richting van acetaatvorming gestuurd zonder toegenomen sulfaatreductie en methaanvorming. Met andere woorden geen van deze stoffen was effectief als osmoprotectant ter vermindering van de acute NaCl toxiciteit voor sulfaatreducerende korrelslibben bemonsterd uit een methanol omzettende UASB reactor.

Hoofdstuk 8: In tegenstelling tot de onsuccesvolle adaptatie van thermofiel (55-70°C) methanoloxiderende sulfidogene biomassa aan NaCl, beschreven in **Hoofdstuk 4, 5 en 6,** gaf de groei van een zouttolerante mesofiele SRB populatie aanwezig in het ongeadapteerde entslib (hetzelfde als gebruikt in **Hoofdstuk 4**) een redelijk hoge sulfaatreductiesnelheid (tot 3.7 g $SO_4^{2-}.L^{-1}.dag^{-1}$), dit bij een hoog zoutgehalte van 50 g $NaCl.L^{-1}$ and 1 g $MgCl_2.L^{-1}$ in

mesofiele UASB reactoren (pH 7.0 ± 0.2; HVT van 8 tot 14 uur) gevoed met acetaat, propionaat of ethanol bij een overmaat sulfaat (CZV/SO_4^{2-} of 0.5). Een aanzienlijke sulfaatreductiesnelheid van 1.40 g $SO_4^{2-}.L^{-1}.dag^{-1}$ was aanwezig bij zoutconcentraties van 70 g $NaCl.L^{-1}$ en 1 g $MgCl_2.L^{-1}$ (overeenkomend met een geleidbaarheid van 85 tot 90 $mS.cm^{-1}$). Hoewel acetaat één van de eindproducten was, waren zowel ethanol als propionaat geschikte substraten voor sulfaatreductie. Pogingen om de acetaatoxiderende SRB *Desulfobacter halotolerans* in het slibbed te houden of laten ingroeien waren onsuccesvol, dit omdat de bacterie uitspoelde zonder duidelijke kolonisatie van de UASB slibkorrels.

Hoofdstuk 9: In een ondergedompelde anaërobe membraan bioreactoren (SAMBaR; pH 7.2 ± 0.2; HVT van 8 tot 36 uur), enkel geënt met de zouttolerante sulfaatreducerende bacterie *Desulfobacter halotolerans* en met acetaat en ethanol in het zoute medium (50 g $NaCl.L^{-1}$ en 1 g $MgCl_2.6H_2O.L^{-1}$; geleidbaarheid 60-70 $mS.cm^{-1}$) werden sulfaatreductiesnelheden tot 6.6 g $SO_4^{2-}.L^{-1}.dag^{-1}$ bereikt. Gebaseerd op de waargenomen redelijk constante en hoge specifieke sulfaatreductiesnelheid van 5.5 g $SO_4^{2-}.gVSS^{-1}.dag^{-1}$, kan worden geconcludeerd dat de prestatie van de reactor gelimiteerd was door de lage hoeveelheid biomassa (0.85 $gVSS.L^{-1}$) aanwezig op het eind van het experiment. Tevens bleek dat sulfaatreducerende ondergedompeld anaërobe membraan bioreactoren, bij een zekere vaste flux, voor langere tijd (ongeveer 100 dagen) kunnen opereren zonder chemische reiniging van de membranen, vooropgesteld dat deze flux substantieel lager is dan de experimenteel bepaalde nominale kritische flux (18-21 $L.m^{-2}.h^{-1}$). Zowel het discontinue opereren als het terugspoelen van de membranen vertraagde het ontstaan van membraanverontreiniging. Frequent terugspoelen (bijv.1 minuut elke 10 minuten) kan worden aanbevolen als strategie voor de minimalisatie van de membraanvervuiling in sulfaat reducerende MBRs.

DISCUSSIE

Het bedrijven van sulfaatreducerende UASB reactoren onder extreme condities (hoog zoutgehalte en hoge temperatuur)

Het opstarten van UASB reactoren (directe vs. stapsgewijze blootstelling)

De efficiëntie van de strategie van de stapsgewijze toename van de zoutconcentratie ter acclimatisering van het slib aan hoge zoutgehaltes, waarschijnlijk resulterend in de geleidelijke selectie van zouttolerante micro-organismen in een initieel ongeadapteerd entslib, zoals voorgesteld door Omil *et al.* (1995) and Feijoo *et al.* (1995), lijkt discutabel. De resultaten dit onderzoek **(Hoofdstuk 4, 5, 6 vs. Hoofdstuk 8)** geven aan dat het ontwikkelen van een gewenste metabole eigenschap (sulfaatreductie bij hoge zoutconcentraties) onafhankelijk is van de biomassa acclimatiseerstrategie. Stapsgewijze adaptatie van

thermofiele sulfidogene methanol omzettende biomassa aan een milieu met een hoge osmolariteit doet zich in de UASB reactoren zowel bij 55°C **(Hoofdstuks 6)** als bij 70°C **(Hoofdstuk 4)** niet voor, dit omdat er waarschijnlijk geen methanol omzettende zouttolerante thermofiele SRB aanwezig waren in het thermofiel entslib gebruikt in dit onderzoek (Tabel 1). Daarentegen laat **Hoofdstuk 8** zien dat het direct blootstellen van het slib (hetzelfde als gebruikt in **Hoofdstuk 4**) aan zeer hoge zoutconcentraties (50 g $NaCl.L^{-1}$) de groei van een mesofiele propionaat en ethanol omzettende zouttolerante SRB populatie stimuleerde, welke in een UASB reactor tot een hoge sulfaatreduktiesnelheid in staat was (tot 3.6 $gSO_4^{2-}.L^{-1}.dag^{-1}$). Daarom is de sleutel tot het succesvol behandelen van afvalwater met een hoge zoutconcentratie het investeren van voldoende tijd voor de groei van het beoogde micro-organisme in de biomassa. Als zodanig is de hierboven beschreven strategie voor zoutadaptatie d.m.v. een stapsgewijze toename van de zoutconcentraties totaal irrelevant. De beoogde extremofiele bacterie (in dit geval zouttolerant) heeft geen baat bij een stapsgewijze toename van de zoutconcentratie omdat het organisme zijn fysiologie (en grenzen) niet zal veranderen als functie van de zoutconcentratie. De volgende conclusie kan daarom worden getrokken: adaptatie van de SRB populatie aan hoge zoutconcentraties moet bij de beoogde zoutconcentratie worden gedaan, hetgeen zal resulteren in de snelle groei van een zouttolerant slib.

Tabel 1. Beschrijving van slibsoorten gebruikt als entslib voor het in dit proefschrift beschreven onderzoek.

Hoofdstuk	pH	T (°C)	Substraat	Morfologie	Opmerkingen	Referentie
3	7.0	55	Methanol (geen sulfaat toegevoegd)	Witte, goed gevormde en bezinkbare korrels	Methanol syntrofisch omgezet via H_2/CO_2. Slib 130 dagen niet blootgesteld aan sulfaat in de vorige reactor run.	Paulo *et al.*, (2001)
5	6.0	30	Zetmeel, sucrose, lactaat, propionaat, acetaat (CZV/SO_4^{2-} ratio 10)	Zwarte, goed gevormde korrels en disperse vlokken	Slib verkregen uit de reactor beschreven in **Hoofdstuk 5** is gebruikt voor de experimenten in **Hoofdstuk 6 en 7.**	Lens *et al.*, (2003)
4, 8	6.9	30 - 37	Zetmeel, acetaat, propionaat, butyraat en formaat (CZV/SO_4^{2-} ratio 9.5)	Zwarte, goed gevormde korrels en disperse vlokken	CZV en sulfaat verwijderingefficiëntie ongeveer 70 % and 95 %. Een van de UASB reactoren in **Hoofdstuk 8** is geënt met de zoutolerante SRB *D. halotolerans*.	Oude Elferink *et al.*, (1998)

Het opstarten van thermofiele (55 tot 65°C) en extreem thermofiele (70°C of hoger) reactoren bij de beoogde temperatuur met mesofiel entslib is een andere voorbeeld, hierbij neemt de beperkende factor niet stapsgewijs toe om een succesvol opstarten en bedrijven van de reactor te bewerkstelligen **(Hoofdstuk , 4, 5 and 6)** . Het opstarten van een (extreem) thermofiele reactor, geënt met mesofiel slib, bij de beoogde temperatuur verloopt over het algemeen snel en stabiel **(Hoofdstuk 4)**. Het opstarten bij de beoogde temperatuur resulteert in een snelle selectie van (extreem) thermofielen. Deze organismen koloniseren het oppervlak, alsook spleten en/of holtes van de mesofiele korrel (van Lier,1995). Verder onderzoek is echter nodig om aan te tonen of deze procedure van directe blootstelling effectief is in het stimuleren van de groei van de beoogde micro-organismen in de aanwezigheid van twee of drie remmende factoren. Deze situatie doet zich bijvoorbeeld voor in sterk gebufferde afvalwaters (bicarbonaat), waar een verontreinigde waterstroom met een hoge pH en zoutgehalte (en in sommige gevallen met hoge temperatuur) moet worden behandeld.

Substraat competitie in sulfaatreducerende reactoren

Dit onderzoek toont aan dat de substraatcompetitie tussen SRB, MPA and AB sterk afhankelijk is van het substraat en de in het experiment opgelegde operationele condities. In dit proefschrift werd het onderdrukken van methaan en acetaat productie nagestreefd. Het is gebleken dat methaan productie gemakkelijk kan worden onderdrukt in thermofiele methanol gevoede reactoren, dan wel door het bedrijven van de reactor bij temperaturen gelijk of hoger dan 65°C of door bij 55°C bedreven reactoren bloot te stellen aan een korte (2 dagen) temperatuur (65 – 70°C) schok **(Hoofdstuk 4)**. De methanogenese kan ook makkelijk worden onderdrukt in mesofiele propionaat en methanol gevoede reactoren, vooropgesteld dat er een hoge zoutconcentratie aanwezig is in de reactor **(Hoofdstuk 8).** Echter, het lijkt er op dat acetaatvorming, met de uitzondering van methanol gevoede reactoren bedreven bij 70°C, onvermijdelijk is, zowel in thermofiele **(Hoofdstuk 4, 5 en 6)** als in mesofiele **(Hoofdstuk 8 en 9)** reactoren. Hoewel waterstof een sleutelrol bleek te spelen als intermediair voor sulfaatreduceerders in thermofiele methanol gevoede bioreactoren waren de acetogenen bacteriën bij temperaturen tussen 55°C en 65°C in staat te concurreren met waterstof producerende organismen **(Hoofdstuk 3 en 4)**. Dit probleem is nog meer evident in mesofiele reactoren omdat dan de op propionaat of ethanol groeiende SRB incomplete oxideerders zijn, waardoor acetaat een onvermijdelijk eindproduct wordt **(Hoofdstuk 8 en 9)**.

Dit proefschrift beschrijft een situatie waarbij de acetaat en methaan productie in methanol gevoede sulfaatreducerende UASB reactor bedreven bij 70°C volledig wordt onderdrukt **(Hoofdstuk 4)**, hetgeen de gewenste situatie is, met andere woorden geen verlies van methanol en geen ongewenste bijproducten. Het is voor het eerst dat een volledig sulfaatreducerend slib gecultiveerd is in een thermofiele sulfaatreducerende reactor. Volgens

Weijma (2000) kan de acetaatvorming niet worden onderdrukt in thermofiele (65°C) methanol gevoede sulfaatreducerende reactoren zonder dat vorming van sulfide ook afneemt. Weijma (2000) suggereert dat de microbiologische populatie verantwoordelijk voor sulfaatreductie direct dan wel indirect betrokken is bij de vorming van acetaat. De optimale elektronenstroom naar sulfaatreductie met methanol bij 70°C, zoals gevonden in het hier beschreven onderzoek, induceert dat er praktische methoden zijn om de acetaat productie in bioreactoren te onderdrukken zonder de sulfide productie negatief te beïnvloeden. Tevens blijken er verschillende microbiologische populaties betrokken te zijn bij de acetaat en sulfide productie in reactoren bedreven bij 70°C.

Recalcitrantie van acetaat in sulfaatreducerende bioreactoren: aanpak van het probleem

De resultaten van dit onderzoek suggereren dat acetaat (wanneer geproduceerd) in sulfaatreducerende bioreactoren een eindproduct is i.p.v. een metabool intermediair, ongeacht de bron van het entslib (Tabel 1), het type substraat, temperatuur, zoutconcentratie, pH of de opgelegde operationele condities, zoals OLR of HVT. Het ophopen van acetaat in de sulfaatreducerende bioreactoren is zeer ongewenst omdat het verdere behandelingsstappen nodig maakt, zowel wanneer het behandelde water moet worden hergebruikt als wanneer het sulfide biologisch moet worden omgezet naar elementair zwavel (Janssen, 1997). Verder betekent, acetaat vorming uiteraard een verlies van deze elektronendonor, in SRB gebaseerde systemen voor de verwijdering van zwaveloxyanionen waaraan een exogene elektronendonor wordt toegediend.

De accumulatie van acetaat in sulfaatreducerende reactoren is beschreven voor zowel thermolfiele (bijv. Weijma, 2000) en mesofiele (bijv. Omil *et al.*, 1997) systemen. Er zijn slechts enkele onderzoeken m.b.t. het acetaat metabolisme in thermofiele (Rintala, 1997) en mesofiele (Visser, 1995) systemen. De in dit en vele andere onderzoeken beschreven onsuccesvolle acetaatoxidatie in sulfaatreducerende reactoren kan worden toegeschreven aan de zeer lage groeisnelheid van de acetaatoxiderende SRB (Omil *et al.*, 1998) en MPA (review door Paulo, 2002). Volgens Omil *et al.* (1998), gebaseerd op de theoretische groeikinetiek van acetotrofe SRB, is er een lange tijd (1000 dagen) nodig voor de ontwikkeling van een substantiële acetaatoxiderende SRB populatie. Naast deze bekende lage groei van acetaatoxiderende SRB, zou ook de activiteit van deze trofische groep negatief beïnvloed kunnen worden door de hoge heersende sulfide concentraties in sulfaatreducerende reactoren (O'Flaherty and Colleran, 2000). Het is aangetoond dat mesofiele acetotrofe SRB veel gevoeliger voor toxiciteit van sulfide zijn dan de hydrogenotrofe SRB (O'Flaherty and Colleran, 2000). Daarom kan geconcludeerd worden dat de acetaatoxiderende SRB de meest gevoelig groep micro-organismen is in sulfaatreducerende bioreactoren. De recalcitrantie m.b.t. het omzetten van acetaat is ook waargenomen voor methanol gevoede thermofiele (55°C) methanogene UASB

reactoren, waar de acetoclastische methanogenen gemakkelijk werden onderdrukt en uitgespoeld onder ongunstige condities (Paulo, 2002). Drie alternatieven kunnen worden toegepast om de acetaatophoping en de recalcitrantie hiervan in sulfaatreducerende reactoren op te lossen:

(4) Aangepaste reactorontwerpen (bijv. het opdelen in compartimenten van het slibbed) hetgeen resulteert in (a) de bescherming van de acetotrofe SRB voor een ongunstige (en remmende) omgeving (b) simulering van hun groei.

(5) Stimulering van de acetaatoxidatie via een alternatieve metabole route, zoals bijvoorbeeld denitrificatie na toevoegen van de alternatieve elektronenacceptor nitraat (Lens *et al.*, 2000).

(6) Keuze van elektronendonoren (bijv. formaat of waterstof, **Hoofdstuk 4**) en operationele condities (bijv. Bedrijven van methanol gevoede UASB reactoren bij 70°C, **Hoofdstuk 4**) die de acetaatproductie in sulfaatreducerende bioreactoren onderdrukken (of in ieder geval niet stimuleren).

Sulfaatreductie onder extreme condities: technologische oplossingen

Sleutelrol van het entsib voor het bereiken van een succesvolle behandeling van extreme afvalwaters.

Het zoals in dit onderzoek beschreven succesvol bedrijven van bioreactoren geënt met ongeadapteerd korrelslib bij zeer hoge temperaturen (tot 75°C, **Hoofdstuk 4**) en zeer hoge zoutconcentraties (tot 70 g $NaC.L^{-1}$, **Hoofdstuk 8**), laat zien dat korrelslib een redelijk aantrekkelijk entmateriaal is voor het behandelen van afvalwater bij extreem hoge temperaturen en zoutconcentraties. Zouttolerantie werd echter niet gevonden in thermofiele UASB reactoren, ongeacht het type entmateriaal: mesofiel **(Hoofdstuk 4)** of thermofiel **(Hoofdstuk 5, 6 en 7)** slib; ongeacht het type substraat: methanol, acetaat, waterstof of ethanol **(Hoofdstuk 4)**; en verschillende antagonistische zouten of osmoprotectantia **(Hoofdstuk 7)**. In tegenstelling tot hetgeen werd gevonden onder thermofiele condities, werd een redelijk hoge sulfaatreductiesnelheid bij hoge zoutconcentraties bereikt in bioreactoren bedreven onder mesofiele condities **(Hoofdstuk 8)**, terwijl hetzelfde entslib werd gebruikt als voor de thermofiele UASB reactoren **(Hoofdstuk 4)**. Verder kon een bevredigende sulfaatreductie onder mesofiele condities en hoge zoutconcentratie (> 50 g $NaCl.L^{-1}$) slechts worden bereikt wanneer ethanol en propionaat als elektronendonor werden gebruikt en niet met waterstof, acetaat of methanol **(Hoofdstuk 8)**.

Vanzelfsprekend kan worden aangenomen dat de diversiteit (en hoeveelheid) aan extremofiele micro-organismen aanwezig in de tegenwoordig beschikbare korrelslibben laag is, omdat extreme condities over het algemeen niet voorkomen in praktijkreactoren waaruit

deze korrelslibben kunnen worden verkregen. Daarom is het aannemelijk dat de verscheidenheid in de beschikbare korrelslibben m.b.t de milieubeperkingen, zoals zoutconcentratie, temperatuur en pH, en de mogelijkheid verschillende substraten te oxideren beperkt zal zijn, hetgeen m.b.t. pH en substraat ook is vastgesteld in **Hoofdstuk 8**. De resultaten in dit proefschrift geven aan dat het succesvol gebruik van korrelslibben voor de behandeling van afvalwaters met hoge zoutconcentraties en zeer hoge temperaturen sterk wordt bepaald door het gebruikte entmateriaal, dat wil zeggen de herkomst van het slib en het substraat waarmee het werd gevoed. Om de aanwezigheid van de beoogde micro-organismen, welke uiteindelijk de haalbaarheid van het proces bij de specifieke extreme condities zal bepalen aan te tonen, moeten er experimenten worden uitgevoerd met verschillende slibsoorten, milieucondities en substraten. Batch activiteitstesten zijn een krachtig hulpmiddel om alle variabelen gezamenlijk te testen, deze testen geven vaak relevante informatie over de metabole capaciteit van het slib en de milieugrenzen waarbinnen het slib kan worden gebruikt, op deze manier kan een gepaste voorselectie voor het entslib worden gemaakt. Het is uiteindelijk noodzakelijk langdurige continu experimenten uit te voeren met de voorgeselecteerde slibsoort(en) in labschaal en pilootschaal reactoren, omdat in de reactoren behoorlijk verschillende milieucondities heersen in vergelijking met batchtests (bijv. hydraulische condities, menging, substraat en nutriënten concentratie en beschikbaarheid). Tevens worden de batch activiteitstesten uitgevoerd bij een oneindige celverblijftijd, hierbij wordt de mogelijkheid van het uitspoelen van cellen, zoals over het algemeen het geval is in bioreactoren, verwaarloosd. M.a.w de batch activiteitstesten geven geen informatie m.b.t. de immobilisatie van micro-organismen.

Gebruik van gespecialiseerde cellen in korrelslibreactoren

Hoewel de resultaten van dit onderzoek laten zien dat de metabole flexibiliteit en microbiële diversiteit van korrelsib voor de behandeling van sulfaatrijke afvalwaters onder extreme condities niet onderschat moeten worden, resteren er nog een aantal belangrijke vragen. Een relevante vraag is bijvoorbeeld of de reeds aanwezige SRB betere fysiologische eigenschappen hebben (m.b.t. metabole grenzen en substraatomzetting) dan de SRB tot dusverre geïsoleerd uit natuurlijke milieus waar deze extreme condities heersen (review door Lens *et al.*, 2002).

Het gebruik van gespecialiseerde micro-organismen, verkregen uit een extreem milieu, in een bioreactor **(Hoofdstuk 9)** zou uiteindelijk kunnen leiden tot veel betere prestaties van de bioreactor dan wanneer de groei van reeds aanwezige organismen in het korrelslib wordt gestimuleerd door ze bloot te stellen aan een extreem milieu **(Hoofdstuk 8)**. Zoals reeds verklaart (zie vorige paragraaf), is de diversiteit (en aantal soorten) en de resulterende metabole flexibiliteit van extremofielen aanwezig in anaerobe korrelslibsoorten waarschijnlijk

redelijk laag, met als gevolg dat de behandeling van extreme afvalwaters waarvoor verschillende samenwerkende bacteriën die verschillende functies uitvoeren nodig zijn (bijv. het fermenteren van substraat en mineralisatie van intermediairen) kan worden belemmerd door het gebrek aan specifieke syntrofe extremofielen (hetgeen normaal is voor de meeste anaërobe omzetting van complexe substraten). Hierom is het, onafhankelijk van de mogelijkheden van korrelslibben, noodzakelijk dat onderzoek wordt gedaan naar zowel de fysiologie en ecologie van extremofielen (geïsoleerd uit specifieke extreme milieus en gekweekt in laboratoria) als de toepassing van deze organismen in bioreactoren, dit om het scala van extreme afvalwaters dat in bioreactoren kan worden behandeld te verbreden. Vooral de wisselwerking tussen extern toegevoegde bacteriën en slibkorrels moet zorgvuldig onderzocht worden. Het is bijvoorbeeld niet duidelijk of deze micro-organismen enkel in suspensie groeien of dat ze samenklonteren via reversibele adhesie, immobilisatie (irreversibele adhesie) of koloniseren (irreversibele adhesie + groei) om granulaire microsystemen te vormen. Verder is de mate waarin ze worden opgenomen (geïntegreerd) in bestaande korrels tijdens de vermeerdering van slibkorrels (groei slibbed) onbekend. De rol van deze factoren moet worden opgehelderd om de 'engineering' van slibkorrels d.m.v. van de ingroei van de micro-organismen met de gewenste metabole en fysiologische eigenschappen voor de succesvolle behandeling van afvalwater onder extreme condities mogelijk te maken.

Retentie van gespecialiseerde organismen in bioreactoren voor de behandeling van "extreme" afvalwaters

Samen met discussie over het gebruik van korrelslib of gespecialiseerde cellen voor de behandeling van afvalwater onder extreme condities, is de keuze van de reactorconfiguratie even belangrijk. Vanzelfsprekend is na de keuze van het juiste korrelslib met de benodigde metabole eigenschappen voor de behandeling van een specifiek afvalwater (zie paragraaf 'sleutelrol van het entslib'), het gebruik van conventionele hoogbelastbare anaërobe reactoren (UASB, expanded granular sludge bed - EGSB, interne circulatie – IC of gepakt bed reactoren) de logische keuze. Een alternatief om extra niches voor de kolonisatie van gespecialiseerde micro-organismen in het reactormilieu te bieden zijn hybride reactor configuraties (bijv. UASB + gepakt bed), hetgeen niet is geprobeerd in dit proefschrift. In het geval de retentie van micro-organismen problematisch blijft, kunnen alternatieve reactor configuraties zoals membraan reactoren **(Hoofdstuk 9)** worden overwogen.

Het concept van ondergedompelde anaërobe membraan bioreactoren (SAMBaR) zoals beschreven in **Hoofdstuk 9** lijkt aantrekkelijk. Zoals aangetoond in dit proefschrift kunnen zeer hoge specifieke sulfaatreductiesnelheden (tot 6.6 g SO_4^{2-}.$gVSS^{-1}$.dag^{-1}) worden bereikt in deze compleet gemengde reactoren waar de biomassa (de zouttolerante *D. halotolerans*) in

suspensie groeit en de biomassa kan tevens effectief in het systeem worden gehouden. Het lijkt er echter op dat de meeste micro-organismen aanwezig in korrleslib dat zich ontwikkelt in conventionele hoogbelastbare anaërobe korrelslib reactoren niet actief zijn, waardoor relatief lage sulfaatreductiesnelheden worden bereikt (**Hoofdstuk 9)**. Concluderend, uit de resultaten behaald in dit onderzoek, die lieten zien dat anaërobe slibkorrels waarschijnlijk geen adequate matrix zijn voor het efficiënt invangen en immobiliseren van exogene micro-organismen **(Hoofdstuk 8)**, moeten reactoren gebaseerd op gesuspendeerde groei (zoals SAMBaR) worden toegepast. Dergelijke innovatieve technieken dienen verder te worden ontwikkeld voor het gebruik van extremofiele micro-organismen in biotechnologische toepassingen. Dit kan bijvoorbeeld door de groei van celculturen met een hoge cel dichtheid in reactor types gebaseerd op pH geïnduceerde continu groei van micro-organismen bij hun maximale specifieke groeisnelheid-μ_{max} (Paulo *et al.*, 2003), gekoppeld aan een zeer hoge biomassa retentie m.b.v. membranen, zouden de in **Hoofdstuk 9** bereikte sulfaatreductiesnelheden waarschijnlijk verder kunnen verhogen, leidend tot kleinere reactoren met toegenomen specifieke sulfaatreductiesnelheden.

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