Anaerobic degradation of methanethiol in a process for Liquefied Petroleum Gas (LPG) biodesulfurization

Promotoren

Prof. dr. ir. A.J.H. Janssen
Hoogleraar in de Biologische Gas- en waterreiniging

Prof. dr. ir. A.J.M. Stams
Persoonlijk hoogleraar bij het laboratorium voor Microbiologie

Copromotor

Prof. dr. ir. P.N.L. Lens
Hoogleraar in de Milieubiotechnologie UNESCO-IHE, Delft

Samenstelling promotiecommissie

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Dr. H.J.M. op den Camp	Radboud Universiteit, Nijmegen, Nederland
Prof. dr. ir. H. van Langenhove	Universiteit Gent, België

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Anaerobic degradation of methanethiol in a process for Liquefied Petroleum Gas (LPG) biodesulfurization

R.C. van Leerdam

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Abstract

Due to increasingly stringent environmental legislation car fuels have to be desulfurized to levels below 10 ppm in order to minimize negative effects on the environment as sulfur-containing emissions contribute to acid deposition ('acid rain') and to reduce the amount of particulates formed during the burning of the fuel. Moreover, low sulfur specifications are also needed to lengthen the lifetime of car exhaust catalysts. The research presented in this thesis focuses on the biological desulfurization of Liquefied Petrol Gas (LPG). Currently, LPG is mainly desulfurized by physical-chemical methods that absorb volatile sulfur compounds present (mainly hydrogen sulfide and thiols) into a strong caustic solution, whereafter the thiols are partially oxidized to disulfides whilst the dissolved hydrogen sulfide is discharged as a 'spent sulfidic caustic'. Disadvantages of this physical-chemical method are the relatively high energy and caustic consumption and the production of a hazardous waste stream.

As an alternative, a new three-step biotechnological LPG desulfurization technology has been studied, that produces elemental sulfur as an end-product from the bio-conversion of hydrogen sulfide (H_2S) and methanethiol (MT). The new process involves: (i) extraction of the sulfur compounds from the LPG phase into a (bi)carbonate-containing solution; (ii) anaerobic degradation of MT to H_2S, CO_2 and CH_4 and (iii) partial oxidation of H_2S to elemental sulfur. The formed sulfur particles are removed from the system whilst the sulfur-free alkaline process water is re-used in the extraction process. The sulfur can be used for the production of sulfuric acid and hydrogen sulfide or for agricultural applications.

In this research attention is paid to the feasibility of the second process step, *i.e.* the anaerobic treatment step as the first and third process step are already well described. Anaerobic degradation of MT appeared to be possible with a variety of anaerobic (reactor) sludges and sediments, both under methanogenic and sulfate-reducing conditions. The related compounds dimethyl disulfide and dimethyl sulfide were degraded as well, in contrast to ethanethiol and propanethiol, which were not degraded anaerobically. In the new LPG biodesulfurization process higher thiols are converted to their corresponding oily disulfides that have to be skimmed off from the reactor solution and can be sent for disposal, *e.g.* to an incinerator.

The fifty percent inhibition concentration of MT, ethanethiol and propanethiol for methanogenic activity of anaerobic granular sludge on methanol and acetate was found between 6 and 10 mM (pH 7.2, 30°C). Hydrogen sulfide inhibited anaerobic MT degradation at concentrations below 10 mM, depending on the pH and the source of the inoculum. Dimethyl disulfide inhibited MT degradation already at concentrations below 2 mM. In a lab-scale upflow anaerobic sludge blanket (UASB) reactor that was inoculated with anaerobic granular sludge originating from a full-scale reactor treating paper mill wastewater, MT degradation was possible up to a volumetric loading rate of 17 mmol $MT \cdot L^{-1} \cdot day^{-1}$ (pH 7.0-7.5, 30°C, < 0.03 M total salts). MT degradation with this inoculum was inhibited by sodium concentrations exceeding 0.2 M. Initially, MT-degrading methanogenic archaea related to the genus *Methanolobus* were enriched in the reactor. Later, they were outcompeted by methanogens belonging to the genus *Methanomethylovorans*, which were mainly present in small aggregates (10-100 μm) in between larger particles.

Estuarine sediment from the Wadden Sea was used to inoculate an anaerobic reactor operated at Na^+ concentrations of 0.5 M. The maximum volumetric degradation rate achieved amounted to 37 mmol $MT \cdot L^{-1} \cdot day^{-1}$ at pH 8.2-8.4 and 22 mmol $MT \cdot L^{-1} \cdot day^{-1}$ at pH 8.9-9.1 (30°C). MT degradation at pH 10

was not possible with this inoculum. In activity tests, no inhibition of MT degradation was observed till 0.8 M Na^+. Initially, *Methanosarcina mazei* was the dominant MT-degrading methanogen, but after about 1.5 years of continuous reactor operation, methanogens related to *Methanolobus taylorii* became dominant, probably due to the pH shift to pH 9.0 in the reactor.

In a UASB reactor inoculated with a mixture of estuarine and salt lake sediments from the Soap Lake (USA) and the Kalunda Steppe (Russia) it was possible to degrade MT at pH 10, at a maximum volumetric loading rate of 13 mmol $MT \cdot L^{-1} \cdot day^{-1}$ (30°C, 0.8 M Na^+) in the presence of methanol as a co-substrate. The methanogenic archaea responsible for the degradation of MT were related to *Methanolobus oregonensis*.

Thiols that are not degraded in the anaerobic reactor of the novel LPG desulfurization process are directed to the third process step, *i.e.* the aerobic bioreactor. Our research shows that here MT will react with biologically produced sulfur (both 1-16 mM; pH 8.7 and 10.3; 30-60ºC) to form poly-sulfur compounds, *i.e.* polysulfide ions and dimethyl polysulfides. The first reaction step is a S_8 ring opening by nucleophilic attack to form $CH_3S_9^-$. The reaction rate depends on the MT and bio-sulfur concentrations, pH and temperature. The activation energy of this reaction was determined to be 70 $kJ \cdot mol^{-1}$ at pH 8.7 and 16 $kJ \cdot mol^{-1}$ at pH 10.3. The $CH_3S_9^-$ ion is unstable and leads to shorter-chain sulfur compounds. The main end-products formed are polysulfides (S_3^{2-}, S_4^{2-}, S_5^{2-}), dimethyl polysulfides [$(CH_3)_2S_2$, $(CH_3)_2S_3$] and H_2S. Also long-chain dimethyl polysulfides [$(CH_3)_2S_{4-7}$] are formed in trace amounts (μM level). Excess MT results in complete methylation of the initially formed inorganic polysulfides. An increased molar MT/S ratio results in the formation of relatively more $(CH_3)_2S_2$ over $(CH_3)_2S_3$.

Flowsheet simulations of the new LPG desulfurization process reveal that for an acceptable degree of desulfurization (*i.e.* less than 10 ppm in the treated LPG product) the pH in the recycle stream to the extractor column must be higher than 9. This means that the used inocula (estuarine and salt lake sediments) provide good opportunities to be applied in the process.

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General introduction

1.1. Introduction

The combustion of sulfur-containing fossil fuels results in SO_2 emissions to the atmosphere. Oxidation and hydrolysis of SO_2 causes formation of acid compounds that are deposited by rainfall (wet deposition) or by dry deposition [1]. Acid deposition strongly affects the health of numerous ecosystems, like weakly buffered lakes and forest reserves [2] and induces the mobilization of aluminum and other potential toxic metals from soils and sediments [1]. In addition, catalytic converters in automobiles are far more effective when the sulfur level in the fuel is low, as sulfur poisons the catalyst. The last 30 years, an ongoing trend in improved desulfurization technologies at oil refineries and natural gas upgrading facilities is therefore observed. Governments in many countries introduced regulations to reduce sulfur levels in gasoline to very low levels. In the United States, Environmental Protection Agency (EPA) regulations dictated gasoline sulfur levels down to 30 ppm by 2005 and diesel sulfur levels to 15 ppm in highway vehicles by 2006. The EPA is also proposing to reduce sulfur levels in non-road, locomotive and marine diesel fuels to 15 ppm by 2010. In Japan gasoline and diesel sulfur levels of below 50 ppm are required since 2005, and even more stringent requirements are forecasted. In the European Union, sulfur levels in both gasoline and diesel below 50 ppm since 2005 and below 10 ppm in 2009 are required [3]. It should be noted that so called "sulfur free" fuel may still contain up to 10 ppm sulfur.

This chapter overviews the biotransformation of methanethiol (MT) and other volatile organic sulfur compounds (VOSC) as well as their physical-chemical properties. These compounds are present in, amongst other, the light hydrocarbon fractions, such as liquefied petroleum gas (LPG). Desulfurization methods for VOSC are discussed with special attention to the desulfurization of LPG.

1.1.1. Sulfur compounds in crude oils

The sulfur content of crude oil ranges from 0.025 % to more than 8 wt % [2, 4, 5]. Organic sulfur compounds are the most important constituents, but inorganic sulfur, *i.e.* elemental sulfur, hydrogen sulfide and pyrites can also be present [6]. The organic compounds include sulfides (R-S-R), thiols (R-SH), thiophenes, substituted benzo- and dibenzothiophenes, and many more complex molecules [2].

It has been postulated that the major part of the organically-bound sulfur in crude oil was formed by reactions of organic matter with reduced inorganic sulfur species (H_2S and/or polysulfides) [7]. Because of the ubiquity of alkylated benzo- and dibenzothiophenes in practically all crude oils, these compounds represent the bulk of the sulfur in crude oil. The majority of the alkylated benzothiophenes have a boiling point of 220°C up to 300°C and the alkylated dibenzothiophenes have a boiling point up to 350°C [8]. The sulfur compounds present in LPG will be described in the next section.

1.1.2. Sulfur compounds in liquefied petroleum gas (LPG)

LPG is one of the top products from the crude oil distiller unit at refineries and consists of a mixture of low molecular weight hydrocarbon compounds. Raw natural gas often contains excess propane and butanes, which must be removed to prevent their condensation in high-pressure pipelines. These streams are known as condensates. LPG is also generated and collected during cracking of heavy hydrocarbons. Therefore, LPG can be considered as by-product and its exact composition and properties will vary greatly with the crude oil source and cracking process. In the United States and Canada, LPG consists primarily of propane (≥ 85% v/v), whilst in many European countries the propane content in LPG can be below 50% and the main compounds are butanes. Under moderate pressures (10-15 bar) LPG exists in a liquid state at ambient temperatures [9] and has a density between 0.5 and 0.7 kg/L [10, 11].

Some common sulfur compounds in LPG are H_2S, MT, ethanethiol (ET), propanethiol (PT), carbonyl sulfide (COS), dimethyl sulfide (DMS) and dimethyl disulfide (DMDS) (Table 1.1) [4, 11]. They are naturally present in crude oil or are formed by decomposition of higher sulfur compounds during crude distillation and cracking operations. Pyburn *et al.* [12] measured a total thiol concentration in propane samples from tank cars up to 30 ppmw.

Table 1.1. Examples of common sulfur contaminations in some LPG sources.

Sulfur compound	LPG[1] (ppmw)	LPG[2] (ppm)	LPG[3] (ppmw)	LPG[4] (ppm-mol)	propane[4] (ppm-mol)	*n*-butane[4] (ppm-mol)	*n*-butane[5] (ppm-mol)	LPG[6] (ppmw)
H_2S	50 - 2000		60		1000		traces	2000
CH_3SH	0 - 250	701	432	1200 - 1500	500 - 600	1500 - 1900	≤ 1000	1000
CH_3CH_2SH			217	4000 - 5000	5 - 10	8000 - 10000	≤ 5000	500
CH_3SCH_3		615						
$CH_3CH_2SCH_3$		329						
$CH_3CH_2CH_2SH$		34						
COS	0 - 50	34			500		traces	
CH_3SSCH_3	< 10	40						

1. Typical commercial LPG feed containing 28 wt% propane and 71% n-butane (Shell); 2. Pt. Gedio Makmur (Jakarta, Indonesia); 3. Manieh and Ghorayeb [11]; 4. Shell; 5. Axens; 6. Beychok [10]

VOSC are undesired in LPG because of their aggressive odor, although for LPG as a car fuel no official European sulfur norm exists. For further catalytical treatment of propane and butane, VOSC must be removed. The degree of removal depends on downstream requirements and product specifications. Industrial LPG product specifications are becoming more stringent. Ten ppmw sulfur used to be acceptable for many catalytic processes, *e.g. e*-butene isomerization to isobutene. But already in 1990, catalytic processes required 5, 2 or even < 1 ppmw of sulfur [13]. According to Lampert [14] desulfurization of LPG and natural gas to levels below 200 ppbv (in many applications to less than 20 ppbv) is necessary to improve the performance of fuel reforming.

Two common tests to detect the presence of thiols in petroleum products are the doctor test and the copper strip corrosion test. The doctor test involves formation of lead mercaptide followed by conversion with free sulfur to organic disulfide and lead sulfide. Conducted on a test-tube scale, these reactions constitute the doctor test for the detection of thiols in liquid petroleum products. The product is said to be doctor sweet when no black precipitate, *i.e.* lead sulfide, is present [15, 16].

The copper strip corrosion test is one of the specifications for propane. The test assesses the relative degree of corrosivity. Sulfur compounds present in propane can cause failure of this test. A classification number from 1a till 4c is assigned [17]. The threshold value for a failure (*i.e.* 2a) is about 0.35 ppmw H_2S as sulfur. However, other sulfur compounds in propane influence the outcome of the test. Pyburn *et al.* [12] concluded that H_2S and sulfur are the corrosive agents in propane and they act synergistically on the test. Also polysulfide contributes to copper corrosion. Thiols, in the presence of sulfur, enhance corrosion. However, in the presence of H_2S, thiols inhibit corrosion, which implies that the test is susceptible for interpretation errors.

1.1.3. Other VOSC-producing industries

Various other industries are faced with VOSC-containing fuels, (waste)water or gas streams. Owing to their low odor threshold (Table 1.2) VOSC contribute to odor pollution upon the emission of very small amounts. VOSC are mainly produced in those processes where organic matter is heated or decays under anaerobic conditions [18]. VOSC transformations are divided in biological and chemical processes.

Biological processes

Concentrations of 30 ppmv DMS and 4 ppmv H_2S were measured in the waste gas from an aerobic brewery wastewater treatment plant [19]. The volatile sulfur compounds (VSC) are mainly formed by (facultative) anaerobic microorganisms in oxygen-deficient parts of the plant. Also in waste gas from the municipal wastewater treatment plants, (thermal) sludge treatment plants, bio-industry (*e.g.* pig slurry odor, animal rearing stations) and in swine and dairy manure wastewater [20] VOSC are present. Iranpour *et al.* [21] found an increase of the MT concentration in the biogas of an anaerobic digester from about 2 ppm to 4 ppm after increasing the digester temperature from 53°C to 57°C. Novak *et al.* [22] noticed MT and DMS as the major odor compounds produced from dewatered sludge cakes. Composting facilities are known to cause odor problems as a result of microbial activity in anaerobic sites [23]. Anaerobic conditions, in addition to elevated temperatures, during the composting process (*e.g.* wet regions or poor mixing of starter material) favor the VSC formation, while their emission is strongly decreased upon aeration [19]. Methanethiol was found in landfill-gas in concentrations of up to 44 ppm [24].

Chemical processes and industrial applications

The Kraft paper production process involves a chemical treatment of wood chips in a cooker with a pulping solution of NaOH and Na_2S to remove the lignin bonding material from the cellulose fibers [25]. During the cooking process, H_2S, MT, DMS, and DMDS are formed by a reaction of the methoxyl groups of lignin with the cooking liquid solution. About 2-3% of the cooking liquor sulfur is transformed into volatile sulfur compounds. Gas effluent concentrations for MT, DMS and DMDS of 94, 17, and 22 ppmv, respectively were reported [19].

1.2. Physical-chemical characteristics of volatile organic sulfur compounds

Thiols are the sulfur analogues of alcohols, *i.e.* the oxygen in the alcohol is replaced by sulfur. Until 1930, these compounds were named mercaptans, because ethanethiol is able to remove mercury from solutions as a crystalline solid [16]. Particularly in commerce, the name mercaptan is still widely used.

H_2S and methanethiol are gases at room temperature and atmospheric pressure, while the other VOSC mentioned in Table 4.2 are volatile liquids under these conditions. The most important characteristics of these compounds are their low odor threshold value (ppbv range), high toxicity and potential corrosive effect [19, 26]. As the odor threshold is in the ppbv range, VOSC will contribute to odor problems when only very small amounts are emitted.

In the ppb range, MT, DMS and DMDS are important flavor components in foodstuffs such as tea, coffee, cocoa, beer, wine, cheese, milk, strawberries, radishes, oysters and many cooked vegetables, but at higher concentrations it contributes to spillage [19, 26]. Trace amounts of MT and DMS (and H_2S) are emitted from decaying proteins and may serve, due to their unpleasant odor, as a warning signal of toxins present in spoiled foods. However, the sense of smell for H_2S is readily fatigued by continuous exposure [27]. MT ($1\text{-}6\cdot10^{-9}$ g/L) and ET are also found in normal human breath [28]. DMS is partially responsible for the smell of the sea [29].

Table 1.2. Physical and chemical properties of some VOSC.

Sulfur compound	Boiling point (°C) [16, 19, 30]	Melting point (°C) [16, 30]	Odor threshold (ppbv) [29, 31, 32]	Odor characterization [33-35]	$H_{25°C}$ (-) * [36, 37]	pKa (25°C) [10, 30, 38]
Hydrogen sulfide (H_2S)	-60.7	-85.5	8.5-1000	Rotten eggs	0.41	6.90; 12.92
Methanethiol (CH_3SH)	6.2	-123.0	0.9-8.5	Disgusting, decayed cabbage	0.10	10.30
Ethanethiol (CH_3CH_2SH)	35.0	-147.9	0.01-35 [13,14]	Skunk	0.15	10.39
n-Propanethiol ($CH_3CH_2CH_2SH$)	67.8	-113.1	3.1 [15]	Rotten cabbage, skunk	0.17	10.44
Dimethyl sulfide (CH_3SCH_3)	37.3	-98	0.6-40	Unpleasant, decayed vegetables	0.073	-
Dimethyl disulfide (CH_3SSCH_3)	109.7	-85	0.1-3.6	Irritating, putrification, foul	0.045	-

* Dimensionless Henry coefficient at 25°C

1.2.1. Toxicity of VOSC and H_2S to animals and humans

MAC values (maximum concentration value in the workplace) for volatile (organic) sulfur compounds are lower than or equal to 20 ppmv (Table 1.3). According to Verschueren [35], severe toxic effects of H_2S for man appear at 200 ppmv after a one minute exposure period, while an 800 ppmv concentration can cause immediate death. At concentrations of 50 to 500 ppmv, H_2S primarily acts as a sensory and respiratory irritant, whereas exposures above 500 ppmv can cause respiratory and central nervous system paralysis [27]. According to the latter author, increased sensitivity to all forms of light serves as an early warning sign of excessive exposure. Because H_2S is readily detoxified in the body, the primary treatment of exposed persons consists of rapid removal from the contaminated environment [19]. Inhalation studies with rats showed that MT is more toxic than higher thiols and H_2S is even more toxic than MT (Table 1.3). The relatively low toxicity of DMS suggests that methylation of MT can be an effective detoxification methods, which was shown for rats. Little is known about the effect of human exposure to very low concentrations of H_2S or MT, although recent studies indicated that adverse health effects of malodorous sulfur compounds occur even at lower concentrations [39].

Table 1.3. Toxicological properties of some volatile (organic) sulfur compounds.

Sulfur compound	LC_{50}[a] inhalation rats (ppm/4h) [16, 32]	LD_{50}[b] oral rats (mg/kg) [16]	MAC[c] (ppmv) [18, 29, 40]
H_2S	444	-	1.6
CH_3SH	675-1664	-	0.5
CH_3CH_2SH	4970	1034	0.5
$CH_3CH_2CH_2SH$	7300	1730	-
$CH_3(CH_2)_3SH$	4460	2575	-
CH_3SCH_3	40,250	-	20
CH_3SSCH_3	805	-	<20

-: not reported
a) Concentration, which is lethal to 50% of the animals tested
b) Dose, which is lethal to 50% of the animals tested
c) Maximum concentration value in workplace conditions

1.2.2. Chemical preparation and usage of thiols

The bulk of the alkanethiols manufactured on a commercial scale are prepared by addition reaction of H_2S with olefins or by the substitution reaction of H_2S with alcohols. Lesser amounts are prepared by converting alkyl halides to the corresponding thiols with sodium or ammonium hydrosulfide [16, 41].

Industrial applications of thiols are their use for the synthesis of amino acids, tetrahydrothiophene and agricultural chemicals. MT is used in the first step of the commercial synthesis of methionine, an amino acid used as poultry feed supplement [16]. Lower thiols are also used as intermediates in the manufacture of insecticides, acaricides, herbicides and defoliants (causes plant leaves to fall off) [16]. DMS is used as solvent for inorganic substrates [19, 42].

Sulfur compounds are added as odorants in natural gas and LPG [19, 42]. In case of leaking they serve as warning agents to protect against hazards. MT [43] or ethanethiol are the odorants in LPG and in natural gas a blend of thiols, *e.g.* tertiair butanethiol and DMS is used or tetrahydrothiophene [16, 44].

1.2.3. Chemical oxidation of methanethiol

The first step in chemical thiol oxidation is the formation of a disulfide molecule. Disulfides are so resistant to further oxidation that under mild oxidizing conditions they are the major, if not the only products [28]. The oxidation of pure thiols with molecular oxygen at low temperatures is a slow reaction. In the presence of metal ions (*e.g.* Cu^{2+} or Fe^{2+}), basic catalysts and/or olefins, however, thiol oxidation by air proceeds rapidly [45]. The oxidation is also catalyzed by UV light, and other initiators of radical reactions. Factors which influence the oxidation rate include temperature, pH, buffer medium, type of catalyst, oxygen tension

and the nature and concentration of the thiol to be oxidized [28]. The rate of autooxidation increases with pH. Capozzi and Modena [46] proposed the following scheme for the overall reaction in the presence of base B^- and oxygen:

$$RSH + B^- \leftrightarrows RS^- + BH \quad (1)$$
$$RS^- + O_2 \leftrightarrows RS\cdot + O_2\cdot^- \quad (2)$$
$$RS^- + O_2\cdot^- \leftrightarrows RS\cdot + O_2^{2-} \quad (3)$$
$$2\ RS\cdot \leftrightarrows RSSR \quad (4)$$
$$O_2^{2-} + 2BH \leftrightarrows ½\ O_2 + 2\ B^- + 2\ OH^- \quad (5)$$

leading to the following overall reaction:

$$RSH + ½\ O_2 \rightarrow RSSR + H_2O \quad (6)$$

Several of the petroleum refinery sweetening processes that convert highly odorous thiols to less odorous disulfides involve oxidation of this type (also see section 1.4.1). A large excess of base and/or prolonged reaction times in aqueous solutions causes oxidation beyond the disulfides level to for example sulfonic acids [46].

1.3. Microbial cycling of volatile organic sulfur compounds

In the global sulfur cycle, volatile sulfur compounds play a role in the exchange of sulfur compounds from atmospheric with the terrestrial, oceanic, estuarine and freshwater compartments [40]. Annually, vast amounts of volatile sulfur compounds are released into the atmosphere by a combination of anthropogenic, geochemical and biological processes (Table 1.4). The global significance of VOSC became clear when Lovelock *et al.* [47] detected low (around 0.1 µg/L) but significant amounts of DMS in ocean waters. Later, Visscher *et al.* [48] reported DMS concentrations from 1-10 nM for a variety of coastal marine environments up to 200-300 µM in sediment pore waters, respectively. Lomans *et al.* [49] report volatile sulfur compounds like MT, DMS and DMDS (ng/L range) in freshwater.

Table 1.4. Emission rates of volatile (organic) sulfur compounds from natural and anthropogenic sources to the atmosphere [26].

	Sulfur compounds release (Tg S/year)						
Source	SO_2	H_2S	DMS	DMDS	CS_2	COS	Total
Oceanic		0-15	38-40	0-1	0.3	0.4	38.7-56.7
Salt Marsh		0.8-0.9	0.58	0.13	0.07	0.12	1.7-1.8
Swamps		11.7	0.84	0.2	2.8	1.85	17.4
Soil and plants		3-41	0.2-4.0	1	0.6-1.5	0.2-1.0	5.0-48.5
Burning of biomass	7	0-1		0-1		0.11	7.1-9.1
Volcanoes/fumaroles	8	1		0-0.02	0.01	0.01	9.0
Total	15	16.5-70.6	39.6-45.4	1.3-3.4	3.8-4.7	2.7-3.5	78.9-142.6

The amount of DMS that is emitted from natural systems and eventually reaches the atmosphere is only a minor fraction of the DMS formed biologically [50]. The major part (90%) of the DMS produced is degraded by anaerobic and aerobic microorganisms before it can reach the atmosphere. Microbial conversions therefore effectively control the fluxes of VOSC from natural environments to the atmosphere [40].

DMS and related methylated thiols play significant roles in natural ecosystems. DMS generated in the open ocean has been implicated as the major source of cloud condensation nuclei present in the marine atmosphere, with subsequent global effects on climate [29, 51].

Several mechanisms for the microbial formation of VOSC have been identified. These include the degradation of sulfur containing amino acids, degradation of dimethylsulfoniopropionate [DMSP, $(CH_3)_2S^+(CH_2)_2COO^-$], reduction of dimethylsulfoxide, reduction of DMDS and methylation of sulfide and MT.

Formation of MT and DMS originates from amino acids like S-methyl-methionine, ethionine, S-methyl-cysteine, both under aerobic and anaerobic conditions [52-54]. Both cysteine and methionine have been shown to be present in protein extracted from activated sludges and anaerobically digested sludges [55]. Methionine is first deaminated and subsequently demethylated to α-ketobutyrate, ammonia and MT. S-methyl-L-cysteine is degraded to pyruvate, ammonia and MT. Conversion of S-methyl-methionine results in the formation of mainly DMS [56] whereas degradation of cysteine results in the formation of H_2S.

Several strains with high DMDS-forming ability were isolated from activated sludge. They belonged to the genera *Lactobacillus*, *Corynebacterium*, *Pseudomonas*, *Alcaligenes* and *Achromobacter*. DL-methionine and S-methyl-L-cysteine were the precursors for DMDS formation [57]. Mechanisms for DMDS formation have not yet been investigated, but Kelly and Smith [26] suggested that DMDS formation is at least partly a result from autooxidation of MT to DMDS.

Ethanethiol was formed from degradation of S-ethylcysteine and from the reaction between ethionine and MT by the methionine auxotroph *Saccharomyces cerevisiae* [58]. Addition of ethionine to anoxic sediments resulted in the production of ET [59].

In marine, estuarine and salt marsh systems formation of MT and DMS originates mainly from the degradation of DMSP, a common osmolyte in many algae and halophilic plants that can alleviate salt stress and prevent freezing [60]. DMSP can be formed from methionine after addition of a methylgroup, deamination and decarboxylation [61]. It can be degraded by cleavage through the action of the enzyme DMSP-lyase resulting in the formation of acrylate (H_2C=CH-COOH) and DMS [62]:

$$(CH_3)_2S^+CH_2CH_2COO^- \rightarrow (CH_3)_2S + CH_2 = CHCOO^- + H^+ \quad (7)$$

Several aerobic [63-66] and anaerobic bacteria [40, 67], phytoplankton [68-70], flowering plants [71], and marine fungi [72] are able to degrade DMSP in this way. In addition, DMSP can be demethylated by aerobic and anaerobic microorganisms to form 3-methylmercaptopropionate (MMPA), *e.g.* [62, 73-75]:

$$(CH_3)S^+CH_2CH_2COO^- + XH \rightarrow CH_3SCH_2CH_2COO^- + XCH_3 + H^+ \quad (8)$$

Subsequently, MMPA can be either demethiolated resulting in the formation of MT and propionate, or demethylated resulting in the formation of 3-mercaptopropionate (MPA) [76, 77]. MPA can be degraded both aerobically as well as anaerobically [73, 77].

The reduction of dimethyl sulfoxide (DMSO), an oxidation product of DMSP is another commonly occurring mechanism of DMS formation is. Low concentrations of DMSO (1-200 nM), which probably originate from marine phytoplankton were found in ocean surface waters [78]. Among various aerobic and anaerobic bacteria, plants and animals DMSO reducing capacity is widespread [79-81]. DMSO is reduced to DMS by several marine sulfate-reducing bacteria [23]. Glindemann *et al.* [82] measured DMS concentrations of 10-200 μg/L in the influent to the aeration tank of a sewage treatment plant as a result of DMSO reduction. This high DMS concentration caused severe odor problems in the neighborhood.

In addition, MT and DMS formation occurs during degradation of methoxylated aromatic compounds (Ar-O-CH_3) by the methylation of sulfide and MT by some homacetogenic bacteria isolated from various habitats, *e.g.* [51, 83, 84]:

$$Ar\text{-}O\text{-}CH_3 + H_2S \rightarrow Ar\text{-}OH + CH_3SH \quad (9)$$

$$Ar\text{-}O\text{-}CH_3 + CH_3SH \rightarrow Ar\text{-}OH + CH_3SCH_3 \quad (10)$$

This type of anaerobic methylation of sulfide during the degradation of methoxylated aromatic compounds appears to be distributed among different genera, *e.g. Holophaga*, *Sporobacterium* and *Termitobacter* [40]. Because methoxylated aromatic compounds are degradation products of lignin, a highly abundant biopolymer on earth, this mechanism for VOSC production is likely to be important in freshwater systems that generally contain more lignin than marine and estuarine systems.

Visscher *et al.* [85] hypothesized that DMS and MT in microbial mats are formed by the reaction of photosynthetically produced low-molecular weight organic carbon and biogenic hydrogen sulfide derived from sulfate reduction. These observations suggest that DMSP or S-containing amino acids are not the only dominant precursors of DMS in intertidal sediment systems. They proposed the following mechanisms:

$$XCH_3 + HS^- + H^+ \rightarrow XH + CH_3SH \quad (11)$$

$$CH_3SH + XCH_3 \rightarrow CH_3SCH_3 + XH \quad (12)$$

where X is a 'generic' methyl group donor (low-molecular weight organic carbon).

Stets *et al.* [84] observed the formation of ethylmethyl sulfide (μM level) by adding ethanethiol to peat slurries. Addition of methanol or syringic acid stimulated the thiol methylation potential and DMS accumulation, suggesting that these compounds could be methyl donors.

Figure 1.1 shows an overview of the cycling of some reduced volatile (organic) sulfur compounds. The anaerobic degradation of MT (reaction 3), which is the main topic of this thesis, will be discussed in section 1.3.1. in more detail.

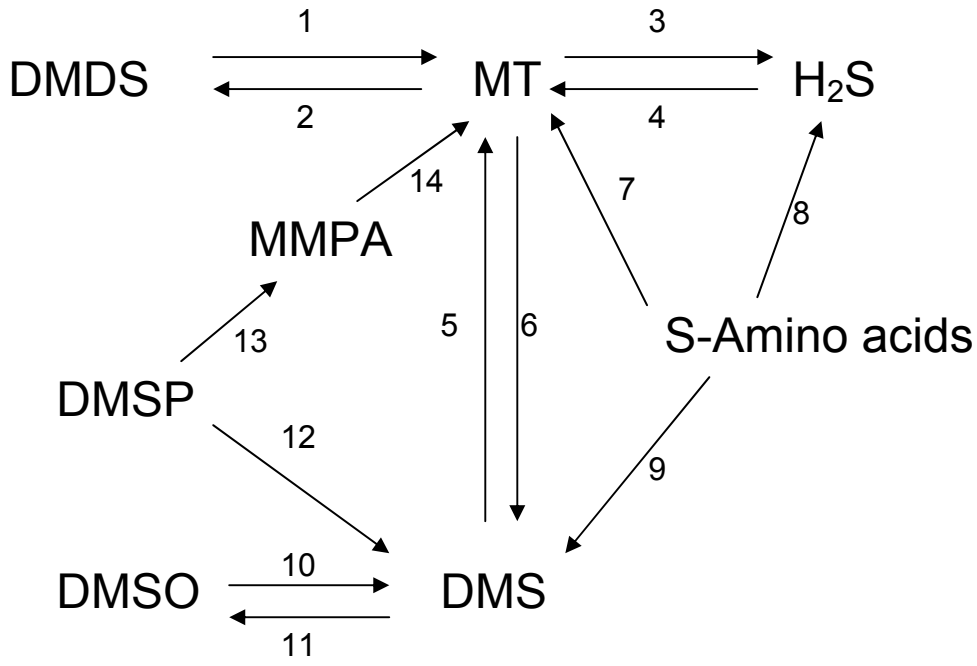

Fig. 1.1. Simplified scheme for the formation and degradation of some reduced volatile organic sulfur compounds [26, 29, 53, 62]. 1. DMDS reduction; 2. chemical MT oxidation (aerobic); 3. MT fermentation (eqn 14, page 13) or sulfate reduction; 4. H_2S methylation (from lignin compounds) (see text); 5. demethylation of DMS by monooxygenase; 6. thiol transmethylation; 7-9. VOSC formation from amino acid degradation; 10. DMSO reduction; 11. DMS oxidation; 12. DMSP cleavage resulting in the formation of acrylate (H_2C=CH-COOH) and DMS; 13. aerobic or anaerobic DMSP demethylation to form 3-methylmercaptopropionate (MMPA); 14. demethiolation of 3-methylmercaptopriopionate (list of abbreviations on page 38).

1.3.1. Microbial degradation of VOSC

Microbial degradation of VOSC occurs under both aerobic and anaerobic conditions (Table 1.5).

Table 1.5. Microbial mechanisms of microbial VOSC degradation [40].

Oxidation	DMS/MT + O_2 → MT + CH_2O → CO_2 + SO_4^{2-}
	DMS/MT + O_2 → CO_2 + $S_2O_3^{2-}$
Phototrophic oxidation	DMS/MT → DMSO
Oxidation to DMSO	DMS + O_2 → DMSO
Denitrification	DMS/MT + NO_3^- → N_2 + CO_2
Methanogenesis	DMS/MT → CH_4 + CO_2 + HS^-
Sulfate reduction	DMS/MT + SO_4^{2-} → CO_2 + HS^-

Aerobic degradation

A large variety of microorganisms isolated from sewage treatment plants, marine sediments, soil and biofilters is able to degradate MT and DMS aerobically. They belong to the genera

Thiobacillus, Methylophaga and *Hyphomicrobium* [86-92]. De Bont *et al.* [88] elucidated the mechanism for DMS degradation for several *Thiobacillus* and *Hyphomicrobium* strains. *Hyphomicrobium* strains oxidize DMS to MT and formaldehyde. MT is subsequently oxidized to formaldehyde, hydrogen peroxide and sulfide. The oxidation of sulfide results in the formation of sulfuric acid. Part of the formaldehyde produced is incorporated into the cell biomass through the serine pathway [40]. Therefore, hyphomicrobia are C_1-compound metabolizing heterotrophs.

Thiobacilli are generally chemolithoautotrophs. Unlike *Hyphomicrobium* strains *Thiobacillus* strains use the Calvin cycle to fix carbon dioxide as their carbon source [26]. Thiobacilli are capable of VOSC oxidation like DMDS, DMS and MT as is the case for H_2S. Oxidation of DMS results in the formation of sulfide and formaldehyde, which is further oxidized to sulfuric acid and carbon dioxide.

Provided that an additional carbon source is present, a variety of bacteria is capable of oxidizing DMS to DMSO [93]. Also the non-biological photochemical oxidation of DMS to DMSO is a major sink for DMS in the overlying water column of microbial mats [85]. Further oxidation of DMSO to dimethyldisulfoxide ($DMSO_2$) that is mediated by haloperoxidases has been found in several marine microorganisms [94].

Higher thiols can also be degraded aerobically. MT oxidase is a key enzyme in the metabolism of methylated sulfur compounds, including MT and ET, by *Hyphomicrobium* EG [95]. Kelly and Smith [26] mentioned the isolation of an aerobic bacterium that grew on several VOSCs, including ET. Later, a *Thiobacillus* strain was isolated that could oxidize diethyl sulfide, ethylmethyl sulfide, dipropyl sulfide, MT, ET, 1-PT, and 1-butanethiol, as well as the corresponding disulfides with NO_3^- or O_2 as the electron acceptor [96].

Anaerobic degradation

The main anaerobic MT- and DMS-degrading groups of microorganisms are methanogenic archaea, sulfate-reducing bacteria, anoxygenic phototrophs and denitrifying bacteria are [40, 61, 97-101]. Anoxygenic phototrophic sulfur bacteria normally use sulfide as electron donor, but are also able to oxidize DMS to DMSO, using DMS as electron donor [102, 103].

Methanogenic conversion of MT and DMS was first illustrated in slurries prepared from lake sediments in which DMS was converted to methane and carbon dioxide with a stoichiometry of 3 to 1 [100, 101]. DMS is first reduced to form methane and MT, and MT is subsequently degraded to methane, carbon dioxide and H_2S [98]:

$$2\ (CH_3)_2S + 3\ H_2O \rightarrow 3\ CH_4 + HCO_3^- + H^+ + 2\ H_2S \qquad (13)$$

$$4\ CH_3SH + 3\ H_2O \rightarrow 3\ CH_4 + HCO_3^- + 4\ HS^- + 5\ H^+ \qquad (14)$$

Initially, it was suggested that MT could only be co-metabolized by methanogens in the presence of DMS. However, in 1992 a methanogen capable of growth on MT alone was isolated [97]. Since then, various methanogens have been isolated with DMS or MT from marine, estuarine, salt marsh, and salt lake sediments (Table 1.6).

Table 1.6. Comparison of the main characteristics of all known DMS- and MT-degrading anaerobic microorganisms (after Lomans *et al.* [40]).

Name (type strain)	Origin	Substrate	pH *	T (°C) *	NaCl (M) *	Reference
Ml. taylori (GS-16^T)	Francisco Bay, Oregon, USA(E)	Me, Ma, MeS	8.0 (6.0–9.0)	29–37 (4–40)	0.5 (0.2–1.4)	[98, 104, 105]
Ml. bombayensis (B-1^T)	Arabian Sea, Bombay, India (M)	Me, Ma, MeS	7.2 (6.0–8.5)	37 (20–40)	0.5 (0.25–2.0)	[106]
Ml. oregonensis (WAL1T)	Alkali Lake, Oregon, USA (ASL)	Me, Ma, MeS	8.1–9.1 (7.6–9.4)	35–37 (20–40)	< 0.5 (0.1–1.5)	[107]
Ms. siciliae (T4/M^T)	Sicily, Italy (M)	Me, Ma, MeS	6.5–6.8 (5.8–7.2)	37–40 (20–48)	0.4–0.6 (0.1–1.5)	[108, 109]
Ms. siciliae (HI350^T)	Oil well, Mexico (M)	Me, Ma, MeS	6.5–6.8 (5.8–7.2)	40 (20–48)	0.4–0.6 (0.1–1.5)	[108]
Ms. siciliae (C2J^T)	Scripps Canyon, California, USA (M)	Me, Ma, MeS, Ac	6.0–7.0 (5.0–8.0)	35 (20–45)	0.2–0.6 (0–0.6)	[110]
Ms. acetivorans (C2A^T)	Scripps Canyon, California, USA (M)	Me, Ma, MeS, Ac	6.5–7.0 (5.5–8.0)	35–40 (15–45)	0.1–0.4 (< 1.0)	[111]
Ms. semesiae (MD1^T)	Mtoni Creek, Tanzania (MF)	Me, Ma, MeS	6.5–7.5 (6.2–8.3)	30–35 (18–39)	0.2–0.6	[112]
Msa. zhilinaeae (WeN5^T)	Bosa Lake, Egypt (ASL)	Me, Ma, MeS	9.2 (8.2–10.3)	45	0.7 (0.2–2.1)	[113, 114]
Msa. zhilinaeae (Z-7936^T)	Lake Magadi, Kenya (SL)	Me, Ma, MeS	8.7–9.2 (7.8–10.2)	37 (20–48)	0.4 (0.2–2.2)	[115]
Strain MTP4 (MTP4^T)	Bordeaux, France (SM)	Me, Ma, MeS, Ac	6.9–7.6 (6.1–8.0)	30 (12–35)	0.035–0.40	[97]
Mm. hollandica (DMS1^T)	Dekkerswald, The Netherlands (F)	Me, Ma, MeS	6.5–7.0 (6.0–8.0)	34–37 (12–40)	0.04 (0–0.4)	[116]
Mm. hollandica (ZB^T)	Baldegger Lake, Switserland (F)	Me, Ma, MeS	Not reported	1–38 (30)	Not reported	[117]

The origin of inoculum is given by the following symbols: ASL, alkaline salt lake sediment; E, estuarine sediment; F, freshwater sediment; M, marine sediment; MF, mangrove forest sediment; SL, salt-lake sediment; SM, salt marsh sediment. The substrates for methanogenesis: Me, methanol; Ma, methylamines; MeS, methylated sulfur compounds; Ac, acetate. Abbreviations of taxa: *Ml., Methanolobus*; *Mm., Methanomethylovorans*; *Ms., Methanosarcina*; *Msa., Methanosalsus*.
*) Values given represent the optimum pH, temperature, and salt concentration for growth (ranges given within parentheses).

Ni and Boone [118] demonstrated that many of the other DMS-degrading methanogens could also grow on MT as sole carbon and energy source. These methanogens belong to the genera *Methanosarcina*, *Methanolobus* and *Methanosalsus* [59, 97, 98, 114, 119] (Table 1.6).

Lomans *et al.* [116] were the first to isolate a methanogen from freshwater sediment, *Methanomethylovorans hollandica*, capable of degrading DMS and using it as sole carbon and energy source. It is an obligately methylotrophic methanogen only capable of growth on methanol, methylamines, MT and DMS [116].

From incubations with DMDS amended sediment slurry, Kiene *et al.* [98] concluded that DMDS is reduced to 2 MT. Subsequently, MT is metabolized to sulfide, methane and carbon dioxide by methylotrophic methanogens. Suspensions of *T. thioparus* strain E6, produced MT from DMDS by a NADH-stimulated reduction under anaerobic conditions [91].

Methanogenesis from DMS and MT seems likely to occur in a similar way to that of structural analogs like methanol [51, 120] since both compounds are disproportionated into methane and carbon dioxide. However, since cell extracts of methylotrophic methanogens grown on methanol or trimethylamine have been found to be unable to convert DMS or MT, it is most likely that these substrates are converted by distinct inducible enzymes [121].

Degradation via sulfate reduction

Kiene *et al.* [98] concluded from inhibitory studies that sulfate reducers and methanogens were competing for DMS when it was present at low concentrations (< 10 μM) in marine and estuarine sediments. At higher concentrations, DMS appeared to be a substrate for methanogens only. Despite the evidence for a possible role of sulfate-reducing bacteria in the degradation of DMS and MT, only three pure cultures of sulfate-reducing bacteria growing on MT or DMS have been isolated from a thermophilic digestor [99]. The strains, which belong to the genus *Desulfotomaculum,* oxidize MT and DMS to carbon dioxide and sulfide.

Anaerobic degradation of higher thiols

Methyl sulfur compounds have been found to serve as sulfur source in several methanogenic archaea incapable of methane formation from these compounds. Rajagopal and Daniels [122] used among others MT, ET, 1-PT, 1-butanethiol, DMS and diethyl sulfide as sulfur source for growth of several methanogenic archaea and found optimal growth between 1-2 mM of the sulfur compounds.

So far, degradation of higher thiols, such as ET and 1-PT, has never been reported under methanogenic or sulfate-reducing conditions. Suflita *et al.* tested the biodegradation of eight organothiol compounds (pentanethiol till octanethiol and some aromatic compounds). Degradation occurred in nitrate-reducing cultures, but not in sulfate-reducing or methanogenic cultures.

Methanogens in Big Soda Lake sediments produced ethane and ethane, stimulated by ET addition [59], but these were very low production rates (about 1 nmol ethane · [150 ml slurry]$^{-1}$ · day^{-1}) and accounted for less than 1% of the initial ET concentration. Addition of 1-PT caused a slight increase in the amount of propane evolved.

ET and 1-butanethiol were hardly degraded (7-9%) by the sulfate-reducing bacterium *Desulfovibrio desulfuricans* M6 during a 5 days incubation at 30°C [123]. Kurita *et al.* [124] claimed to have anaerobic degradation of 1-butanethiol by anaerobic bacteria isolated from sludges of reservoir bottom of an oil well district (Nagaoka, Japan). However, this was only based on the formation of dark brown FeS precipitate in the medium after anaerobic incubation for 2 days (30°C, pH 7.2).

1.3.2. Inhibition of microorganisms

The sulfhydryl groups of thiols may inhibit enzyme functioning by steric hindrance and ionic or hydrophobic interactions [125]. Sulfhydryl compounds may also disrupt intramolecular disulfide bonds in neutral or alkaline solutions and thus alter the tertiary structure of the protein, rendering it catalytically inactive [126, 127]:

$$R_1\text{-S-S-}R_1 + R_2S^- \leftrightarrows R_1\text{-S-S-}R_2 + R_1S^- \quad (15)$$

Organic sulfur compounds are well known nitrification inhibitors. DMS and DMDS are weak inhibitors, while ET and allylsulfides (allyl: CH_2=CH_2-CH_2-R) are strong inhibitors of the oxidation of ammonium to nitrite. Inactivation of ammonia monooxygenase occurred during the process of oxidation of the sulfur compounds [128, 129].

A decrease in microbial activity of activated sludge in a wastewater treatment plant by DMS and DMDS at gas concentrations of 30 ppmv was reported. However, continuous exposure to these compounds resulted in adaptation of the sludge and increased tolerance limits [19].

Londry and Suflita [130] found that thiols inhibited methanogenesis at concentrations above 5 mM. They tested the toxicity of a mixture of long-chain (C_5–C_8) and cyclic thiols with lactate as the substrate. MT and DMS are indicators of toxicity or stress on biomass. Numerous microorganisms accumulate and release sulfur compounds in response to environmental stress [131]. A variety of high-molecular-weight thiols are detoxified in a methylation reaction catalyzed by thiol S-methyl-transferase. A similar mechanism is used in the detoxification of H_2S and MT to DMS. DMS is a relatively nontoxic compound [132].

1.4. Desulfurization of VOSC-containing fuels, wastewater and gas streams

Various biotechnological and physical-chemical technologies have been developed to treat VOSC-containing fuels, wastewater and gas streams. Traditional methods for odorous gases treatment include the use of chemical scrubbers and adsorption with activated carbon. Three types of bioreactors are commonly used for biotechnological waste gas treatment: bioscrubbers, biotrickling filters and biofilters. Desulfurization of thiol-containing light hydrocarbon streams is normally done by caustic extraction and chemical oxidation.

The following section discusses the common physical-chemical techniques used for VOSC removal: chemical scrubbing, caustic extraction, ammonia washing, stripping, wet air oxidation, advanced oxidation and adsorption. Alternative biological methods that can be used for the treatment of VOSC containing streams, are: biofiltration, biotrickling filtration and bioscrubbing, membrane bioreactor, aerobic treatment of spent caustics and anaerobic treatment.

1.4.1. Physical-chemical treatment

Chemical scrubbing of VOSC-containing gases

The aim of a scrubber is to remove (scrub) pollutants or particulates from industrial exhaust gas streams into a liquid phase by intense mixing of the gas stream with the liquid stream. The mass transfer of VOSC from a gas phase to an aqueous phase depends on the concentration of the pollutants in both phases, the air/water distribution coefficient (Henry, Table 1.2) and the mass transfer resistance of the scrubber [133]. The transfer of VOSC from a gas stream into an aqueous phase requires a high water-to-gas ratio, because of their high Henry-coefficients. The scrubbing efficiency of VOSC can be enhanced, however, by making the water alkaline, by oxidation of the absorbed VOSC to more water soluble compounds or by the use of other scrubbing liquids [133] that have a higher VOSC solubility than water. Alkaline scrubbing can be applied for the removal the light thiols MT, ET and PT (pKa = 10.3-10.5 – Table 1.2) provided that the pH of the scrubbing liquid is at least 11 [134]. Desorption of thiols from the liquid phase is possible due to the reversibility of the reaction.

Hypochlorite is an efficient oxidant for the chemical scrubbing of VOSC [135, 136]. MT is oxidized to methane sulfonic acid ($MeSO_3H$) via DMDS as an intermediate [134]. Good odor removal was obtained from composting waste gases with a two-stage packed tower scrubber using acid (pH 2.5) in stage I and hypochlorite (pH 6.7) as oxidizing agent for VOSC in stage II. DM(D)S (15 ppmv), thiols (2 ppmv), amines (20 ppmv) and ammonia (300 ppmv) were removed up to 99% [137].

Hydrogen peroxide (H_2O_2) is also used as an oxidant for the chemical scrubbing of VOSC. In aqueous solutions at room temperature, hydrogen peroxide converts DMS to DMSO. $DMSO_2$ is formed at higher temperature and excess H_2O_2. MT and DMDS are oxidized by H_2O_2 to methyl sulfonic acid [138]. Complete removal was reported of amines and thiols in rendering waste gases using an alkaline (pH > 9.6) hydrogen peroxide (H_2O_2) solution (0.1-0.8 g/L) in scrubbing stage I and an acid (pH < 5.0) H_2O_2 solution (0.5-0.8 g/L) in scrubbing stage II [139].

Treatment with an alkanolamine solution is a commonly used method to remove H_2S and CO_2 from various gas streams [140]. Examples of amines employed are monoethanolamine, diethanolamine, diglycolamine, and methyldiethanolamine [141].

Analogously, Yan [142] presented a method for thiol removal from gasoline by ammonia washing as an alternative for caustic extraction. Thiols were washed from an oil phase into an ammonia liquid. In the ammonia liquid phase the thiol reacted as follows:

$$RSH + NH_4OH \leftrightarrows RSNH_4 + H_2O$$

The produced $RSNH_4$ easily dissolves in ammonia, so the thiols can be separated from the oil phase. Optimal thiol removal with ammonia were reported at 1.5 to 3 wt% ammonia, a gasoline/solvent ratio of 20:1 and an extracting time of 20 minutes. The advantage compared to caustic washing is that the process operates at lower pH values and no alkaline residue, which needs to be treated, is produced. However, caustic washing has a higher efficiency than ammonia washing. Even at optimal conditions, thiol removal with ammonia never exceeded 62% [142].

Caustic liquid-liquid extraction of hydrocarbon streams

Liquid-liquid extraction is a process for separating components in solution by their distribution between two immiscible liquid phases [36]. Strong alkaline sodium hydroxide solutions (5-15 wt%) [10, 15] are used to extract hydrogen sulfide and acidic organic sulfur compounds, such as thiols, from liquid hydrocarbon streams. The degree of desulfurization obtained is primarily dependent on the hydrocarbon portion (R) of the thiol molecule, which affects the solubility of the compound in the caustic solution. The extraction of thiols includes a mass transfer step from the organic to the water phase and a dissociation reaction where a thiolate is formed:

$$R\text{-}SH\ (org) \leftrightarrows R\text{-}SH\ (aq) \leftrightarrows\ RS^-\ (aq) + H^+\ (aq) \qquad (16)$$

A low carbon number for the R portion of the RSH molecule, a low temperature [143], excess NaOH and higher RSH concentration drive the equilibrium of the two reactions to the right. A large volume of dilute alkali is more efficient than a small volume of concentrated alkali when a large stoichiometric excess of caustic soda is used for an extraction (both the same molar quantity of caustics) [144].

The most efficient way of removing thiols is in a multistage countercurrent extraction column, which may contain a series of extraction trays. The thiol containing feedstock is introduced in the first mixer settler and caustic is introduced into the last mixer settler. Each mixer-settler stage provides for intimate contacting of the charge stock and caustic and then for separation of the two immiscible phases. In a perfect theoretical stage of contact, dynamic equilibrium is reached, but in practice this never happens [145] due to too little contact time. Depending on the feedstock properties and the required product specifications, the number of extraction stages have been optimized at five to seven [144]. This ensures maximum

desulfurization with a minimum caustic circulation rate [145]. However, continuous countercurrent extraction is only economic if the extraction liquid can be continuously regenerated.

The resulting sulfide- and thiol-containing waste stream is called spent-sulfidic caustic [146]. These spent sulfidic caustics typically have a pH greater than 12, sulfide concentrations exceeding 2 to 3 wt %, and a large amount of residual alkalinity. The usual spent caustics are derived from LPG extraction or debutanized gasolines containing low amounts of H_2S, ET and heavier thiols, thiophenols and phenols [10]. Depending on the source, spent caustics may also contain, amines, and other organic compounds that are soluble or emulsified in the caustic [147, 148].

Currently, most spent sulfidic caustics are sent off-site for commercial recovery or reuse (*e.g.* in pulp and paper mills), treated in wet-air oxidation processes, treated by ozonation, disposed by deep-well injection [147-149], incinerated [149] or biotreated [150].

Stripping

Minami *et al.* [151] removed more than 80% of the MT, DMS and DMDS from evaporator condensate, discharged from a kraft pulp production process, by stripping with gas from a digester. The gas can be treated by methods described in this section (1.4.1 and 1.4.2).

Wet air oxidation

Wet oxidation is the oxidation of soluble or suspended components in water using oxygen as the oxidizing agent. When air is used as the source of oxygen the process is referred to as wet air oxidation. The oxidation reactions occur at temperatures of 150ºC to 320ºC and pressures from 10 to 220 bars [152]. Wet air oxidation for on-site treatment of spent sulfidic caustics is commercially available, but the capital investment costs and the operating costs are considerable [146, 153]. Wet air oxidation is typically employed as a waste treatment technology when the waste is non-conducive to incineration or biological treatment [149]. It is an ideal process for pretreatment of wastes that are difficult to degrade in conventional biological treatment facilities. Wet air oxidation is commonly used for treating spent (sulfidic) caustics from gasoline sweetening, gasoline and LPG prewashing and from gasoline and LPG thiol extraction. Sulfides and thiols are oxidized to sulfate [149]. In addition to the primary end-products SO_4^{2-}, CO_2 and H_2O, carboxylic acids (*e.g.* acetic acid) and other partially oxidized short chain organics are formed. This wastewater stream can be treated in a biological treatment system [150].

Advanced oxidation

Several advanced oxidation methods are used for the oxidation of VOSC. Ozone (O_3) is a powerful oxidant, especially when it is decomposed into OH-radicals in aqueous solutions, initiated by OH^- ions. Sulfur-containing compounds are efficiently oxidized with ozone in a

water phase [154], while gas phase oxidation reactions are usually too slow to be of interest, except for H_2S. Ozone oxidation products of VOSC include sulfonic acids, sulfones, acids and sulfonic anhydrides [133]. Ozonation of MT results in the accumulation of methane sulfonic acid ($MeSO_3H$) via DMDS as an intermediate [154].

Currently, wet scrubbers using oxidizing chemicals, such as chlorine dioxide (ClO_2), are applied to remove volatile organic compounds generated in for example the poultry rendering industry. Thiols and disulfides rapidly react with chlorine dioxide. The pH affects the reaction rates significantly [155, 156]. However, the use of chlorine dioxide (ClO_2) as an oxidant is more expensive in comparison with hypochlorite and ClO_2 produces toxic chlorine in the exit gas [19].

Yan *et al.* [157] report the removal of odorous gasses containing H_2S, MT and DMS by a combination of oxidation by pulsed corona plasma and an active carbon filter.

Lampert [14] presented a selective catalytic oxidation method for the desulfurization of natural gas and LPG for fuel cell reformer applications. The fuel is mixed with air and contacted with a monolith supported sulfur tolerant catalyst at atmospheric pressure and a temperature of 250-270°C. Organic and inorganic sulfur species in the fuel are selectively oxidized to sulfur oxides. Subsequently, the sulfur oxides are adsorbed on a high capacity adsorbent. For liquid petroleum gas at maximum allowable sulfur levels (120 ppmw or 165 ppmv), the residual sulfur is less than 160 ppbv [14].

Adsorption

Activated carbon (AC) is the most often used adsorbent material for the removal of volatile sulfur compounds [158]. The method is based on physical adsorption of the compounds on a high specific surface (750-1500 m^2/g) with a high fraction of the total pore volume in the micropore range (< 2.5 nm diameter) [158]. Other available adsorbents are *e.g.* silica gel, zeolites, activated alumina, synthetic resins and clay minerals. Sugiura [159] used sepiolite, a clay mineral, and coconut-shell activated carbon as adsorption material for MT removal from air.

Removal of dissolved DMS, DMDS and CS_2 with AC was very effective [154]. Katoh *et al.* [160] used a wet AC fibre at room temperature to remove H_2S, MT and DMS from air. After breakthrough DMDS was detected in the outlet of the AC column in an experiment with MT only, while MT was completely oxidized to methane sulfonic acid in a mixed supply with H_2S. Methane sulfonic acid was detected in the drain water. DMSO and $DMSO_2$ were formed from DMS. The presence of H_2S in the waste gas strongly affected the breakthrough time of MT on AC [161].

AC can be impregnated with NaOH or KOH to optimize the adsorption of H_2S and MT [158]. This is used at many wastewater treatment plants for H_2S removal and other compounds. However, NaOH and KOH also react with CO_2 in the air to form carbonates, which decreases the process performance. The use of ammonia gas as a catalyst in

combination with impregnation of the AC with base offers an advantage of about a factor 8 over virgin carbon and a factor 3 to 6 over KOH impregnated carbon [158].

Activated carbon provides a catalytic surface for oxidation and can also be impregnated with oxidation catalysts such as metallic oxides or noble metals. Thiols are readily oxidized to disulfides. When such oxidation occurs on carbon, the almost doubled molecular weight yields an increased capacity for physical adsorption [158]. Also the addition of a few ppmv of ozone to sulfur-containing process gases prior to the AC adsorption increased the breakthrough time of the AC filter considerably [133].

Molecular sieves

Molecular sieves are adsorbents composed of aluminosilicate crystalline polymers (zeolites). They efficiently remove low concentrations of polar or polarizable contaminants such as H_2O, methanol, H_2S, CO_2, COS, thiols, sulfides, ammonia, aromatics and mercury down to trace concentrations [162] at ambient pressure and temperature [14]. However, sulfur adsorption capacities are low, typically less than 2 g S/100 g adsorbent for natural gas and less than 1 g S/100 g for LPG. This requires large adsorption inventories and frequent replacements of the molecular sieves. Also, since they accumulate heavier hydrocarbons, the spent adsorbents are hazardous and require special handling [14]. Molecular sieves are widely used for removal of sulfur compounds from LPG. Their use is required as some feedstocks contain other nonacidic, non-thiol sulfur compounds, such as DMS and CS_2, which cannot be removed by caustic extraction.

For high thiol feedstocks or for low sulfur requirements, a two-step treating approach should be considered. A Merox or Sulfrex unit (see below) is placed first in line to reduce the sulfur to around 20 ppmw. The molecular sieve removes the residual thiol, reentry sulfur and any other sulfur down to less than 1 ppmw.

Combined technology: Merox process for LPG desulfurization

Several commercial processes for thiol removal from hydrocarbon steams have been developed, such as the Merox process, the Sulfrex process and the Mericat process [163]. They are all based on the caustic extraction of thiols from the hydrocarbon to a water phase and subsequently a chemical oxidation to disulfides.

The Merox process is a catalytic process for thiol extraction or sweetening of hydrocarbon fuels with compositions ranging from natural gas to diesel fuel. The extraction version of the Merox process is shown in Figure 1.2. Some of the bigger NGL (natural gas liquids, *i.e.* condensates) Merox units treat more than 100,000 BSD (barrels per stream day) (= $15.9 \cdot 10^3$ m^3/day) of propane, butane, natural gasoline or a combination of these. The smaller NGL Merox units treat as little as 300 BSD (48 m^3/day) of natural gasoline.

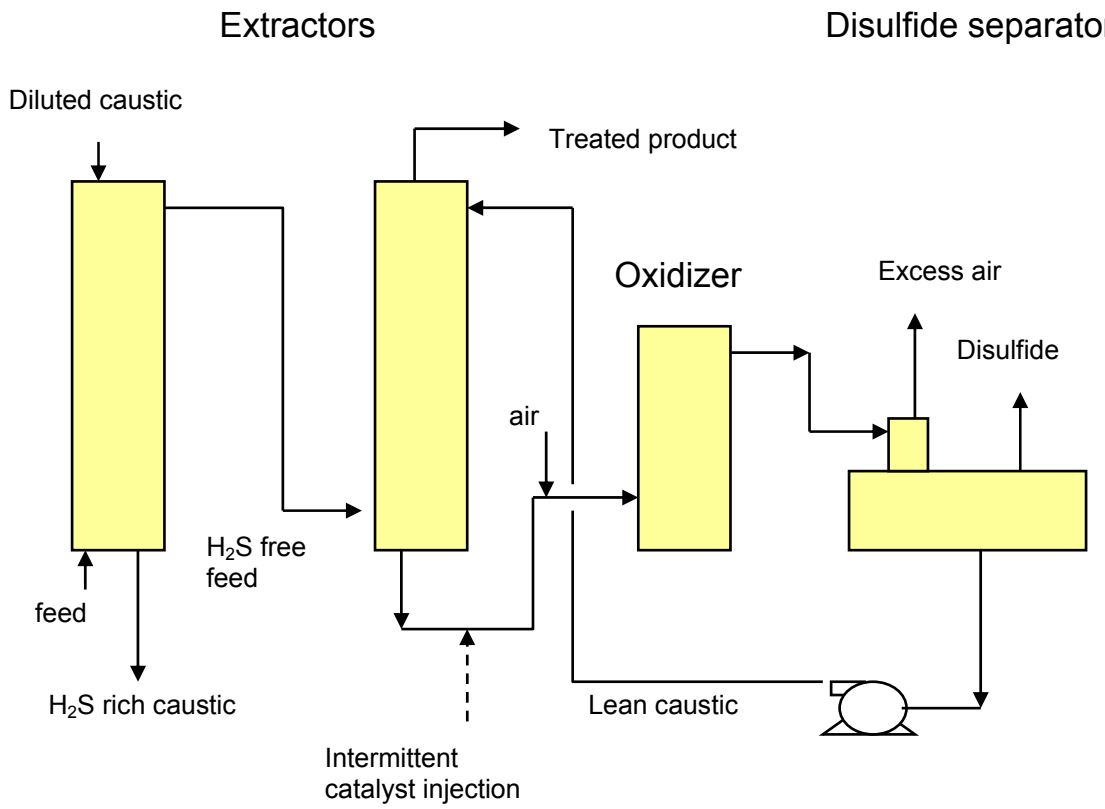

Fig. 1.2. Schematic representation of the Merox process for thiol extraction.

If H_2S is present in the feed, the extractor column is preceded by a diluted caustic (2 wt % NaOH) prewash for H_2S removal. In the next step, strong caustic solutions (20 wt % NaOH) are used to extract low molecular weight thiols from the hydrocarbon feedstock. After extraction, the treated product flows through a sand filter coalescer to reduce the sodium content to less than one ppmw. In the presence of a catalyst and air, the thiols are oxidized to disulfides in the oxidation reactor (Fig.1.2) according to equation 16:

$$2\ RS^-\ (aq) + 0.5\ O_2\ (g) + H_2O\ (l) \rightarrow RSSR\ (org) + 2\ OH^-\ (aq) \qquad (16)$$

This reaction is irreversible and accelerated by higher temperatures, higher concentrations of air, better mixing and increased catalyst activity (see section 1.2.3). The disulfide formed is insoluble in the caustic solution. The oxidizer effluent flows to the separator. The oxidized solution exiting the top of the oxidizer column flows to the disulfide separator where spent air, disulfide oil/wash solvent and caustic solution are separated. The spent air is usually vented to an existing fired heater near the site, while the disulfide oil/wash solvent stream is decanted and sent to a hydrotreating unit to form H_2S and CH_4. The caustic stream leaving the disulfide separator is circulated back to the extraction column. The Merox process can treat the natural gas product to less than 5 ppm-mol [13]. For different

applications (natural gas, LPG, gasoline), the configuration of the process can be slightly different.

Sweetening

Sweetening is used for thiols that are caustic insoluble or when the thiol content is so low that extraction is not economical (Fig. 1.3). The only purpose of sweetening is to mitigate odor problems by catalytic oxidation of thiols to disulfides. This reaction takes place in a fixed bed reactor [145]. It is applicable to feed stocks boiling above the butane boiling range. Air is injected directly into the feedstock. The sweetening reaction can be written as follows:

$$2\ RSH\ (org) + 0.5\ O_2\ (g) \rightarrow RSSR\ (org) + H_2O\ (l) \qquad (17)$$

The oxidation is carried out in the presence of an aqueous alkaline solution, such as sodium or potassium hydroxide. The disulfide formed remains in the hydrocarbon phase and therefore no reduction in total sulfur content takes place. The sweetened product exits the reactor and flows to the caustic settler. The sweetened product is separated from the caustic solution, which is then reused.

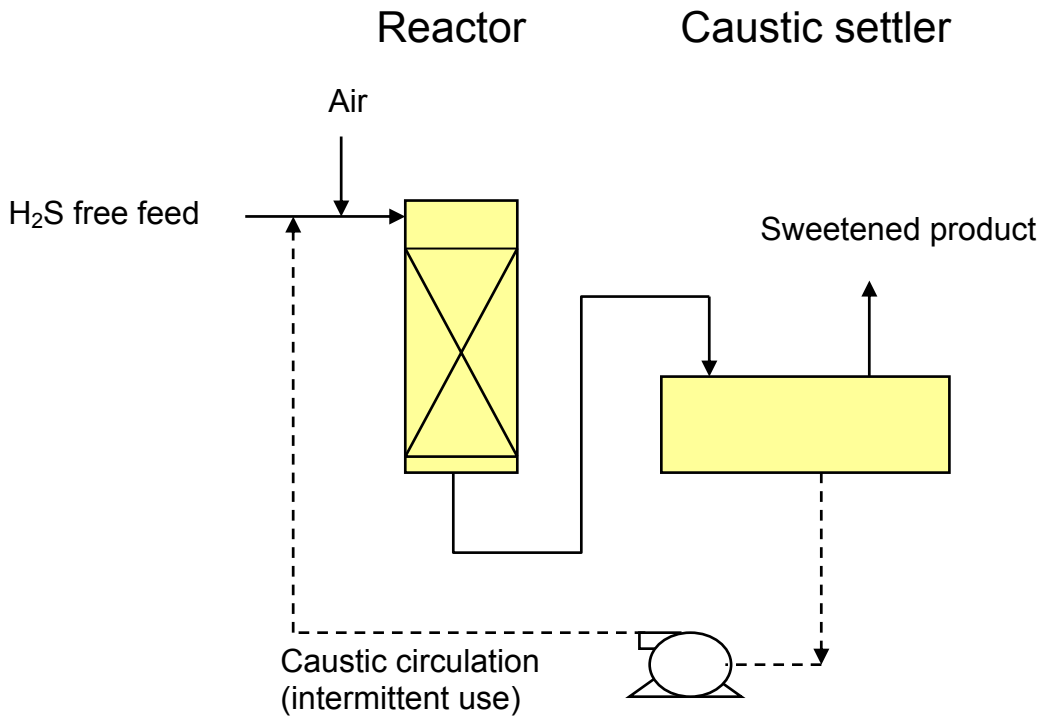

Fig. 1.3. Schematic representation of the Merox sweetening process.

Depending on the feedstock properties and product specifications, a pre- and post-treatment section may be included in the flow scheme. A caustic prewash may be included upstream of

the reactor to remove H_2S or naphthenic acids. Post-treatment may include a sand filter, or in case of jet fuels, a water wash, salt filter or clay filter.

1.4.2. Biological treatment

For compounds that are sufficiently water soluble and biodegradable, biological treatment methods can be considered as an attractive alternative to physical-chemical methods. In the last 25 years, biological abatement technologies are becoming more popular because of low costs, operational simplicity and because they are intrinsically clean technologies [164].

Biofiltration

Biofilters are regularly used to treat off-gases in the bio-industry, *e.g.* in composting and rendering plants. The gas is humidified and forced to flow through a bed packed with an organic carrier material (*e.g.* compost, peat or bark) on which pollutant-degrading microorganisms are attached as a biofilm. In a biofilter, compounds with an dimensionless air-water distribution coefficient (Henry) up to 10 can be removed because of the high gas residence time (30-60 s) and the high specific gas/liquid surface area (300-1000 $m^2 \cdot m^{-3}$) in a biofilter [164]. In addition, the apolar fraction in the organic carrier material promotes sorption and subsequent biodegradation of the apolar compounds. As a drawback of this method, however, large reactor volumes are required to obtain the high gas residence times mentioned and the operational control of the technique is limited due to the absence of a recirculating water phase.

Numerous aerobic microorganisms able to degrade MT, DMS and DMDS were isolated. Among these, mainly methylotrophic *Hyphomicrobium* spp. and autotrophic *Thiobacillus* spp. have been used to inoculate biotechnological waste gas treatment systems. These microorganisms convert the methylated sulfur compounds into sulfate and carbon dioxide (see section 1.3).

Due to the absence of a recirculating water phase in a traditional biofilter and the limited buffer capacity of common carrier materials, biofiltration of acidifying compounds results in an acidification of the bioreactor. For most compounds, including VOSC, acidification will result in an inhibition of the biofiltration process.

The removal efficiency for VOSC in biotechnological waste gas treatment is rather low and variable, in contrast to the biological removal of H_2S and numerous volatile organic non-sulfur compounds [165, 166]. Smet *et al.* [167] observed very low elimination capacities (< 0.01 $kg \cdot m^{-3} \cdot d^{-1}$) for DMS in a wood bark and compost lab-scale biofilter. In a compost biofilter inoculated with *Hyphomicrobium* MS3, a decrease of the elimination capacity for the odorant DMS was observed when the pH of the carrier material dropped below 6, while the DMS biofiltration was almost completely inhibited (28% of the maximum EC) at a pH of 4.7 [168].

If the biofilter is used to oxidize hydrogen sulfide only, lower pH values (< 3) can be tolerated since acidophilic thiobacilli are able to degrade this compound [164]. H_2S-oxidizing microorganisms are inherent in raw biofilters contrary to the microorganisms degrading VOSC [169].

Pinjing *et al.* [170] developed a packed bed filled with microorganisms immobilized in beads for removing H_2S and MT, which are the important odorous components emitted from wastewater treatment facilities. They found that the cultures able to metabolize H_2S and MT can be enriched from activated sludge. The suitable pH ranges were 2-3 and 6-8 for H_2S and MT, respectively. Therefore, they recommended a two-stage biofilter in series under different pH values to efficiently remove H_2S and MT from waste gases.

Bioscrubbing and biotrickling filtration

In a bioscrubber, the waste gas is contacted with water in a spraying tower with inert packing and the off gas compounds are absorbed in the water phase. The wastewater is subsequently treated in bioreactor [164]. In a biotrickling filter the waste gas is forced through a packed bed filled with a inert carrier material covered with an active biofilm. Biotrickling filters can be regarded as an intermediate between biofilters and bioscrubbers. In contrast to biofilters, bioscrubbers and biotrickling filters are only capable of removing compounds with an air-water distribution coefficient lower than 0.01 [164, 171]. Therefore, removal of VOSC will be critical (Table 1.2). However, a big advance of these liquid systems is that the recirculating water phase allows a better control of the reaction conditions (pH, temperature, addition of nutrients, removal of accumulated salts) [133].

In the first quarter of a carrier-packed biological deodorization reactor, H_2S (> 99%) and MT (70-80%) were removed from the raw waste gas. For this reason, this section of the packed bed was considered to be essential in the removal of sulfur odorants. The pH in this section was very low (pH 1–2). *Thiobacillus thiooxidans* KS1 was the dominant strain isolated from this section of the reactor [172].

For both H_2S and VOSC, high (> 90%) elimination efficiencies were reported [173] using a two-stage trickling filter with separate degradation of H_2S and the VOSC. A low pH allowed efficient H_2S biodegradation in the first stage, while the VOSC were removed in the second stage (pH 6-7).

Ruokojärvi *et al.* [174] developed a two-stage biotrickling filter inoculated with refinery sludge for removing a mixture of H_2S, MT and DMS from waste gases. The first filter (pH 2) removed most of the H_2S and some MT and DMS. The second filter (pH 6.5) removed the rest of the MT and most of the DMS. The total maximum loads of the whole two-stage biotrickling filter were 1150 g $S \cdot m^{-3} \cdot day^{-1}$ for H_2S, 879 g $S \cdot m^{-3} \cdot day^{-1}$ for DMS and 66 g $S \cdot m^{-3} \cdot day^{-1}$ for MT. The average removal efficiencies for all gases tested were 99% or higher.

DMS removal with a membrane bioreactor

Little research is done on the use of membrane bioreactors for VOSC removal. De Bo *et al.* [175] reported DMS removal from waste air in a membrane bioreactor. Van Langenhove *et al.* [176] reported DMS degradation in an aerobic membrane bioreactor inoculated with a pure *Hyphomicrobium* VS culture. A maximum specific DMS degradation rate of 160 mg DMS · g^{-1} biomass · day^{-1} was observed between pH 6.0 and 7.0.

Biological treatment of spent sulfidic caustics

Biological treatment of spent sulfidic caustics may be an inexpensive disposal option. H_2S and MT were successfully biotreated by an enrichment immobilized culture in a fluidized-bed column bioreactor [146]. The starting material for the enrichment culture was activated sludge from a refinery aerobic wastewater treatment system and sludge from an industrial anaerobic digester. Both H_2S and MT were completely oxidized to sulfate. Excessive build-up of sulfate (> 12 g/L) in the bioreactor medium resulted, however, in an upset condition evidenced by excessive MT breakthrough. *Thiobacillus thioparus* was involved in the oxidation of both compounds [146].

Both Subramaniyan *et al.* [153] and Sipma *et al.* [150] report the simultaneous oxidation of H_2S and MT into sulfate in aerobic bioreactors inoculated with mixtures of thiobacilli and activated sludge. In the latter study, oxidation of 10 mM sulfide and 2.5 mM MT to sulfate from a synthetic spent caustic was achieved in a bubble column reactor with carrier material at a hydraulic retention time of 6 h. Addition of 7.5 mM phenol, a common pollutant of spent caustics, did not adversely affect the biological oxidation process and phenol was completely removed from the effluent. Also different spent caustics solutions from refineries were successfully treated and sulfide and MT were oxidized to sulfate.

Treatment of actual thiol-containing spent sulfidic caustic occurred at a lower rate than predicted by operation on MT and H_2S alone, indicating that the caustic contained other inhibitory compounds [153].

Anaerobic treatment of VOSC-containing wastewater in digesters

Wastewaters from Kraft pulping industry contain MT, DMS, DMDS en H_2S. In a digester inoculated with thermophilic anaerobic sludge, DMDS was anaerobically converted to MT and subsequently partially degraded to H_2S. Optimal conditions were reached at 50°C and pH 6.5. To maintain the degradation activity in the digester, H_2S concentrations below 5 mg/L were required [177]. The currently known MT- and DMS-degrading methanogenic archaea were already discussed in section 1.3.1.

Biotechnological process for H_2S removal

A biological method for H_2S removal from anaerobically treated wastewaters has been developed at Wageningen University. Later, this process has been further developed to

desulfurize various gas streams, whereafter modifications of the process became known as the THIOPAQ and Shell-Paques process. The processes are based on the biological oxidation of H_2S to elemental sulfur [178]. The Shell-Paques process is a competitive process at sulfur loads between 0.1 and 50 tons/day [179, 180]. At sulfur loads above 50 tons/day, amine-based processes are most economical [181].

The Shell-Paques process consists of two integrated parts: a high pressure absorption column (about 60 bar) for the absorption of H_2S and a bioreactor (operated under atmospheric conditions) in which sulfide is oxidized to elemental sulfur or sulfate (eqns 18 and 19) by using chemolithoautotrophic bacteria belonging to the genus *Thiobacillus* [181]. Under sulfide limitation in a bioreactor thiobacilli can compete successfully with the chemical oxidation of sulfide because of their high affinity for this compound. Since the formation of sulfate yields most energy, this reaction is carried out preferentially by the microorganisms. The formation of sulfur will only proceed under oxygen-limiting conditions or at high sulfide loading rates, whereas sulfate is the main product in the presence of an excess amount of oxygen [181, 182]. It is possible to reach a selectivity between 90 and 95% for the formation of elemental sulfur by applying oxygen-limiting conditions [183, 184].

$$2\ HS^- + O_2 \rightarrow 2\ S^0 + 2\ OH^- \quad (18)$$

$$2\ HS^- + 4\ O_2 \rightarrow 2\ SO_4^{2-} + 2\ H^+ \quad (19)$$

The effluent from the bioreactor is recycled via a settler to the absorption column to remove the majority of the sulfur particles. The washed elemental sulfur has a purity of more than 99%. The remainder is biomass. Recently, it was shown that this process could be operated under halo-alkaline conditions (pH 10, $\geq$ 2 M Na^+) [185].

The application of biological desulfurization, using the Shell-Paques system, is however limited to waste streams containing exclusively inorganic sulfur compounds. For the biological desulfurization of LPG also thiols must be degraded.

An integrated new process for LPG desulfurization

A three-step process for the removal of H_2S and thiols from LPG has been proposed to successfully treat thiol-containing hydrocarbon streams [186, 187]. This process (Fig. 1.4) involves: (i) extraction of the sulfur compounds from the LPG phase into a (bi)carbonate-containing solution; (ii) anaerobic degradation of thiols to H_2S, CO_2 and CH_4 and (iii) partial oxidation of H_2S to elemental sulfur. In a settler, the sulfur particles are removed by gravity separation and the cleaned alkaline process water is reused in the extraction process. A concentrated Na_2CO_3 stream is needed to maintain a constant pH in the process. The sulfate production (eqn 19) necessitates a continuous bleed stream [179], which is replenished by a make-up water stream. In this thesis, the anaerobic conversion step is studied in more detail, with special attention to the anaerobic degradation step. In the extractor column (10-15 bars)

H_2S and MT are transferred from the LPG to the alkaline water phase, where partial dissociation of the acidic compounds takes place:

$$H_2S\ (LPG) \leftrightarrows H_2S\ (aq) \leftrightarrows HS^-\ (aq) + H^+\ (aq) \quad (20)$$

$$CH_3SH\ (LPG) \leftrightarrows CH_3SH\ (aq) \leftrightarrows CH_3S^-\ (aq) + H^+\ (aq) \quad (21)$$

Subsequently, MT is degraded to H_2S, CH_4 and HCO_3^- in the anaerobic bioreactor:

$$4\ CH_3SH + 3\ H_2O \rightarrow 3\ CH_4 + HCO_3^- + 4\ HS^- + 5\ H^+ \quad (14)$$

In the aerobic bioreactor, H_2S is biologically oxidized to elemental sulfur or sulfate (eqn 18 and 19). Any thiol that is not biologically degraded in the anaerobic reactor is chemically oxidized to the corresponding disulfide in the aerobic reactor (eqn 6) or reacts with bio-sulfur to (dimethyl)polysulfides [28]. Autooxidation of thiols is a fast process in the presence of trace metals (see section 1.2.3), which are present in this reactor for growth of the microorganisms. DMDS is probably not further degraded to sulfate and bicarbonate in the aerobic reactor, because of the preferential conversion of sulfide compared to DMDS [90] and the oxygen-limiting conditions in the reactor [180]. Although this oxidation leads to a considerable decrease in odor emission it is undesirable: the produced disulfides are extremely hydrophobic and might re-dissolve into the treated hydrocarbon product, when the regenerated caustic is recycled. To prevent disulfides from entering the extractor column it is necessary to include a filter (*e.g.* an active carbon filter or molecular sieve) in the recycle stream to remove them. In addition, solid bio-sulfur particles that are not removed in the settler are removed by filtration.

The main advantages of this process, compared to the Merox process (section 1.4.1.), are (i) the absence of expensive catalysts, (ii) absence of waste materials (*i.e.* disulfides) and (iii) the production of reusable elemental sulfur.

1.5. Outline of the thesis

The aim of the study presented in this thesis is to develop a new process for the biological desulfurization of liquid hydrocarbon streams, in particular for liquefied petroleum gas (LPG) in which lower thiols (MT, ET and PT) are abundantly present. The research project was financed by the Chemical Sciences (CW) and Technology Foundation (STW), the applied science division of The Netherlands Organization for Scientific Research (NWO). It was carried out at the sub-department of Environmental Technology and at the Laboratory for Microbiology of Wageningen University and Research Centre (The Netherlands). Several subtopics were defined:

- computer simulation of the new process to determine the boundary conditions (*i.e.* pH and salt concentration) for proper thiol extraction and for the bioreactors;
- anaerobic degradation of volatile organic sulfur compounds at increased salinity and pH;
- toxicity of thiols in anaerobic degradation processes;
- characterization of the methanethiol-degrading communities in the reactor sludges;
- chemical reactions between bio-sulfur and thiols.

In **Chapter 2** the MT-degrading community is characterized in a lab-scale up-flow anaerobic sludge bed (UASB) reactor inoculated with granular sludge at low salinity and neutral pH. The toxicity of thiols and H_2S in anaerobic processes is described in **Chapter 3**. **Chapters 4 and 5** describe the anaerobic MT degradation in an UASB reactor inoculated with Wadden Sea sediment. Effects of salinity and pH on the reactor performance and on the microbial community are presented. **Chapter 6** describes the anaerobic MT degradation and microbial community analysis at elevated pH values (pH 10) in an UASB reactor inoculated with a mixture of salt lake sediments. The boundary conditions for the new biological LPG desulfurization process are determined by flowsheet simulations. Chemical reactions between bio-sulfur and thiols are discussed in **Chapter 7**. Finally, the results of this study are summarized and an outlook is given in **Chapter 8**.

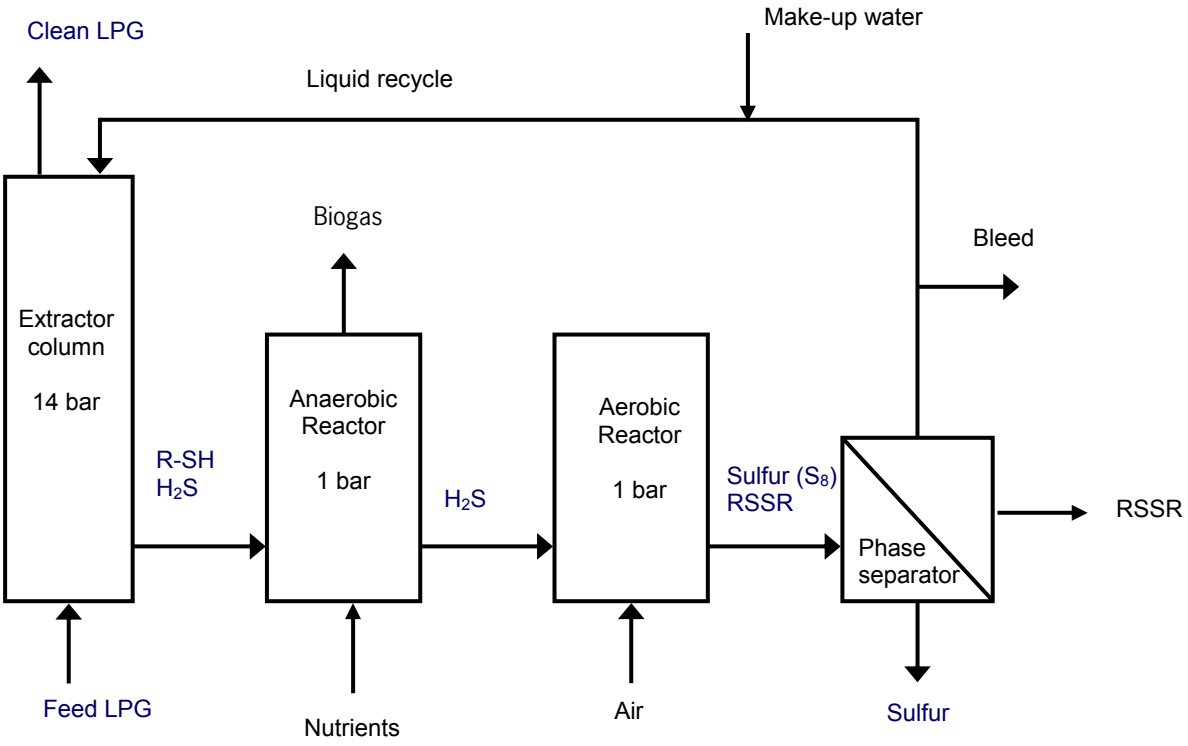

Fig. 1.4. Integrated process for combined anaerobic-aerobic desulfurization of LPG [186, 187] studied in this PhD-thesis.

List of abbreviations

AC	Activated carbon
DMDS	Dimethyl disulfide, CH_3SSCH_3
DMS	Dimethyl sulfide, CH_3SCH_3
DMSO	Dimethyl sulfoxide, $CH_3(SO)CH_3$
DMSP	Dimethyl sulfoniopropionate, $(CH_3)_2S^+(CH_2)_2COO^-$
ET	Ethanethiol, CH_3CH_2SH
LPG	Liquefied Petroleum Gas
MMPA	Methyl mercaptopropionate, $CH_3SCH_2CH_2COO^-$
MT	Methanethiol, CH_3SH
ppm-mol	parts per million, on molar basis
ppmv	parts per million, on volume basis
ppmw	parts per million, on weight basis
PT	n-Propanethiol, $CH_3CH_2CH_2SH$

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2

Degradation of methanethiol by methylotrophic methanogenic archaea in a lab-scale upflow anaerobic sludge blanket reactor*

Abstract
In a lab-scale upflow anaerobic sludge blanket reactor inoculated with granular sludge from a full-scale wastewater treatment plant treating paper mill wastewater, methanethiol (MT) was degraded at 30°C to H_2S, CO_2 and CH_4. At a hydraulic retention time of 9 h, a maximum influent concentration of 6 mM MT was applied, corresponding to a volumetric loading rate of 16.5 $mmol \cdot L^{-1} \cdot day^{-1}$. The archaeal community within the reactor was characterized by anaerobic culturing and denaturing gradient gel electrophoresis analysis, cloning, and sequencing of 16S rRNA genes and quantitative PCR. Initially, MT-degrading methanogenic archaea related to members of the genus *Methanolobus* were enriched in the reactor. Later, they were outcompeted by *Methanomethylovorans hollandica*, which was detected in aggregates but not inside the granules that originated from the inoculum, the microbial composition of which remained fairly unchanged. Possibly other species within the *Methanosarcinacaea* also contributed to the degradation of MT, but they were not enriched by serial dilution in liquid media. The archaeal community within the granules, which was dominated by *Methanobacterium beijingense*, did not change substantially during the reactor operation. Some of the species related to *Methanomethylovorans hollandica* were enriched by serial dilutions, but their growth rates were very low. Interestingly, the enrichments could be sustained only in the presence of MT and did not utilize any of the other typical substrates for methylotrophic methanogens, such as methanol, methyl amine, or dimethyl sulfide.

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Introduction

Organosulfur compounds present in petroleum and other fossil fuels are receiving considerable attention because of their potential direct and indirect negative effects on the environment. Thiols and H_2S, the major (organo)sulfur compounds in liquefied petroleum gas (LPG), are extracted using alkaline solutions. Currently, thiol-containing LPG (mainly methanethiol [MT]) is treated by the Merox process, in which H_2S and thiols are extracted from the LPG separately. The thiols are then catalytically oxidized to water-insoluble disulfide oil [1].

Alternatively, H_2S and thiols can be extracted simultaneously from LPG, which results in a solvent stream loaded with H_2S and MT. The thiols present in the solvent are converted in an anaerobic bioreactor to H_2S, CO_2, and CH_4 [2]. In a second reactor, H_2S is biologically oxidized to elemental sulfur, a process that has been studied in detail [3, 4] and is already being applied. After separation of the elemental sulfur, the solvent is reused for the extraction process.

Under anaerobic conditions, MT is degraded by methanogens, but oxidation by sulfate reducers has also been reported [5, 6]. Kiene *et al.* [7] showed that sulfate reducers compete with methanogens for MT and dimethyl sulfide (DMS), but only at concentrations below 10 μM. Theoretically, conversion of MT to acetate or to H_2, CO_2, and H_2S, as described for methanol [8, 9], is also possible, but this has never been found for methylated sulfur compounds. Several MT-degrading methanogens have been isolated from marine, estuarine, and soda lake sediments, and more recently also from freshwater sediments [10, 11]. These methanogens were usually isolated on DMS, methylated amines, or methanol, but never on MT. It is assumed that DMS-degrading methanogens also degrade MT, and this was demonstrated for several species [12]. Finster *et al.* [13] proposed a stoichiometry for the anaerobic conversion of MT, similar to the methanol metabolism of *Methanosarcina barkeri* (eqn 1). The anaerobic degradation of DMS (eqn 2) is assumed to proceed via MT as the intermediate [5].

$$4\ CH_3SH + 3\ H_2O \rightarrow 3\ CH_4 + HCO_3^- + 4\ HS^- + 5\ H^+ \qquad \Delta G^{0'} = -51.0\ kJ/mol\ CH_4 \qquad (1)$$

$$2\ CH_3SCH_3 + 3\ H_2O \rightarrow 3\ CH_4 + HCO_3^- + 2H_2S + H^+ \qquad \Delta G^{0'} = -52.2\ kJ/mol\ CH_4 \qquad (2)$$

MT-degrading methanogens can be used for the treatment of waste streams rich in MT and related volatile organic sulfur compounds (VOSC). In previous studies, the anaerobic degradation of MT by granular sludge was studied in a lab-scale upflow anaerobic sludge blanket (UASB) type reactor [2, 14]. In these studies, however, the microbial diversity and activity of the anaerobic community were not assessed. Here, we present the results of reactor experiments in which we focused on the microbiology of MT degradation. We studied the

metabolic properties of the reactor sludge, followed the microbial community in the reactor over time, and enriched MT-degrading microorganisms.

Materials and methods

Lab-scale reactor

Two continuous flow reactor experiments were carried out in a laboratory-scale UASB reactor with a liquid volume of 1.6 liters. The reactor was fed with an oxygen-free synthetic influent at pH 12 containing 50 to 100 mM MT (stock solution) mixed with a nutrient solution at pH 4 (see below) using peristaltic pumps (Watson Marlow, Falmouth, Cornwall, United Kingdom). The stock solutions were stored in closed glass bottles in which the volume of the consumed liquid was replaced by N_2.

The nutrient solution contained the following macronutrients: 9.35 mM NH_4Cl, 0.86 mM K_2HPO4, 0.30 mM $MgCl_2$, 1.61 mM KCl, 1.36 mM $CaCl_2$, and 15 mg/L yeast extract. Trace elements were added from a stock solution (1 ml/L) according to the method of Paulo *et al.* [15]. An influent containing 2 to 6 mM MT was composed by mixing the MT stock solution with the nutrient solution near the inlet of the reactor.

The superficial liquid upflow velocity in the reactor was maintained at 1.0 m/h for the first 100 days and was then increased to 1.5 m/h to improve the mixing by applying external circulation of the reactor liquid. The influent and circulation flows were 4.2 to 4.8 L/day and 188 to 283 L/day, respectively. The hydraulic retention time was 8 to 9 h. The reactor was operated at 30°C using a thermostat bath. The pH of the reactor liquid was measured with an H_2S resistant Flushtrode pH electrode (Hamilton Flushtrode; Hilkomij B. V., Rijswijk, The Netherlands) and controlled between 7.2 and 7.5 by adding sodium hydroxide or hydrochloric acid from 0.1 M stock solutions. Between the two reactor experiments, the reactor was stopped for 69 days. The sludge was kept in the reactor anaerobically at ambient temperature.

Sludge characteristics

The reactor was inoculated with 96 g volatile suspended solids (VSS) of fresh anaerobic granular sludge (dry weight) from a full-scale anaerobic bioreactor treating paper mill wastewater (Eerbeek, The Netherlands). The VSS content of the sludge was 66% of the dry weight. The granule strength was measured with a tension-and-compression test apparatus (Overload Dynamics S900; Overload Dynamics BV, Schiedam, The Netherlands) according to the method of Hulshoff Pol [16]. Microscopic analysis of sludge and batch samples was performed on an Olympus BH2 epifluorescence microscope. To measure the sludge washout, the concentrations of VSS and total suspended solids (TSS) were determined in effluent samples.

Batch incubations

To enrich for MT-degrading methanogens and to study the activity of the reactor sludge, reactor sludge samples were incubated in 120-ml serum flasks filled with 50 ml bicarbonate-buffered medium with 0.1 g/L yeast extract and a headspace composed of 1.7 bar N_2/CO_2 (80:20) [17]. To localize specific methanogenic activities, the sludge was separated into a suspended and a granular fraction by decanting the suspended fraction of the granules. The granules were washed three times with freshly prepared anaerobic medium. Bromoethane sulfonate was used to inhibit methanogenesis. Substrates and bromoethane sulfonate were added from sterile stock solutions, except for H_2, which was added to the batches by flushing the bottles with H_2/CO_2 (80:20). Methanogenic activities were quantified by measuring the CH_4 produced over time. Sterile controls were prepared by sterilizing the batches for 20 minutes at 120°C after addition of the sludge. The batches were incubated in duplicate at 30°C in the dark.

DNA extraction and 16S rRNA gene amplification

Samples withdrawn from the reactor were fixed in 60% (vol/vol) ethanol containing 25 mM NaCl. Cells from enrichment cultures were collected by centrifugation at 17,500 × *g*. DNA was extracted using a FastDNA SPIN kit (for soil) (Q Biogene, Cambridge, United Kingdom) and quantified with a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, Del.). The 16S rRNA genes were amplified from genomic DNA using *Taq* DNA polymerase (Invitrogen, Breda, The Netherlands) with primers targeting conserved domains (Table 2.1). The primers were purchased from Eurogentec (San Diego, CA). 16S rRNA genes were amplified using the following thermocycling program: predenaturation at 94°C for 5 min.; 35 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 40 s, and elongation at 72°C for 90 s; and postelongation at 72°C for 5 min. To amplify bacterial (V6 to V8) and archaeal (V2 to V3) 16S rRNA gene fragments for denaturing gradient gel electrophoresis (DGGE) analysis, the annealing temperature was 56°C and the elongation step was 60 s. The PCR (Polymerase Chain Reaction) product size was checked by electrophoresis in 1.2% (wt/vol) agarose gels stained with ethidium bromide using a 100-bp DNA ladder (MBI Fermentas, Vilnius, Lithuania). Quantitative real-time PCR amplification was performed with universal primers for bacteria and archaea [18, 19] (Table 2.1) using the Bio-Rad iQ SYBR green supermix (Bio-Rad Laboratories) according to the manufacturer's instructions. Each of the primer sets was optimized with respect to the annealing temperature and time required for extension. Real-time PCR amplification was performed in a Bio-Rad iCycler programmed for 10 min. at 95°C for initial heat activation, followed by 45 cycles of denaturation for 15 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. For archaea, the annealing and extension steps were combined (30 s at 60°C). DNA samples for standard curves were prepared by amplifying group-specific cloned 16S rRNA genes using vector-targeted primers

and purifying the products with the Bioké PCR purification kit. DNA standards were quantified with a Nanodrop spectrophotometer.

Table 2.1. PCR-primers used for DGGE-analysis, ARDRA, cloning and sequence analysis (SA). Primers marked with an asterisk contained the 40bp GC-clamp '5-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGG-3' when used for DGGE analysis.

Primer	Sequence ('5 → '3)	Application(s)	Ref.
Arch-109(T)f	ACTGCTCAGTAACACGT	DGGE, SA	[20-22]
Uni-515r*	ATCGTATTACCGCGGCTGCTGGCAC	DGGE, SA	[21, 23]
Bac-968f*	GAACGCGAAGAACCTTAC	DGGE, Cloning	[24]
Bac-1401r	CGGTGTGTACAAGACCC	DGGE, Cloning	[24]
Arch-109f	AC(G/T)TGCTCAGTAACACGT	Cloning	[20]
Uni-1492r	CGGCTACCTTGTTACGAC	Cloning	[25]
Pg1	TGGCGGCCGCGGGAATTC	ARDRA	Promega
Pg2	GGCCGCGAATTCACTAGTG	ARDRA	Promega
T7	AATACGACTCACTATAGG	SA	Promega
Sp6	ATTTAGGTGACACTATAG	SA	Promega
Uni-519f	CAGC(A/C)GCCGCGGTAA(G/A/T/C)(A/T)C	SA	[23]
Arch-915r	GTGCTCCCCCGCCAATTCCT	SA	[26]
Arch-787f	ATTAGATACCC(G/C)(G/T/C)GTAGTCC	qPCR	[19]
Arch-1059r	GCCATGCACC(A/T)CCTCT	qPCR	[19]
Msc-380f	GAAACCG(C/T)GATAAGGGGA	qPCR	[19]
Msc-749r	ACCCGTTCTGGTAAGACG	qPCR	This study
Eub-341f	CCTACGGGAGGCAGCAG	qPCR	[18]
Eub-534r	ATTACCGCGGCTGCTGGC	qPCR	[18]

DGGE

Denaturing gradient gel electrophoresis was performed in 8% (wt/vol) polyacrylamide (37.5:1 acrylamide-bisacrylamide) gels containing a 30 to 50% or 30 to 60% denaturing gradient for archaeal and bacterial 16S rRNA gene amplicons, respectively. A 100% denaturing solution contained 7 M urea and 40% (vol/vol) formamide. Gelbond (Amersham Biosciences, Little Chalfont, United Kingdom) was used as a physical support. Electrophoresis was performed in 0.5 × TAE buffer (0.02 M Tris, 0.01 M acetic acid, and 0.5 mM EDTA, pH 8) at 85 V and 60°C for 16 h using a DCode System (Bio-Rad, Hercules, CA). Silver staining and development of the gels were performed according to the method of Sanguinetti *et al.* [27].

Cloning and sequencing

For cloning and sequencing, 16S rRNA gene amplicons were purified with a Bioké PCR purification kit (Leiden, The Netherlands) and cloned into *Escherichia coli* XL1 blue (Stratagene, Amsterdam, The Netherlands). The pGEM-T Easy vector system (Promega, Madison, WI) was used to transform the 16S rRNA gene amplicons into *E. coli* and to select for positive clones using ampicillin selection and blue/white screening. The clones were screened by amplified rRNA gene restriction analysis, using the restriction enzymes MspI, CfoI, and AluI (Promega, Madison, WI). The restriction fragments were analyzed by

electrophoresis in 12% (wt/vol) agarose gels (Elchrom, Cham, Switzerland) and ethidium bromide staining. Sequence analysis was performed using the pGEM-T vector-targeted sequencing primers Sp6 and T7 and the 16S rRNA gene-targeted internal primers Uni-519f and Arch-915r (Table 2.1). Phylogenetic trees were constructed in ARB, using the neighbor-joining method (*E. coli* positions 125 to 1469) and the Felsenstein correction [28, 29]. Sequences were aligned with FastAligner, followed by manual alignment according to secondary-structure models.

Analytical techniques

MT, DMS, dimethyl disulfide (DMDS), and ethanethiol (ET) in the reactor effluent were analyzed by high pressure liquid chromatography (Separations, Hendrik Ido Ambracht, The Netherlands) using a Chrompack (Bergen op Zoom, The Netherlands) C18 column with a length of 20 cm. The oven temperature was 30°C. The composition of the eluent was 35% acetonitrile and 65% water, and the flow rate was 0.6 ml/min. The injection volume of the samples was 20 µL, and a UV detector (Gynotek Germering, Germany) was used to monitor the VOSC at a wavelength of 210 nm. Total and volatile suspended solids were analyzed according to standard methods [30]. The reactor biogas composition (CH_4, CO_2, N_2, and H_2S) was analyzed on a Packard Becker gas chromatograph, model 433 (Delft, The Netherlands), equipped with two columns connected in parallel (split 1:1): 1.5 m by 1/8 in, Teflon packed with Chromosorb 108 (60 to 80 mesh), and 1.2 m by 1/8 in, stainless steel packed with a molecular sieve of 5 Å (60 to 80 mesh). Helium was used as a carrier gas (45 ml/min). The temperatures were 40°C for the column and 100°C for the injection port and hot-wire detector. The injection volume was 100 µL. The VOSC contents of the headspaces of batch incubations were measured on a Hewlett-Packard CP9000 gas chromatograph equipped with a CP-Porabond Q column (25 m by 0.53 mm) and a flame ionization detector. Volatile compounds were separated on the column in a 1-min linear gradient from 150 to 190°C. For quantitative experiments, 150 µL propane was included in the batches as an internal standard. The total dissolved sulfide was measured photometrically according to the methylene blue method described by Trüper and Schlegel [31]. CH_4 in the headspaces of batches was measured gas chromatographically with a Shimadzu GC-14B gas chromatograph equipped with a thermal-conductivity detector and molecular sieve 13 × (60/80 mesh). The column temperature was 50°C, and the carrier gas was argon at a flow rate of 30 ml/min.

Chemicals

All chemicals used were of analytical grade. A sodium mercaptide solution was supplied by the Arkema Group (Rotterdam, The Netherlands).

Calculations

The removal efficiency of the reactor was calculated in two ways (based on the VOSC or H_2S concentration in the effluent) according to the following equations:

$$1 - \frac{VOSC_{effl}}{VOSC_{\inf l}} \times 100\% \quad (3)$$

$$\frac{H_2S_{effl}}{VOSC_{\inf l}} \times 100\% \quad (4)$$

in which $VOSC_{infl}$ is the VOSC concentration in the influent (MT and some DMDS produced by autooxidation of MT), $VOSC_{effl}$ is the sum of VOSC in the effluent (MT, ET, DMS, and DMDS), and H_2S_{effl} is the H_2S concentration in the effluent. The sludge retention time (τ) is calculated by equation 5:

$$\tau = \frac{Total\ Solids}{TSS_{effl} \times Q_{effl}} \quad (5)$$

where Total Solids is the amount of dry sludge in the reactor (g), TSS_{effl} is the measured total suspended solids in the effluent (g/L), and Q_{effl} is the effluent flow (L/day).

Nucleotide sequence accession numbers

The nucleotide sequences obtained in this study have been deposited in GenBank (accession no. DQ631884 to DQ631889).

Results

Degradation of methanethiol in a lab-scale reactor

Eight days after startup of the reactor at an influent MT concentration of 2 mM, H_2S production started (Fig. 2.1A). The influent MT concentration was increased to 4 mM at day 16 and to 6 mM at day 39. This corresponded to maximum volumetric and specific loading rates (at 6 mM MT in the influent) of 16.5 mmol $MT \cdot L^{-1} \cdot day^{-1}$ and 0.33 mmol $MT \cdot gVSS^{-1} \cdot day^{-1}$. Besides MT, DMDS, DMS (< 0.1 mM), and ET (< 0.05 mM) were also detected in the effluent, and for this reason the total VOSC concentration was used to determine removal efficiencies. During the first reactor run (0 to 163 days), it was difficult to maintain a constant MT concentration in the influent. MT shock loads disturbed the reactor performance substantially (Fig. 2.1). For instance, at day 30, due to failure of one of the pumps, the influent MT concentration increased to 20 mM for 20 h. The effluent H_2S concentration increased to 5

mM, indicating that degradation of MT continued. The reactor performance recovered within a few days after the influent MT concentration was reduced back to 3 mM. In general, MT shock loads resulted in increased VOSC (mainly MT) and H_2S concentrations in the effluent, but the effluent VOSC concentration was always restored (to < 0.5 mM) within 10 days after the influent concentration was lowered to 6 mM MT or less. After 163 days of operation, the reactor was stopped and started up again 69 days thereafter. During the second part of the reactor experiment, in which the influent MT concentration was kept more constant, the reactor performance was sometimes strongly affected by exposure to air. At day 209, the reactor had to be stopped and opened for a short time due to clogging problems. After that, it took 10 days before the removal efficiency was restored to the previous level.

Biogas production and composition

The CH_4 content of the biogas was approximately 60% and was fairly constant throughout both reactor experiments (data not shown). The remaining 40% was N_2, as the influents were kept anaerobically under an N_2 atmosphere. The CO_2 content was around 0.3%, and no H_2S was detected in the biogas.

Part of the CH_4 dissolved in the reactor liquid and left the reactor via the effluent. This was verified qualitatively by gas chromatography, in which a soluble CH_4 peak was identified in effluent samples. The distribution coefficient ($m = C_g/C_l$ [dimensionless], where C_g is the concentration in the gas phase and C_l is the concentration in the liquid phase) of CH_4 at 30°C was calculated from different sources [32-35]. The average m value from these sources was 32. Using this distribution coefficient, the ideal gas law, and a CH_4 concentration of 60% in the biogas, the estimated CH_4 concentration in the effluent was 0.7 mM. For an average effluent flow of 4.4 L/day, this was equivalent to a CH_4 flow of 81 ml/day at 30°C.

A complete conversion of MT to CH_4, CO_2, and H_2S (eqn 1) at an MT load of 16.5 mmol $MT \cdot L^{-1} \cdot day^{-1}$ (MT influent, 6 mM; 4.4 L/day; reactor volume 1.6 liters) provides a theoretical CH_4 production of 490 ml/day. The average daily CH_4 production recorded over the period between days 120 and 160 was 316 ml/day (biogas, 235 ml/day; effluent, 81 ml/day), which accounts for only 65% of the theoretical maximum. The amount of H_2S recovered during the period between days 120 and 160 was also between 60% and 80%.

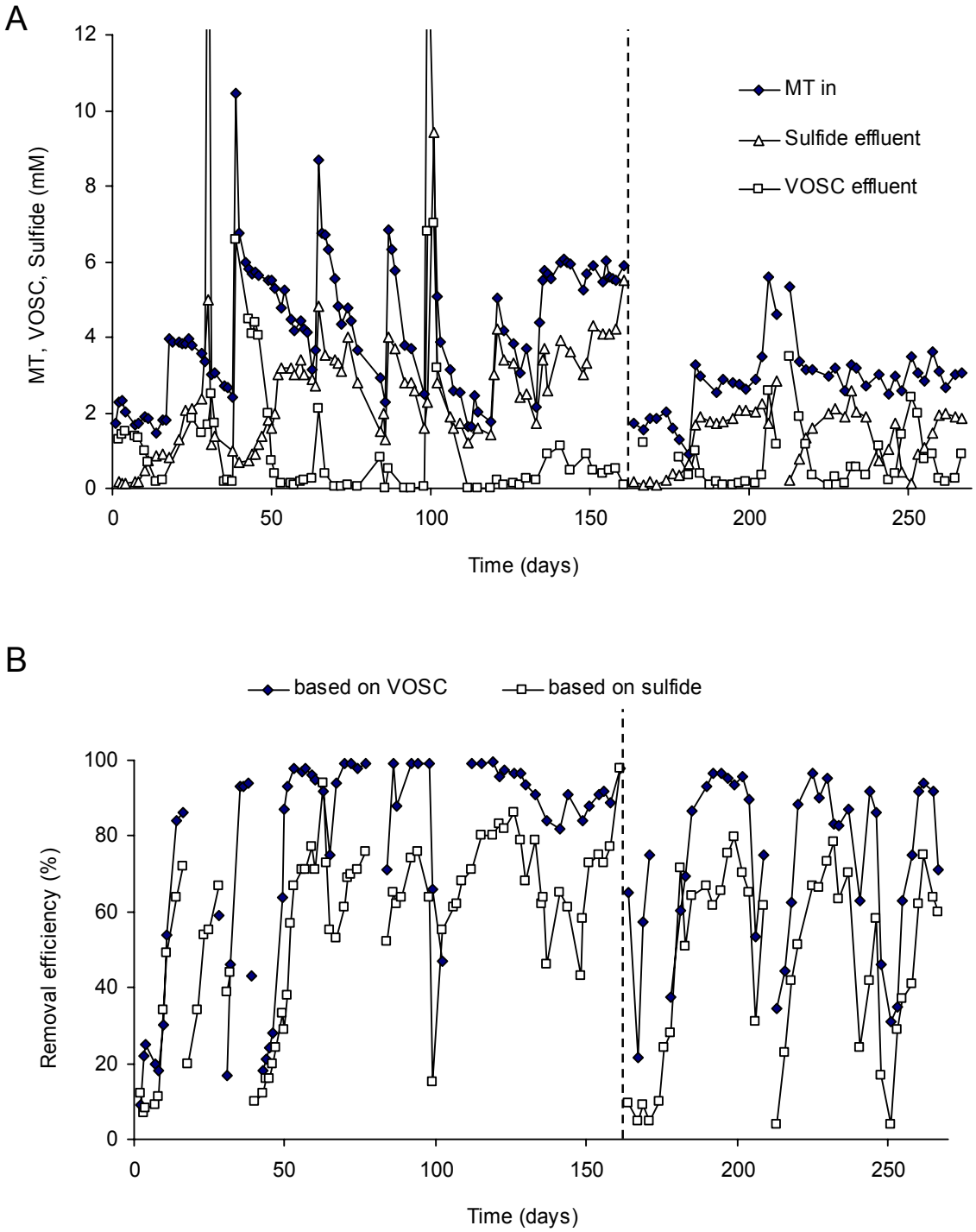

Fig. 2.1. Performance of the MT-degrading UASB reactor. (A) MT influent, VOSC total (the sum of MT, DMS, and DMDS), and H_2S in the effluent. (B) MT removal efficiency. The reactor was stopped after 163 days of operation and restarted 69 days later.

Sludge washout and sludge characteristics

During the second part of the reactor experiment, sludge washout was monitored. It varied between 27 and 165 mg TSS/L. The average amount of sludge in the reactor was around 110 g total solids. The sludge retention time was estimated at between 153 and 787 days, using the minimal and maximal sludge washouts in equation 5. At the start of the reactor experiment,

the granules were black and varied in diameter between 1 and 4 mm. The granule strength of the starting sludge was 3.9 ± 0.1·10^5 N/m^2, which decreased by 36% to 2.5 ± 0.1·10^5 N/m^2. Microscopic examination of the reactor sludge showed that it was composed of granules and a "suspended" fraction containing aggregates varying in size between 10 and 100 µm. Epifluorescence microscopy revealed mainly long (*Methanobacterium*-like) rodshaped methanogens within the granules, while *Methanosarcina*-shaped cells were detected in the suspended fraction of the sludge embedded in aggregates (Fig. 2.2). At the end of the reactor experiment, the suspended sludge fraction comprised approximately 5% of the total reactor sludge.

Sludge activity tests

To localize the MT-degrading activity in the sludge (immediately after sampling at the end of the reactor experiment), a sludge sample was divided into three fractions: total sludge, a granular sludge fraction, and a suspended sludge fraction. Of each fraction, 0.5 g wet sludge was incubated in 120-ml serum bottles with methanogenic medium and either 1 mM MT, 20 mM acetate, or 1.7·10^5 Pa H_2/CO_2 (80:20) as a substrate. The batches with the suspended fractions and those with the total sludge clearly degraded MT faster than the batches with the granular sludge fraction only (Table 2.2). In contrast, the specific CH_4 production rate with acetate was significantly lower with the suspended sludge fraction than with the granular fraction, whereas CH_4 production levels from H_2/CO_2 were comparable for all fractions (Table 2.2).

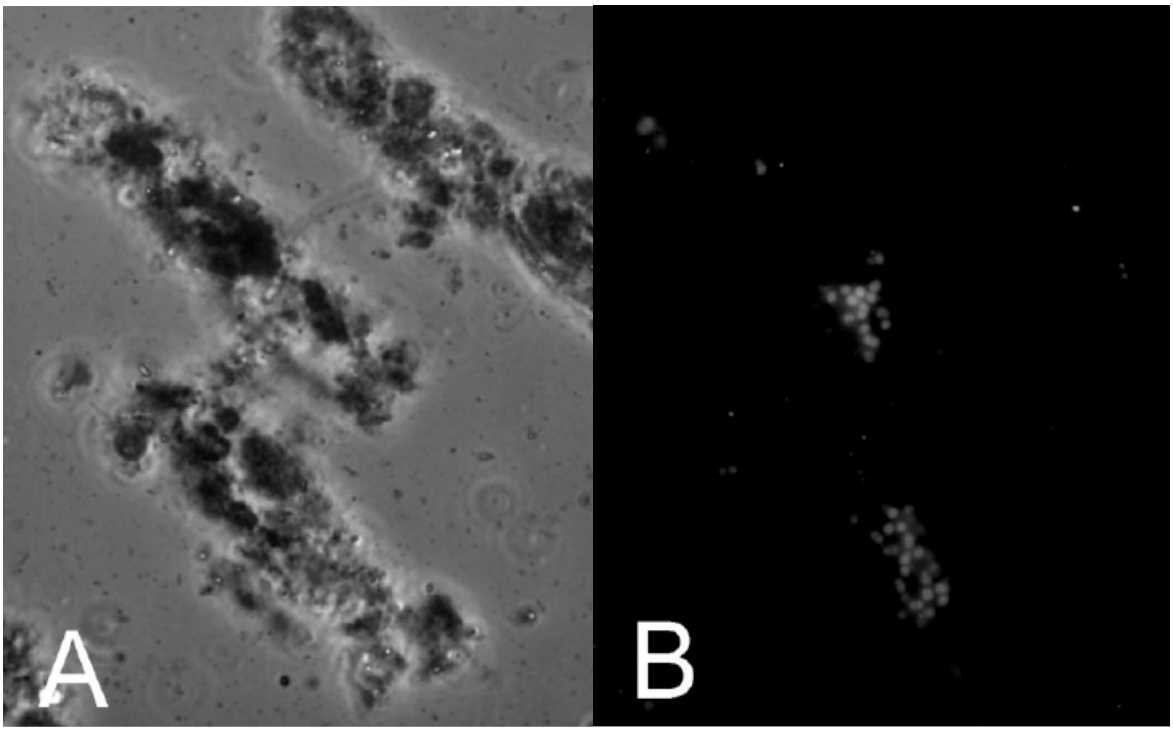

Fig. 2.2. Phase-contrast images of the suspended sludge fraction (A) and localization of methanogenic cells in the same preparation by epifluorescence (B).

Table 2.2. Specific activities of the different sludge fractions from the reactor at the end of the experiment, measured at 30ºC in batch with MT, H_2/CO_2 or acetate as substrate. The values are averages of duplicates (sludge).

Sludge fraction	MT	H_2/CO_2	Acetate
	mmol MT degraded · g VSS^{-1} · day^{-1}	mmol CH_4 produced · g VSS^{-1} · day^{-1}	mmol CH_4 produced · g VSS^{-1} · day^{-1}
Total	1.34	1.68	1.35
Granules	0.134	1.35	1.41
Suspended	2.91	1.40	0.70

Enrichment of MT-degrading methanogenic archaea

To enrich for the MT-degrading methanogens, several liquid dilution series were prepared from sludge from the reactor and from batches used for the sludge activity tests. For dilutions from suspended sludge, degradation of MT coupled to CH_4 formation was observed up to a dilution of 10^{-7} (equal to 10^{-5} % inoculum), and for some even up to a dilution of 10^{-8}. Subculturing from these dilutions was possible only with MT concentrations below 2 mM, and the lag phases were up to 1 month, while complete degradation of MT required up to 2 months. When fresh medium was inoculated from lower positive dilutions, MT was usually completely degraded within 2 weeks. For a dilution series from washed and crushed granules, degradation of MT was not observed beyond a dilution of 10^{-6}. The enrichment did not metabolize DMS, methanol, acetate, trimethylamine, methylamine, H_2, or formate, and it required either yeast extract, peptones, or Casamino Acids for growth. No CH_4 was produced in any of the controls or in batches to which no MT was added (endogenous controls). Further attempts to isolate the MT-degrading microorganisms were not successful.

Community analysis of the sludge samples and enrichment cultures

Four sludge samples were taken from the reactor over time and were used to characterize the composition and dynamics of the microbial community. Sludge from the full-scale wastewater treatment reactor that was used to inoculate the lab-scale reactor, and for which the microbial community was characterized in detail in a previous study [21], was also included in the analyses. Since MT was degraded to CH_4 and H_2S in the reactor and batch tests, we mainly focused on the archaeal community of the reactor. Amplification of the V2-V3 region of the archaeal 16S rRNA genes, followed by DGGE analysis of the DNA isolated from each of the samples, did not reveal any substantial changes in the archaeal community. For the sample from day 126, however, some additional amplicons were clearly visible. Exclusion of granules from the DNA isolation procedure revealed that some archaeal phylotypes had become more abundant over time in the suspended sludge fraction (Fig. 2.3). Some of these amplicons were not visible in the DGGE fingerprint of the original sludge (Fig. 2.3II, A and G to K), while some others did seem to represent species from the original granular sludge (E, L, and M). Some amplicons were detected only in the sample from day 126 and not in the samples taken thereafter (B, C, and D).

To determine the phylogenetic affiliations of the archaeal populations detected by DGGE analysis, DNA from the suspended sludge fraction on day 269 was used to construct a clone library of archaeal 16S rRNA gene fragments (V2 to V8; primers Arch-109f and Uni-1492r). Sequence analyses of 16S rRNA gene inserts of clones that were selected by DGGE analysis revealed that the majority of archaeal populations detected in the suspended sludge fraction of the reactor over time were most closely related to members of the genus *Methanomethylovorans* that were closely related to *Methanomethylovorans hollandica* (Table 2.3). Nevertheless, not all amplicons that appeared in the archaeal 16S rRNA gene fingerprints were represented in the clone library. The sequence of the amplicon (Fig.2.3 II, band E) that was assumed to represent *Methanosaeta concilli* based on a previous study [21] appeared to be most closely related to *Methanobacterium beijingense*, suggesting that the band representing this species might have overlapped with that of *Methanosaeta* in the fingerprint. Therefore, five additional clones representing this band were selected and analyzed, but those clones also contained *Methanobacterium beijingense* 16S rRNA gene fragments. To determine the 16S rRNA gene sequences of species that appeared in the reactor but were not present in the clone library, the corresponding amplicons were excised from DGGE gels, reamplified, and subjected to sequence analysis. Using this strategy, we were able to confirm that the dominant amplicon in the DGGE fingerprints indeed represented *Methanobacterium beijingense*. The amplicons (C and D) representing the archaeal phylotypes that were visible only in the DGGE fingerprint of the sample from day 126 (Fig. 2.3 II) were both more closely related to *Methanolobus taylorii* (Table 2.3). To determine the full 16S rRNA gene sequences of these species, a second clone library (V2 to V8; Arch-109f and Uni-1492r) from the day 126 (suspended sludge) sample was prepared. DGGE analysis showed that both populations of interest were represented by 7 out of 19 clones (Table 2.3). DGGE analysis of the enrichment cultures revealed that archaeal species that were enriched in the reactor also dominated the enrichments (Fig. 2.3II and 2.3III). Moreover, significant differences in the intensities of the dominant bands observed for the enrichments in the absence or presence of DMS suggested that the relative abundances of the different dominant populations differed substantially (Fig. 2.3II and 2.3III).

DGGE analysis of the bacterial population in the reactor, based on the V6-V8 region amplified from bacterial 16S rRNA genes present in the samples (Bac-968f and Bac-1401r), showed that the majority of the amplicons detected in the inoculum (granules) remained constant during reactor operation (Fig.2.3 IV). Some additional amplicons did appear in the DGGE fingerprints, and most of these were also detected in the suspended sludge fraction from the day 269 sample (Fig. 2.3V), the fingerprint of which was very similar to the corresponding fingerprint of the total sludge.

Quantification of archaea and bacteria in the reactor samples revealed that bacteria dominated the reactor sludge, as well as the suspended fraction of the sludge, even if the differences in the average numbers of rRNA gene operons between bacteria and archaea were

taken into account (4.1 for bacteria and 1.5 for archaea). The ratio of both groups in the granules did not change, with 13% (±2.3%) archaea in the inoculum sludge and 15% (±1%) in the total sludge after 269 days. The ratio in the suspended sludge fraction changed in favor of the bacteria, from 33% (± 3.6%) archaea after 126 days to 11% (± 0.7%) after 269 days.

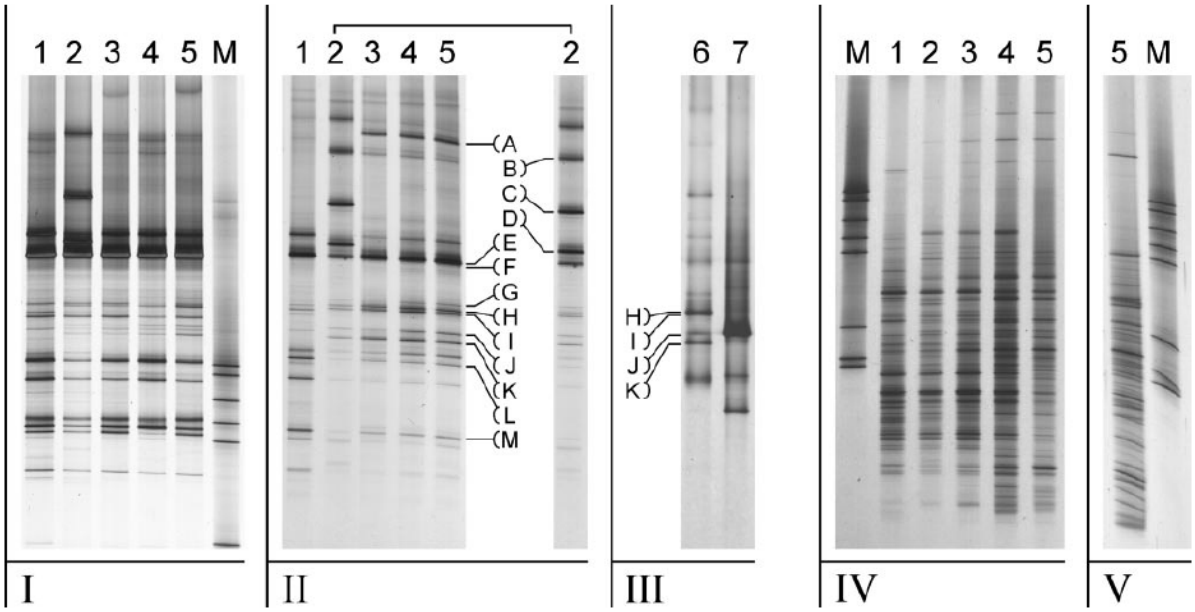

Fig. 2.3. DGGE fingerprints showing changes in the microbial community in sludge from the lab-scale UASB reactor fed with MT. (I) Archaeal community profiles of the total reactor sludge (granular plus suspended fractions). (II) Archaeal community profiles of the suspended fraction of the sludge (granules excluded). (III) Archaeal community profiles of enrichment cultures. (IV) Bacterial community profiles of the total reactor sludge. (V) Bacterial community profile of the suspended fraction of the reactor sludge. Lanes: 1, sludge from the full-scale UASB reactor treating paper mill wastewater at Eerbeek, The Netherlands, sampled on 16 January 2003; 2 to 5, samples from the lab-scale reactor fed with MT sampled at 126 days (lane 2), 187 days (lane 3), 248 days (lane 4), and 269 days (lane 5); 6, enrichment culture on MT; 7, enrichment culture on MT plus DMS; M, marker. (A to M) Amplicons for which the identities of the corresponding populations were determined by sequence analysis of the corresponding cloned 16S rRNA genes or of amplicons excised from the gels.

Discussion

Granular sludge from a full-scale UASB reactor treating paper mill wastewater can be used as the inoculum for a lab-scale UASB reactor to treat MT-containing waste streams, which is in agreement with previously published data [2, 14]. During initial experiments in which the reactor was fed with 6 mM MT during startup, no MT was degraded, most likely due to the toxicity of MT (data not shown). MT was shown to inhibit methanogens at concentrations between 6 and 10 mM (chapter 3). This also might explain why incidental increases in the influent MT concentration seriously affected reactor performance, requiring up to 10 days for full recovery (*e.g.* day 209) (Fig. 2.1). However, sulfide may also have contributed to toxicity effects after shock loads, since the sulfide concentration in the effluent usually increased initially with increased influent MT concentrations (Fig. 2.1A).

The small amounts of DMS and ET detected in the reactor effluent suggest that methylation of MT, and possibly also other reactions, occurred in the reactor. Sipma *et al.* have reported DMS formation from MT in an anaerobic bioreactor treating MT [2], but it has also been found in freshwater sediments and chemostat cultures of *Methanomethylovorans hollandica* [10, 36, 37]. Possibly, the MT-degrading archaea produce DMS from MT themselves by methylation, since MT is also an intermediate of DMS degradation. However, other microorganisms may also be involved in DMS formation from MT [5, 37]. More difficult to explain is the formation of ET, which, unlike MT and DMS, is not a known intermediate of MT degradation. Since ET is coextracted from LPG with MT and H_2S, ideally it would be treated in the same step as MT to produce H_2S, which then can be oxidized to elemental sulfur in the ultimate step of the process [2]. However, batch experiments showed that both ET and PT are not degraded under methanogenic or sulfate-reducing conditions (chapter 3).

Table 2.3. Identities of archaeal 16S rRNA genes retrieved from a lab-scale reactor fed with MT and percent similarities to the closest related sequence in the NCBI database.

Position[a]	Clone[b]	Abundance	Closest related sequences in database (NCBI)	Accession no.	Size (bp)	Similarity (%)
A		0/44[c]	Uncultured archaeon WCHD3-30 *(Methanosarcinaceae)*	AF050612	385	87.8
B		0/44[c]	Artefact/hybrid	-	-	-
C	VII-A11	7/19	Uncultured archaeon clone PL-7A3	AY570661	1355	98.1
			Methanolobus taylorii	U20154	1327	96.4
D	VII-A7	7/19	Uncultured archaeon clone PL-7A3	AY570661	1358	98.2
			Methanolobus taylorii	U20154	1330	96.3
E		10/44	*Methanobacterium beijingense* strain 8-2	AY350742	384	99.7
F		1/44	*Methanosaeta concilli*	X51423	714	99.7
G		1/44	Uncultured archaeon clone MP104-1109-a25	DQ88782	560	99.6
			Methanobacterium beijingense strain 4-1	AY552778	560	98.4
H/I	I-B1	12/44	*Methanomethylovorans hollandica* strain ZB	AY260433	1360	98.9
J	I-G4	6/44	*Methanomethylovorans hollandica* strain ZB	AY260433	1360	98.8
K	I-A10	3/44	*Methanomethylovorans hollandica* strain ZB	AY260433	1361	98.5
L		0/44[c]	Heterogeneous sequence	-	-	-
M		1/44	*Methanosaeta concilli*	X51423	719	99.9
N	I-D9	1/44	*Methanomethylovorans hollandica* strain ZB	-	1363	98.4
N		6/44[d]	*Methanomethylovorans hollandica*	-	> 700	> 98
N		2/44[d]	*Methanobacterium beijingense*	-	> 500	> 97
N		1/44	Uncultured crenarchaeote	AJ576209	700	97.6

a A to K, positions of the amplicons in the DGGE gel (Fig. 2.3); N, not identified in the DGGE gel or position does not correspond to any of the bands in the fingerprint of the reactor sludge.
b Codes for sequences that were deposited in GenBank.
c Not represented in clone library; 16S rRNA gene fragments were retrieved directly from DGGE gels.
d None of these sequences were 100% similar.

Granules can be considered spherical biofilms consisting of densely packed anaerobic microbial consortia. Due to their high settling velocities and the ability to withstand hydraulic shear, they are an essential feature of the UASB process [38, 39]. In previous studies, it was assumed that the microorganisms responsible for MT degradation were located in the granules [2, 14]. Our results showed that the MT-degrading archaea were embedded in small aggregates, which varied in size between approximately 10 and 100 μm, but not in the

granules (Fig. 2.2). Initially, a population related to *Methanolobus* dominated the archaeal community of the suspended sludge fraction (Fig. 2.3), but they were eventually outcompeted by *Methanomethylovorans hollandica*, which was represented by five different 16S rRNA gene sequences. *Methanomethylovorans hollandica* is the only methanogen known to degrade MT and DMS in freshwater environments [10]. The original type strain of this genus (*Methanomethylovorans hollandica* strain DMS1) was isolated on DMS, but like most other DMS-degrading methanogens, it has also been reported to degrade MT. To our knowledge, methylotrophic methanogens have never been isolated on MT, so our experiments revealed that microorganisms enriched on MT do not represent different genera or species than DMS-degrading species. However, the MT-degrading species related to *Methanolobus* that were initially detected in the reactor represent a novel species, and possibly even a new genus, of freshwater methanogens degrading MT. In contrast, the *Methanomethylovorans* enrichment obtained by using MT as the sole substrate degraded only MT and not DMS, methylamine, or methanol, compounds which are degraded by all other DMS-degrading species described so far [5]. Our culture, however, produced traces of DMS, which may explain why the reactor was not dominated by one archaeal species. DNA samples of enrichments on MT were dominated by two of the four *Methanomethylovorans* species found in the reactor. When both MT and DMS were used as substrates in dilution series, one of the other species, which must have been growing on DMS in the reactor as well, dominated. Unfortunately, none of the strains was obtained in pure culture, which would have allowed a more detailed characterization.

Remarkably, the dominant species in the granular sludge was *Methanobacterium beijingense*, a hydrogenotrophic methanogen, while previous studies reported that species of the genus *Methanosaeta* dominated that sludge [21, 40]. One explanation may be that the microbial community in the Eerbeek full-scale reactor changed between our study and previous studies. However, such a dramatic community change in a full-scale reactor would be rather unusual, especially as the composition of the waste stream did not change. Furthermore, the archaeal 16S rRNA gene-based DGGE fingerprint of the original sludge sample used in our study was remarkably similar to the fingerprint presented by Roest *et al.* [21], who used the same primer sets for DGGE analyses. A possible explanation for this unexpected discrepancy could be that the procedure previously used for the selection of clones for sequencing by amplified rRNA gene restriction analysis was not able to distinguish any of the *Methanomethylovorans* species found in the reactor and, in addition, was not suitable for discriminating *Methanosaeta* and *Methanobacterium* from *Methanomethylovorans* (data not shown).

At the end of the reactor experiment, it was estimated that 95% of the biomass was still present in granules. Since the granules were not involved in the degradation of MT, we expect that much higher specific loading rates should be possible, provided that the biomass is retained in the reactor. For instance, the specific activity of suspended sludge in batch (Table

2.2) was ninefold higher than the maximum specific loading rate calculated for the reactor. Future studies, therefore, should also focus on other types of sludge retention or on stimulating the MT-degrading microorganisms to form granules.

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3

Volatile organic sulfur compounds in anaerobic sludge and sediments: biodegradation and toxicity*

Abstract
A variety of environmental samples was screened for anaerobic degradation of methanethiol (MT), ethanethiol, propanethiol, dimethyl sulfide (DMS), and dimethyl disulfide (DMDS). All sludge and sediment samples degraded MT, DMS and DMDS anaerobically. In contrast, ethanethiol and propanethiol were not degraded by the samples investigated under any of the conditions tested. MT, DMS and DMDS were mainly degraded by methanogenic archaea. In the presence of sulfate and the methanogenic inhibitor bromoethane sulfonate, degradation of these compounds coupled to sulfate reduction occurred as well, but at much lower rates. Besides their biodegradability, also the toxicity of MT, ethanethiol, and propanethiol to methanogenesis with methanol, acetate, and H_2/CO_2 as the substrates was assessed. The 50% inhibition concentration of MT on the methane production from these substrates ranged between 7 and 10 mM. The 50% inhibition concentration values of ethanethiol and propanethiol for the degradation of methanol and acetate were between 6 and 8 mM, whereas hydrogen consumers were less affected by ethanethiol and propanethiol, as indicated by their higher 50% inhibition concentration (14 mM). Sulfide inhibited MT degradation already at relatively low concentrations: MT degradation was almost completely inhibited at an initial sulfide concentration of 8 mM. These results define the operational limits of anaerobic technologies for the treatment of volatile organic sulfur compounds in sulfide-containing wastewater streams.

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Introduction

The combustion of sulfur-containing fuels results in SO_2 emission and subsequently in acid deposition. Therefore, technologies to remove sulfur from fuels have received considerable attention in the last few decades [1]. The sulfur content of petroleum from different sources ranges from 0.025 to more than 5% (w/w). In addition to inorganic sulfur species (*e.g.* sulfide, sulfate, sulfite, and thiosulfate), more than 200 sulfur-containing organic compounds have been identified in crude oils. These include sulfides, thiols, thiophenes, substituted benzo- and dibenzothiophenes, and more complex molecules [2, 3]. In this paper, we focus on the anaerobic degradation and toxicity of some short-chain volatile (organic) sulfur compounds that are present in wastewater streams of refineries (*e.g.* as a result of the extraction of liquefied petroleum gas). These compounds include sulfide, methanethiol (MT), ethanethiol, propanethiol, dimethyl sulfide (DMS) and dimethyl disulfide (DMDS).

The processes involved in the microbial production and degradation of DMS gained much attention in the past because of its role in the global sulfur cycle [4]. Under anaerobic freshwater conditions, DMS and MT are mainly degraded by methanogenic archaea [5], but sulfate reducers can also contribute to DMS and MT degradation [6]. The anaerobic degradation of DMS (eqn 1) is assumed to proceed via MT as an intermediate [7]. Many of the methanogens capable of DMS degradation also grow on MT. Finster *et al.* [8] proposed the following stoichiometry for the anaerobic conversion of MT (eqn 2). The autooxidation product of MT, DMDS, is assumed to be degraded anaerobically via two MT molecules [9].

$$2\ CH_3SCH_3 + 3\ H_2O \rightarrow 3\ CH_4 + HCO_3^- + 2\ H_2S + H^+ \quad (1)$$

$$4\ CH_3SH + 3\ H_2O \rightarrow 3\ CH_4 + HCO_3^- + 4\ HS^- + 5\ H^+ \quad (2)$$

Various reports document the treatment of MT and DMS-containing waste streams, both aerobically [10-15] and anaerobically [16-18]. At very low concentrations (μM level), sulfate-reducing bacteria compete for MT and DMS with methanogens in sulfate-rich anoxic sediments [19]. Only three sulfate reducers, all thermophilic *Desulfotomaculum* species capable of growth on MT or DMS, have been isolated so far [20]. In contrast to MT and DMS, little is known about the biodegradation of higher thiols such as ethanethiol and propanethiol. A bacterium, identified as a *Thiobacillus* strain, is able to grow on MT, ethanethiol, and propanethiol as well as on diethyl sulfide, ethylmethyl sulfide, and dipropyl sulfide with NO_3^- or O_2 as electron acceptors [21]. So far, degradation of higher thiols, such as ethanethiol and propanethiol, has never been reported under methanogenic or sulfate-reducing conditions.

Many (organic) sulfur compounds are known to be toxic (*e.g.* to humans). Very little is known about their potential toxicity to microorganisms. Sipma *et al.* [17] reported a 50%

inhibition concentration (IC_{50}) of 6 mM MT for acetoclastic methanogens in anaerobic granular sludge. Besides the thiols, sulfide also is a well-known toxicant in anaerobic degradation processes. O'Flaherty and Colleran [22] reviewed sulfide toxicity mechanisms and reported 50% inhibition of acetoclastic methanogenic archaea between 38 and 252 mg/L total H_2S, depending on the sludge origin and operation conditions. The toxic effect of sulfide on MT degradation has not been reported so far. In this study, the capability of several sludges and sediments to degrade MT, ethanethiol, propanethiol, DMS, and DMDS under methanogenic and sulfate-reducing conditions is determined. Also, the toxicity of MT, ethanethiol, and propanethiol on methanogenesis from methanol, acetate, and H_2/CO_2 as the substrates is studied. Furthermore, the sulfide toxicity of MT degradation is determined.

Materials and methods

Biodegradability tests

Biodegradability tests under methanogenic and sulfate-reducing conditions were performed for MT, ethanethiol, propanethiol, DMS, and DMDS. Serum bottles (120-ml) were filled with 50 ml of bicarbonate-buffered medium with a headspace of 1.7 bar N_2/CO_2 (80:20 v/v) and were sealed with viton stoppers. The medium contained 4,000 mg/L $NaHCO_3$, 1,420 mg/L Na_2SO_4 (in case of sulfate-reducing tests), 408 mg/L KH_2PO_4, 534 mg/L $Na_2HPO_4{\cdot}2H_2O$, 218 mg/L NH_4Cl, 218 mg/L NaCl, 73 mg/L $MgCl_2{\cdot}6H_2O$, 73 mg/L $CaCl_2{\cdot}2H_2O$, 0.5 mg/L resazurin (redox indicator), 240 mg/L $Na_2S{\cdot}7\text{-}9H_2O$ (reductant), and vitamins and micronutrients according to de Bok *et al.* [23]. For the estuarine and salt lake sediments, the medium contained 28.13 g/L NaCl, 0.20 g/L K_2HPO_4, 0.77 g/L KCl, and no $Na_2HPO_4{\cdot}2H_2O$. All other compounds were added in the concentrations listed above. Volatile organic sulfur compounds (VOSCs) were added from stock solutions at final concentrations as indicated in Table 3.1. The final pH of the medium was 7.2. This pH was chosen because most of the sludge and sediment samples tested were sampled from environments with neutral pH.

Bottles with different types of inoculum (1–10%) were incubated without shaking in the dark at the temperatures indicated in Table 3.1. Thiol, DMS, and DMDS degradation was monitored by measuring concentrations of these compounds and methane in the headspace and the sulfide and sulfate concentration in the liquid medium.

Specific degradation rate of methanethiol, dimethyl sulfide, and dimethyl disulfide

The maximum specific degradation rates of MT, DMS, and DMDS with granular sludge obtained from an anaerobic MT-degrading lab-scale bioreactor [24] (chapter 2) were determined at 30°C and pH 7.2. The compounds were added separately to reach initial concentrations of 1 to 7 mM. The sludge (0.5 g wet sludge per bottle) was incubated as described in the *Biodegradability tests* section of *Materials and Methods*. No sulfate was

added in this experiment. Sulfide was measured to monitor the anaerobic degradation. MT, DMS, and DMDS were measured occasionally in the liquid phase as well. The maximal specific degradation rates (mmol sulfide produced per gram volatile suspended solids [VSS] per day) were determined by dividing the maximum (volumetric) sulfide production rate by the VSS content.

Table 3.1. Samples selected for biodegradation studies of volatile organic sulfur compounds (VOSCs) under methanogenic and sulfate-reducing conditions.

Origin biomass	VOSCs (μmol / batch)					T (°C)
	MT	ET	PT	DMS	DMDS	
Granular sludge from Upflow Anaerobic Sludge Bed reactors						
Wastewater from packaging industry (Nieuweschans, The Netherlands)	150	50	50	ND	ND	30
Paper mill wastewater (Sappemeer, The Netherlands)	150	50	50	ND	ND	30
Paper mill wastewater (Hoogezand, The Netherlands)	150	50	50	ND	ND	45
Paper mill wastewater (Eerbeek, The Netherlands)	150	50	50	ND	ND	30
Methanol/glycol wastewater (Emmen, The Netherlands)	150	15	15	15	15	30
Sulfate-reducing sludge						
Zinc factory (Budel, The Netherlands)	50	10	10	ND	ND	30
Ethanol/sulfate wastewater (Emmen, The Netherlands)	150	15	15	15	15	30
Aerobic sludge						
Sulfide oxidizing sludge (Emmen, The Netherlands)	150	15	15	15	15	30
Pilot-plant treating ethylthiourea (Rotterdam, The Netherlands)	150	15	15	15	15	30
Sediment						
Canal (Wageningen, The Netherlands)	50	10	10	ND	ND	30
Estuarine (Den Oever, The Netherlands)	150	50	50	ND	ND	20; 30
Salt lake (Larnaca, Cyprus)	150	50	50	ND	ND	30
Sulfur spring (Miliou, Cyprus)	50	50	50	ND	ND	30
Winogradsky column (Laboratory of Microbiology, Wageningen, The Netherlands)	50	50	50	ND	ND	30
Oil contaminated sediments, mixture (Harbor of Rotterdam, The Netherlands)	50	50	50	ND	ND	30
Other samples						
Sewage treatment plant, fermented sludge (Almere, The Netherlands)	150	15	15	15	15	30
BTEX contaminated groundwater (Harbor of Rotterdam, The Netherlands)	150	15	15	15	15	30

ND = not determined; BTEX = benzene, toluene, ethylbenzene and xylene

Toxicity tests

Toxicity of thiols was determined in duplicate in batch tests at 30°C and pH 7.2 with granular sludge collected from an anaerobic MT-degrading lab-scale bioreactor [24] (chapter 2). MT, ethanethiol, and propanethiol were added in concentrations ranging from 0 to 20 mM. Acetate

(60 mM), methanol (40 mM), and H_2/CO_2 (80:20 v/v) were added as substrates. In each experiment a different combination of toxicant and substrate was applied. The sludge was incubated without sulfate as described in the *Biodegradability tests* section of *Materials and Methods*. The methanogenic activity was determined by the pressure bottle technique according to Colleran and Pistilli [25]. In the experiments with hydrogen, bottles were shaken at 100 rpm, and hydrogen and methane were measured on a gas chromatograph (Hewlett-Packard, Amstelveen, The Netherlands). After complete degradation of the substrate, sulfide was measured in the bottles with methanol and acetate to check for thiol degradation.

A separate toxicity experiment was done to determine the effect of sulfide on the specific MT degradation rate at 30°C. MT was added from a stock solution to reach an initial concentration of 2 mM in the liquid phase. A fraction of MT is present in the gas phase because of the equilibrium of MT between gas and liquid phases. Sulfide was added from a neutralized Na_2S stock solution to reach initial concentrations of 0.3 to 9 mM in the liquid phase. The pH was kept at 7.2 with a $NaHCO_3$ buffer (4 g/L). The concentration of MT in the headspace was measured over time. To all bottles, 100 µl of propane was added as an internal standard. The sludge was incubated as described in the *Biodegradability tests* section of *Materials and Methods*.

Source of inoculum

All experiments described in this paper, besides the biodegradability tests, were carried out with granular sludge (0.5 g wet sludge per bottle) collected from an anaerobic MT-degrading lab-scale bioreactor [24] (chapter 2). It should be noted that tests were done with sludge sampled from the reactor at different times. Dry matter of the sludge was 110 to 130 g/kg and the VSS content was 64 to 68% of the dry weight. The lab-scale reactor was originally seeded with granular sludge obtained from a full-scale Upflow Anaerobic Sludge Bed (UASB) reactor (Industriewater, Eerbeek, The Netherlands) treating wastewater from three paper mills. The original seed sludge was also included in the biodegradability tests (Table 3.1).

Analytical procedures

For the biodegradability tests and the sulfide toxicity experiment, the thiol concentration was measured in the headspace of the bottles with the use of a Hewlett Packard CP9000 gas chromatograph (injection volume 150 µl) equipped with a CP-Porabond Q column (25 m × 0.53 mm; Hewlett-Packard).

For the thiol toxicity experiments, the pressure was monitored as an indicator for gas production with a portable membrane pressure unit, ranging from 0 to 4 bar absolute (WAL, Oldenburg, Germany). Concentrations of thiols, DMS, and DMDS were measured in the liquid phase by high-pressure liquid chromatography (Separations, Hendrik Ido Ambracht, The Netherlands) with a Chrompack (Varian Chrompack International, Bergen op Zoom, The Netherlands) C-18 column of length 20 cm. The oven temperature was 30°C. The

composition of the eluent was 35% acetonitrile and 65% water, and the flow rate was 0.6 ml/min. The injection volume of the samples was 20 µl, and an ultraviolet detector (Gynotek, Germering, Germany) was used to monitor VOSCs at a wavelength of 210 nm.

Sulfide was measured photometrically as described by Trüper and Schlegel [26]. Total and VSS were analyzed according to standard methods [27]. Hydrogen and methane were analyzed on a Hewlett-Packard 5890 gas chromatograph (injection volume 100 µl) equipped with a thermal conductivity detector (Hewlett-Packard) and molecular sieve 25H (60–80 mesh). The column size was 1.5 m by 6.4 mm. Argon was used as carrier gas at a flow rate of 25 ml/min. Temperature of the column was 40°C, injection port 110°C, and detector 125°C.

Chemicals

Organosulfur compounds were obtained from Merck (Darmstadt, Germany) and were at least 99% pure. A 3-M sodium mercaptide (sodium salt of MT) solution of analytical grade was supplied by the Arkema Group (Rotterdam, The Netherlands).

Results

Biodegradability tests

Degradation of VOSCs by anaerobic sediments and sludges was investigated. In all anaerobic samples tested, MT (as well as DMS and DMDS when tested) was completely degraded within three months, but in most batches, the degradation was already complete within three weeks (Table 3.2). Degradation of MT with granular sludge (Fig. 3.1A) started after more than 20 d (data not shown). Disappearance of MT, DMS, and DMDS coincided with methane production, indicating that these compounds were converted by methanogenic archaea (Fig. 3.1B and C). The samples in which no anaerobic MT degradation occurred came from aerobic environments. In batches without DMS and DMDS addition, traces of DMS and DMDS were often detected, but these were ultimately degraded as well.

In batches in which sulfate was present, MT (and DM(D)S when tested) was also degraded by methanogens. In some of the batches with sulfate and the specific methanogenic inhibitor bromoethane sulfonate, MT disappeared after prolonged incubation (two to three months). Methane was not formed in these batches, but more sulfide was produced (data not shown). This indicates that MT (and DM(D)S) was oxidized by sulfate-reducing bacteria.

In none of the batches was ethanethiol or propanethiol degraded, even after extended incubation periods (6–12 months).

Table 3.2. Biodegradation of volatile organic sulfur compounds (VOSCs) under methanogenic (M) and sulfate-reducing (S) conditions.

Origin biomass	VOSCs degraded			Conditions
	MT	DMS	DMDS	
Granular sludge from Upflow Anaerobic Sludge Bed reactors				
Wastewater from packaging industry (Nieuweschans, The Netherlands)	+	ND	ND	M
Paper mill wastewater (Sappemeer, The Netherlands)	+	ND	ND	M
Paper mill wastewater (Hoogezand, The Netherlands)	+	ND	ND	M
Paper mill wastewater (Eerbeek, The Netherlands)	+	+	+	M; S
Methanol/glycol wastewater (Emmen, The Netherlands)	+	+	+	M
Sulfate-reducing sludge				
Zinc factory (Budel, The Netherlands)	+	ND	ND	S
Ethanol/sulfate wastewater (Emmen, The Netherlands)	+	+	+	M
Aerobic sludge				
Sulfide oxidizing sludge (Emmen, The Netherlands)	+	+	+	M
Pilot-plant treating ethylthiourea (Rotterdam, The Netherlands)	-	ND	ND	M
Sediments				
Canal (Wageningen, The Netherlands)	+	ND	ND	M
Estuarine (Den Oever, The Netherlands)	+	ND	ND	M
Salt lake (Larnaca, Cyprus)	+	ND	ND	M
Sulfur spring (Miliou, Cyprus)	+	ND	ND	M; S
Winogradsky column (Laboratory of Microbiology, Wageningen, The Netherlands)	+	ND	ND	M; S
Oil contaminated sediments, mixture (Harbor of Rotterdam, The Netherlands)	+	ND	ND	M; S
Other samples				
Sewage treatment plant, fermented sludge (Almere, The Netherlands)	+	+	+	M
BTEX contaminated groundwater (Harbor of Rotterdam, The Netherlands)	-	-	-	-

ND = not determined; + = degraded; - = not degraded

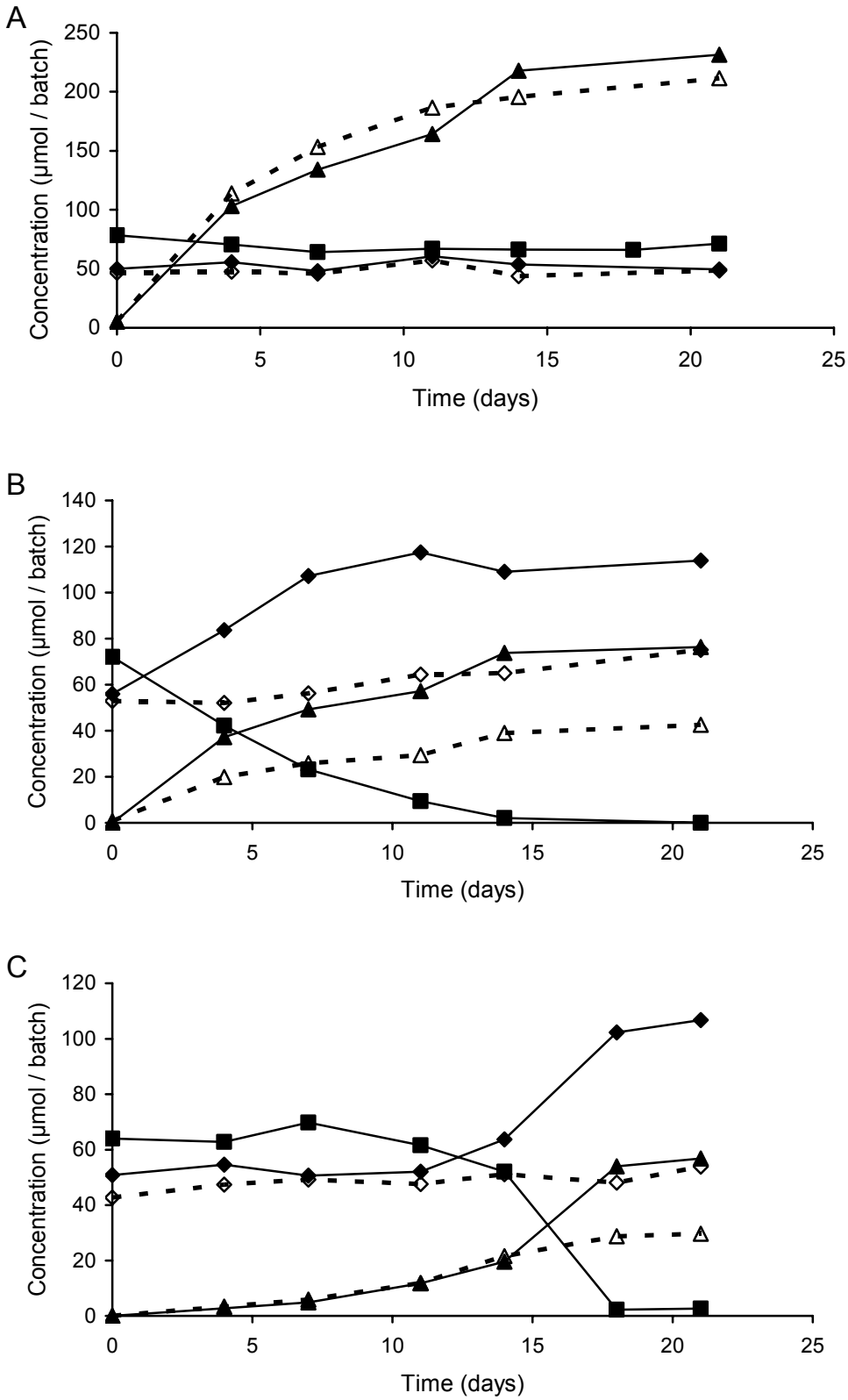

Fig. 3.1. MT degradation by reactor sludge sample from an Upflow Anaerobic Sludge Bed (UASB) reactor fed with ethanol and sulfate (A), an estuarine sediment sample (Den Oever, The Netherlands) (B), and canal sediment sample (Wageningen, The Netherlands) (C) incubated under methanogenic conditions. —■— MT; —▲— methane; —♦— sulfide; broken lines and open symbols: endogenous controls (incubations without thiols). The ethanethiol and propanethiol concentrations remained constant throughout the incubation period (data not shown).

Specific methanethiol, dimethyl sulfide, and dimethyl disulfide degradation rates

The specific degradation rates of MT, DMS, and DMDS by the UASB sludge were determined at 30°C and pH 7.2 (Fig. 3.2). The degradation rates of MT and DMS tend to increase with increasing initial substrate concentration. The maximal specific degradation rates and toxic levels were not yet reached in the concentration range tested. Complete degradation of MT and DMS was reached between days 15 and 30 for all concentrations tested.

DMDS degradation proceeded slowly. Low initial DMDS amounts (to 1 mM) were completely degraded to sulfide within 38 d, with MT as the intermediate compound. In incubations with initial DMDS concentrations of 2 mM and higher, however, 0.5 to 2 mM of DMDS was still present after 50 d of incubation. MT accumulated in concentrations between 2.4 and 6.0 mM and was not further degraded. Because no more than 2 mM sulfide was formed as end product in the latter incubations, DMDS seemed to deteriorate the MT degrading capacity of the sludge.

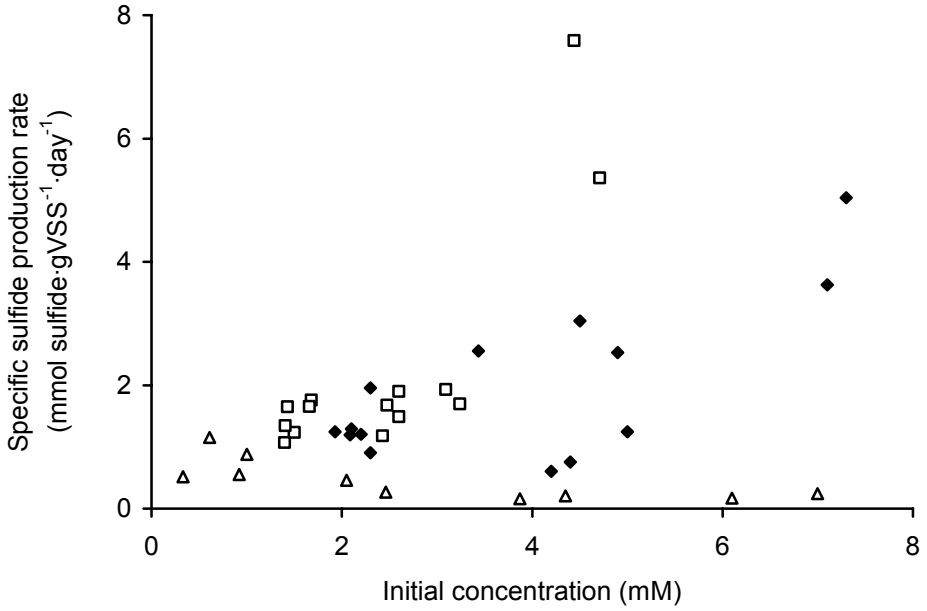

Fig. 3.2. Effect of initial volatile organic sulfur compound concentration on the maximal specific degradation rate of MT (♦), DMS (□) and DMDS (Δ) by granular sludge from an anaerobic MT-degrading lab-scale bioreactor.

Toxicity of thiols

Toxicity of thiols was assessed by the additions of MT, ethanethiol, and propanethiol to the anaerobic granular sludge converting methanol, acetate, or hydrogen. In the absence of thiols, methanogenesis from methanol and acetate (measured as an increase in gas pressure) started within 4 d and from hydrogen within 2 d of incubation (data not shown). The lag phase for methanogenesis from methanol increased because of the addition of MT up to a maximum of 35 d at the highest MT concentration tested (Fig. 3.3). Note that the maximal activities on methanol or acetate differed considerably in the experiments with the different thiols (MT, ethanethiol, propanethiol) because not all experiments could be performed at the same time with the same sludge sample.

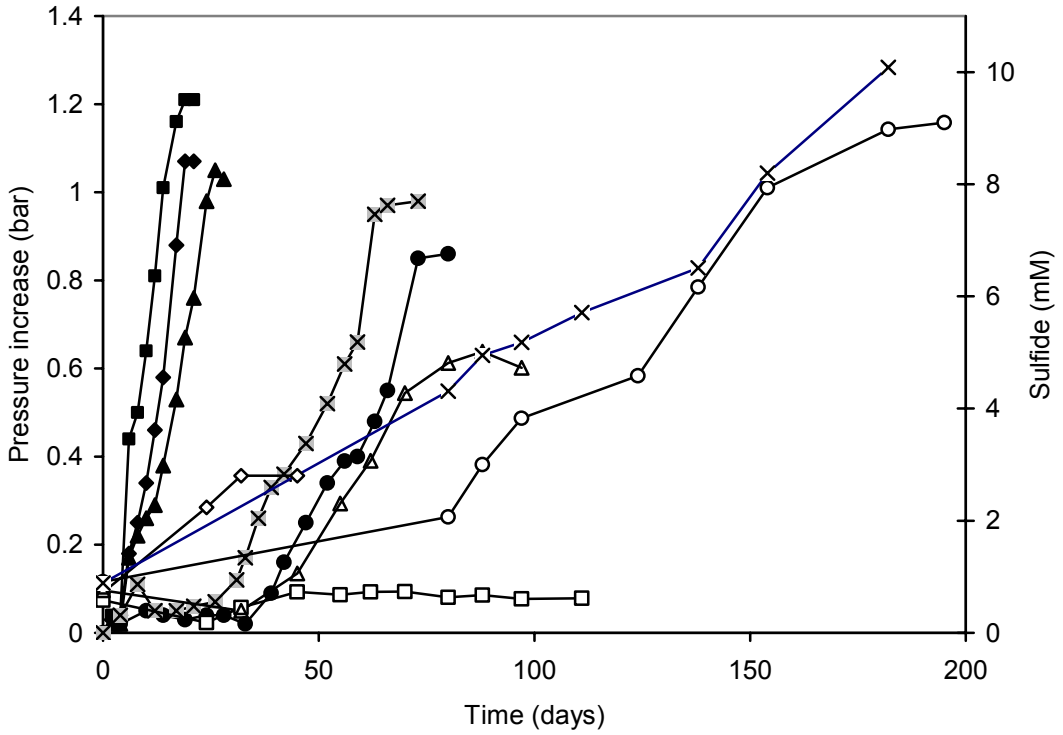

Fig. 3.3. Methanol degradation (indicated as pressure increase, closed symbols) and sulfide formed from MT (open symbols) by granular sludge from an anaerobic MT-degrading lab-scale bioreactor. The initial methanol concentration was 40 mM; the initial MT concentrations were 0 (■), 2.3 (♦), 6.6 (▲), 12 (×), and 17 mM (●).

The IC_{50} values for each thiol (Table 3.3) were determined from data presented in Figure 3.4. Ethanethiol and propanethiol were slightly more toxic for methanol degradation than MT. Acetoclastic methanogens seemed to be slightly more sensitive to thiols (IC_{50} values ranging from 6 to 8 mM) than methylotrophic methanogens.

Hydrogen was consumed rapidly in the three experiments with MT, ethanethiol, and propanethiol. Although the bottles were shaken, it is known that hydrogen mass transfer

limitation often determines the reaction rate [28]. In the presence of MT, hydrogen was completely consumed between day 2 (no MT) and day 13 (14 mM MT). Also, methane production rate clearly decreased at increasing MT concentrations (Fig. 3.4C). In the presence of ethanethiol and propanethiol, almost all hydrogen was consumed between days 2 and 4, independent of the thiol concentration. Ethanethiol and propanethiol inhibited methane production (Fig. 3.4C), but the IC_{50} values were significantly higher than for MT (Table 3.3).

Table 3.3. The 50% inhibition concentrations (IC_{50}s) of MT, ethanethiol, and propanethiol for the methanogenic activity on methanol, acetate, and H_2/CO_2.

	IC50 (mM)		
	Methanethiol	Ethanethiol	Propanethiol
Methanol	10	8	8
Acetate	8	6	7
H_2/CO_2	7	>14	14

To investigate the biodegradation of thiols in the above described toxicity experiments, sulfide was measured at the start of the experiments and after stabilization of pressure (complete methane formation) in the experiments with methanol and acetate. Methanol was completely degraded between days 19 and 73 (Fig. 3.3, closed symbols), depending on the initial MT concentration. MT degradation started already during the methanogenesis from methanol, but the major fraction was degraded afterwards. The lowest initial MT concentration tested (2.3 mM) degraded within 30 d (Fig. 3.3). Sulfide was produced in all tests, even at high initial (17 or 18 mM) MT concentrations. MT degradation was, however, incomplete in incubations with initial MT concentrations exceeding 12 mM. For example, after 129 d of incubation, the residual concentrations of VOSCs (mainly as DMS) were 2.2 mM (12 mM initially) and 6.0 mM (17 mM initially), perhaps because of sulfide toxicity. Calculations on the data presented in Figure 3.3 reveals that the specific rate of methanogenesis from methanol (in mmol $CH_4 \cdot [g\ VSS]^{-1} \cdot d^{-1}$) was 20 to 40 times faster than methanogenesis from MT, showing that MT is a less favorable substrate for methanogens.

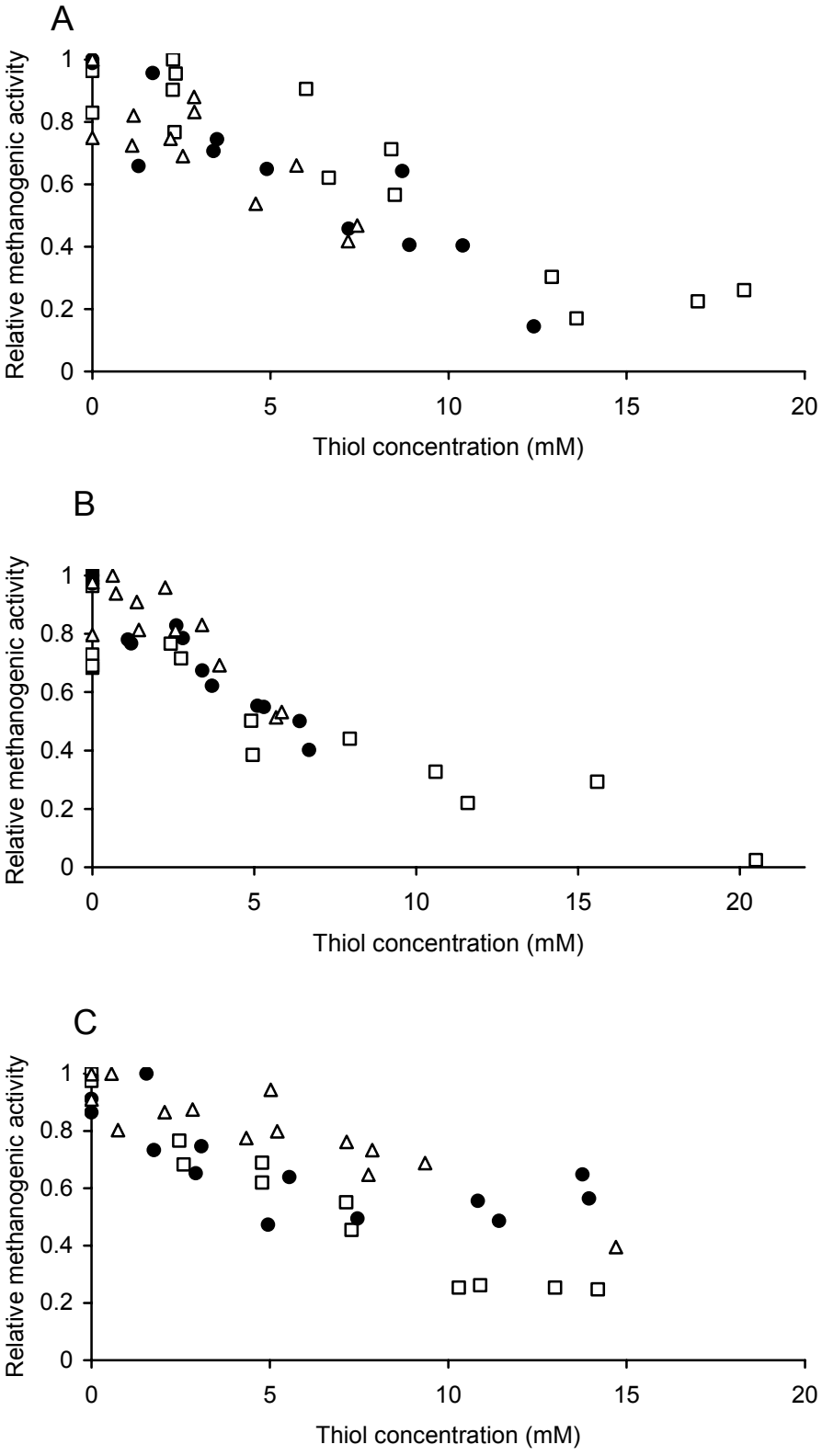

Fig. 3.4. Inhibition effects of MT (□), ethanethiol (●), and propanethiol (Δ) on the methanogenic activity from methanol (A), acetate (B), and H_2/CO_2 (C) by granular sludge from an anaerobic MT-degrading lab-scale bioreactor. Maximal methanogenic activities were 6.5, 15, and 25 mmol $CH_4 \cdot (g\ VSS)^{-1} \cdot d^{-1}$ (methanol); 5.6, 1.1, 12 mmol $CH_4 \cdot (g\ VSS)^{-1} \cdot d^{-1}$ (acetate); and 1.2, 2.9, 2.2 mmol $CH_4 \cdot (g\ VSS)^{-1} \cdot d^{-1}$ (H_2/CO_2) for incubations with MT, ethanethiol, and propanethiol, respectively.

The incubations with MT and acetate showed a similar trend concerning sulfide production. Sulfide concentrations did not exceed 11 mM, and in tests with high initial (> 10 mM) MT concentrations, MT was still not fully degraded after more than 120 d. The specific rate of methanogenesis from acetate was, similar to methanol, 20 to 40 times faster than methanogenesis from MT.

No sulfide was formed from ethanethiol and propanethiol during more than 100 d of incubation. This is in agreement with the results from the biodegradability tests, in which no degradation of ethanethiol and propanethiol was found under anaerobic conditions in any of the tested sludge samples. Ethanethiol and propanethiol were not completely stable, however. Between 0 and 30% of the initial amount had disappeared at the end of the incubations.

During incubation with methanol and MT, considerable amounts of DMS were formed as an intermediate compound by the granular sludge from the UASB reactor (data not shown). This indicates that methylation of MT occurs. For example, at an initial concentration of 6.6 mM MT, 99% of the remaining total VOSCs was DMS (5.6 mM) after an incubation period of 33 d. Only traces of MT and DMDS were detected. All VOSCs were ultimately completely degraded to H_2S and CH_4 in this incubation (6.6 mM MT) after 80 d. In general, initial MT concentrations exceeding 6 mM gave rise to high DMS formation: more than 70% of the VOSCs (MT, DMS, DMDS) was DMS during the degradation of MT. In incubations with acetate and MT, less DMS (30% of the total VOSCs) was found as an intermediate (data not shown).

Sulfide inhibition

The effect of the initial sulfide concentration on the degradation of 2 mM MT was measured at pH 7.2 and 30°C. Degradation of MT started after 20 d at an initial sulfide concentration of 0.3 mM. The lag phase increased to more than 60 d at the highest sulfide concentration tested (Fig. 3.5). As soon as MT degradation started, degradation was complete within 10 d at initial sulfide concentrations of 0.3 and 1.5 mM. Higher initial sulfide concentrations caused lower degradation rates. An initial sulfide concentration of 4.3 mM inhibited MT degradation by 80% compared with MT degradation at an initial sulfide concentration of 0.3 mM. At initial sulfide concentrations of 5.7 mM and higher, MT degradation was still not complete after 112 d. MT was stoichiometrically converted to sulfide (eqn 1). At initial sulfide concentrations of 2.5 mM and higher, traces of DMS were identified in the gas phase.

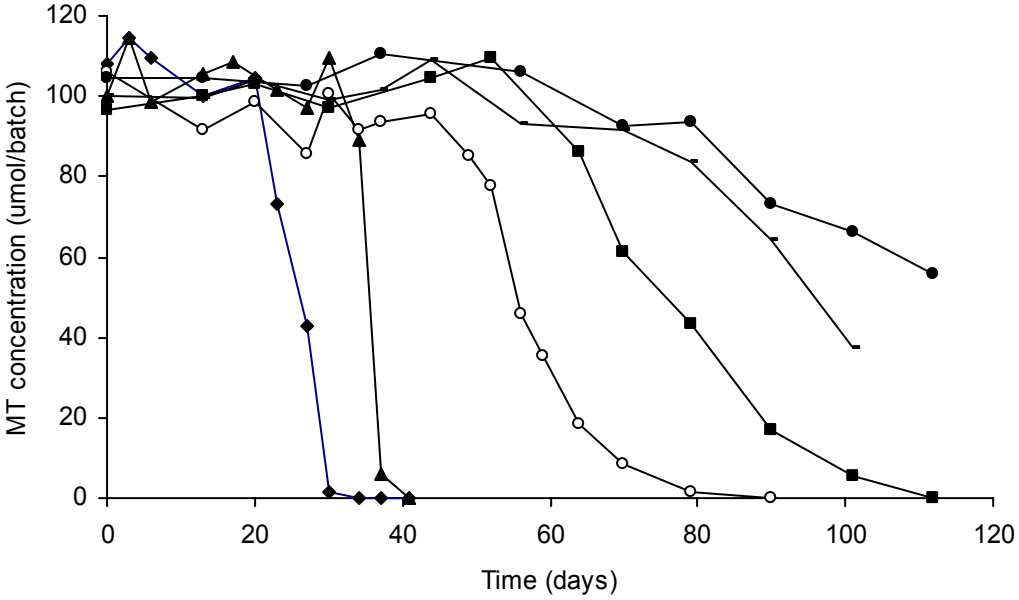

Fig. 3.5. The effect of the initial sulfide concentration on the MT degradation by granular sludge from an anaerobic MT-degrading lab-scale bioreactor. The total initial sulfide concentrations were 0.3 (♦), 1.5 (▲), 2.5 (○), 4.3 (■), 5.7 (-), and 8.4 mM (●).

Discussion

Anaerobic degradation of methanethiol, dimethyl sulfide, and dimethyl disulfide

Under anaerobic freshwater conditions, DMS and MT are mainly degraded by methanogenic archaea [5, 29], but sulfate reducers can also contribute to DMS and MT degradation [6]. Several methylotrophic methanogens capable of degrading MT have been isolated from marine and freshwater sediments [8, 29]. These organisms were isolated on DMS but also degraded MT, which is an intermediate of the conversion of DMS. The anaerobic degradation of MT resembles the degradation of methanol, where the ratio CH_4:CO_2 is also 3:1. The current research confirmed that MT, DMS and DMDS are degraded by methanogenic archaea under anaerobic conditions (Table 3.2; Fig. 3.1) and that these methanogens are present in many anoxic sediments and sludges. Apparently, MT-degrading methanogens were not present in aerobic samples tested, such as groundwater and aerobic sludge. The results also confirmed that MT, DMS and DMDS can be anaerobically oxidized with sulfate as electron acceptor (Table 3.2), provided that methanogens are inhibited by bromoethane sulfonate. At very low concentrations, sulfate-reducing bacteria are thought to compete for these compounds with methanogens in anoxic sediments [19]. Only three sulfate reducers capable of growth on MT or DMS have been isolated so far. They were obtained from a thermophilic anaerobic digester [20]. Alternatively, MT degradation under sulfate-reducing conditions proceeds via interspecies hydrogen transfer [30]. In that case, reducing equivalents, in the

form of hydrogen, are transferred from the MT-degrading methanogen to the hydrogen-consuming sulfate-reducing bacterium.

Sulfide formation from DMDS proceeded slowly compared with MT and DMS (Fig. 3.2). MT was found as an intermediate and was not further degraded. The first step in DMDS degradation is a reductive cleavage into two MT. This conversion is catalyzed by a DMDS reductase that uses nicotinamide adenine dinucleotide as the reductant [9]. This finding is in agreement with Kiene *et al.* [19], who also found that DMDS inhibited the methanogens that degrade MT and that MT conversion was inhibited at lower concentrations by DMDS than by DMS.

Intermediate DMS formation

In incubations with methanol and MT, the main part of MT was first converted to DMS before it was degraded. Zitomer *et al.* [31] mention DMS as an indicator of toxicity or stress on the biomass in anaerobic treatment. In incubations with acetate and MT, less DMS was found as an intermediate. This suggests that methanol serves as a methyl donor for DMS formation, which was also found by others [30, 32]. DMS was found in anaerobic bioreactors fed with MT as the sole substrate [18]. When DMS is formed from two molecules of MT, H_2S is released (eqn 3). The excess reduction equivalents released during the disproportionation of MT is used for the initial reduction of DMS (eqn 4) [6].

$$2\ CH_3SH \rightarrow CH_3SCH_3 + H_2S \quad (3)$$

$$CH_3SCH_3 + 2\ [H] \rightarrow CH_3SH + CH_4 \quad (4)$$

Transient accumulation of DMS in batches incubated with MT also indicated that transfer of the first methyl group during DMS degradation is a reversible process. Formation of DMS was also found in suspensions of strain DMS1^T (T = type strain) cells containing both methanol and MT [29] and in anoxic slurries from a ditch [30]. Higher actual concentrations of MT and sulfide in the batch cultures might make the transfer of a methyl group onto MT energetically more favorable, resulting in the observed accumulation of DMS [29]. All experiments in this study were carried out at pH 7.2 because this was the pH at which the reactor was operated (from which the sludge was sampled) and because most samples tested (Table 3.1) were taken from environments with neutral pH. The methylation of MT to form DMS might, however, also be affected significantly by the pH because of the enhanced reactivity of thiols at elevated pH. Similarly, an increase in the reaction rate for the methylation of 2-mercaptoethanol (p*K*a 9.63) was observed from pH 7.0 to 9.6 [33].

Sulfide toxicity

In the presence of sulfide, MT degradation by MT-adapted sludge proceeded slower (Fig. 3.5), and lag phases increased from 20 d (0.3 mM sulfide) to about 60 d (8.4 mM sulfide). An

initial sulfide concentration of 4.3 mM already inhibited MT degradation by 80% compared with MT degradation at an initial sulfide concentration of 0.3 mM. Sipma *et al.* [17] operated a reactor degrading MT inoculated with sludge from the same origin as used in this research. At an influent concentration of 6 mM MT and an additional sulfide concentration of 6 mM, MT was degraded almost completely (pH 7.3–7.6). An increase to 8 mM sulfide in the influent resulted in MT degradation efficiencies of 50% or less. The toxic effect of sulfide is thought to be caused by undissociated H_2S, which is uncharged and therefore membrane permeable [22]. Once inside the cell, dissociation takes place, and the internal pH is lowered. Thus, the total sulfide concentration is not important for toxicity determination, only the undissociated fraction. This means that pH plays an important role in sulfide toxicity. The concentration of undissociated H_2S causing 50% inhibition of acetoclastic methanogens at pH values between 6.2 and 8.0 varied between 90 and 252 mg/L [34-37]. In our sulfide toxicity experiment, 142 mg/L sulfide (4.3 mM; Fig. 5), which corresponds to 47 mg/L undissociated H_2S (pH 7.2), resulted already in an inhibition of the MT degradation by 80%. This indicates that the methanogenesis with MT as the substrate is much more vulnerable to sulfide compared with acetoclastic methanogenesis.

Degradation of higher thiols

Anaerobic degradation of ethanethiol and propanethiol was not found in the current research under either methanogenic or sulfate-reducing conditions. This confirms that degradation of higher thiols has never been reported under these conditions. The anaerobic degradation of methylethyl sulfide stopped at the formation of ethanethiol (F.A.M. de Bok,Wageningen University, personal communication), which shows that methylotrophic methanogens are only able to demethylate VOSCs. Oremland *et al.* [38] reported anaerobic ethane and ethane formation stimulated by ethanethiol, but these were very low production rates (about 1 nmol ethane·[150 ml slurry]$^{-1}$·d^{-1}), which accounted for less than 1% of the initial ethanethiol concentration. MT degradation by methanogens resembles methanol degradation. This is not the case with ethanethiol and propanethiol. These compounds are difficult to degrade anaerobically, whereas ethanol and propanol are readily degraded.

Batch experiments with several sludge samples were also performed to assess whether MT, ethanethiol, and propanethiol oxidation could be coupled to denitrification, but after prolonged incubation, the thiols were always recovered as disulfides, even in endogenous and chemical controls (data not shown). Kelly and Smith [4] mentioned the isolation of an aerobic bacterium that grew on several VOSCs, including ethanethiol. Later, a *Thiobacillus* was isolated that could not only oxidize diethyl sulfide, ethylmethyl sulfide, and dipropyl sulfide but also MT, ethanethiol, propanethiol, and butanethiol, as well as the corresponding disulfides with NO_3^- or O_2 as the electron acceptor [21]. This organism could 3 be used to treat waste streams rich in organic sulfur compounds, but the strain was never deposited in any of the culture collections and is also not commercially available as far as we know.

Toxicity of thiols on methanogenesis

Our research showed that 50% inhibition of methanogenesis by MT took place at concentrations between 7 and 10 mM for the substrates methanol, acetate, and hydrogen. This is in agreement with observations made by Londry and Suflita [2], who found that thiols inhibited methanogenesis at concentrations above 5 mM. They tested the toxicity of a mixture of long-chain (C5–C8) and cyclic thiols with lactate as the substrate. Sipma *et al.* [17] carried out experiments similar to those described here, with anaerobic sludge originating from a UASB reactor in Eerbeek (The Netherlands), and found an IC_{50} of 6 mM MT, whereas 15 mM MT caused only 34% inhibition for methanol degradation. The current research confirmed that acetoclastic methanogens are more sensitive for lower thiols than methylotrophic and hydrogenotrophic methanogens (Table 3.3). Indeed, acetoclastic methanogens are, in general, recognized as the most sensitive trophic group of the anaerobic consortium [39].

Intermediates were not measured during the degradation of methanol, acetate, and H_2/CO_2. Methanogenesis is, however, the predominant mineralization route for methanol at mesophilic conditions [40]. In the experiments in which H_2/CO_2 was used as a methanogenic substrate, hydrogen was completely consumed before the methane concentration reached its maximal level. Homoacetogens can compete with the methanogens for hydrogen to form acetate [41]; therefore, part of the H_2/CO_2 might have been first converted into acetate. Elucidation of the pathways of hydrogen and acetate degradation requires further research with selective inhibitors for the trophic groups involved.

A possible explanation for the observed toxicity effects is the catalytic site inactivation of enzymes by thiols and disulfides. The active site of an enzyme contains amino acid residues, which are susceptible to covalent modifications [42]. DMDS can react with the thiol group of cysteine, for example, to form a disulfide bond amino acid residue and MT. The other way around, MT or a higher thiol can open a disulfide bond from an enzyme and inactivate the catalytic side. The reactivity of thiols (and therefore maybe also their toxicity) is pH dependent and usually higher at elevated pH. In ionic form the thiol residue (RS^-) serves as a strong nucleophile. The pKa values of MT, ethanethiol and propanethiol have values of around 10.4. Because the thiols were mainly present in their protonated form in this study (pH 7.2), inactivation of enzymes by the breakage of S–S bonds in the secondary structure might have been limited.

Application

The results presented here indicate the possibilities and limitations for anaerobic (pre)treatment of VOSCs-containing wastewaters. Volatile organic sulfur compound–rich wastewaters are produced at refineries during desulfurization of gasoline and liquefied petroleum gas. The possibility of anaerobic treatment depends on the concentration of the

compounds and on other factors like pH and salt concentration. This research showed that MT, ethanethiol, and propanethiol inhibit methanogenic activity by 50% in a concentration range of 6 to 14 mM. An initial sulfide concentration of only 4.3 mM inhibited the MT degradation rate by 80% (Fig. 3.5) compared with the situation with 0.3 mM sulfide. MT, DMS and DMDS were degraded anaerobically, but ethanethiol and propanethiol were not. When the majority of the VOSCs is converted to sulfide, an oxidation step to form elemental sulfur and a separation step afterwards are required to remove the sulfur from the wastewater. Volatile organic sulfur compound-containing wastewater streams from the oil industry could have a high salt concentration and high pH. Because of their possible higher reactivity at elevated pH, it is therefore of great importance to study the degradation of MT, ethanethiol, and propanethiol at alkaline conditions. Higher degradation rates, and on the other hand higher toxicity levels, might seriously affect the feasibility of methanogenic-driven thiol removal.

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4

Anaerobic methanethiol degradation in upflow anaerobic sludge bed reactors at high salinity (≥ 0.5 M Na^+)*

Abstract
The feasibility of anaerobic methanethiol (MT) degradation at elevated sodium concentrations was investigated in a mesophilic (30°C) lab-scale upflow anaerobic sludge bed (UASB) reactor, inoculated with estuarine sediment originating from the Wadden Sea (The Netherlands). MT was almost completely degraded (>95%) to sulfide, methane and carbon dioxide at volumetric loading rates up to 37 mmol $MT \cdot L^{-1} \cdot day^{-1}$, 0.5 M sodium (NaCl or $NaHCO_3$) and between pH 7.3 and 8.4. Batch experiments revealed that inhibition of MT degradation started at sodium (both NaCl and $NaHCO_3$) concentrations exceeding 0.8 M. Sulfide inhibited MT degradation already around 3 mM (pH 8.3).

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Introduction

The combustion of sulfur containing fuels results in SO_2 emission and subsequently in acid deposition. The last 30 years, an ongoing trend is observed in installing better desulfurization technologies at oil refineries and natural gas upgrading facilities. In addition, desulfurization of hydrocarbon streams is needed for the protection of catalysts in certain downstream process units (e.g. reformers), improvement of product quality and protection of car exhaust catalysts from sulfur poisoning.

The sulfur content of crude oils ranges from 0.025 to more than 5 wt.% [1-3]. Liquefied petroleum gas (LPG) is one of the top products from the crude distiller unit at refineries and consists of light hydrocarbon compounds (C_3 and C_4) and light sulfur-containing compounds, such as H_2S, methanethiol (MT) and ethanethiol, are present as sulfur impurities [1, 4]. Even though European sulfur norms for LPG do not yet exist, it is normally desulfurized to levels below 10 ppm.

Sipma *et al.* [5] proposed a three-step process for the removal of H_2S and MT from LPG. The process involves: (i) extraction of the sulfur compounds (MT and H_2S) from the LPG phase into a (bi)carbonate-containing solution; (ii) anaerobic degradation of MT to H_2S, CO_2 and CH_4; (iii) partial oxidation of H_2S to elemental sulfur. The sulfur particles are removed in a settler and the cleaned alkaline process water is reused in the extraction process. Extraction of H_2S and MT from LPG is improved at increased pH values. Sodium (bi)carbonate solutions must provide sufficient alkalinity to run the process at a nearly constant pH value. Levels of at least 0.5 M Na^+ are expected in the process. As these Na^+ concentrations impose high osmotic stress to microorganisms, salt tolerant or halophilic microorganisms are needed to apply this new biotechnological process.

The oxidation of H_2S to elemental sulfur at increased sodium levels has already been studied extensively [6, 7]. Therefore, the present research focuses on the anaerobic degradation of MT at elevated salinity. Ethanethiol it is not considered in the present study, since it was found to be non-biodegradable under anaerobic conditions [8] (chapter 3).

Anaerobic MT degradation occurs in freshwater, estuarine and marine sediments as well as in digested sewage sludge and UASB (upflow anaerobic sludge bed) granular reactor sludge [8-14]. Finster *et al.* [9] described a marine methanogen capable of growing on MT (eqn 1) as the sole energy source and proposed a stoichiometry for the reaction, similar to methanol metabolism of *Methanosarcina barkeri*:

$$4\ CH_3SH + 3\ H_2O \rightarrow 3\ CH_4 + HCO_3^- + 4\ HS^- + 5\ H^+ \qquad (1)$$

Previous research showed the feasibility of anaerobic MT degradation in a mesophilic (30°C) lab-scale UASB reactor at neutral pH (7.0-7.5) and low salt concentrations (~15 mM of total salts dissolved) [5, 15, 16]. The inoculum was an anaerobic granular sludge

originating from a UASB reactor treating paper mill wastewater. The current research focuses on the anaerobic MT degradation at elevated salt (NaCl and $NaHCO_3$) concentrations (0.5 M Na^+) in defined media using estuarine sediment (Wadden Sea, The Netherlands) as inoculum. Furthermore, the effect of Na^+ and sulfide on the MT degradation by the reactor sludge is assessed in batch activity tests.

Materials and methods

Inoculum characteristics

The reactors were inoculated with 6 g VSS (volatile suspended solids) of estuarine sediment (Wadden Sea, East Texel, The Netherlands, sampling date: 1 September 2002). As the majority of the sediment consisted of sand, the VSS content of the dry material was 10%. The particle size distribution of the sediment ranged from 1 μm to 1500 μm. The potential of the sediment to degrade MT anaerobically was already demonstrated in batch experiments [8] (chapter 3).

In addition, anaerobic granular sludge obtained from an anaerobic MT-degrading lab-scale bioreactor (30°C, pH 7.2-7.5, ~15 mM total salinity) [15] (chapter 2) was used for a batch experiment. The typical diameter of these granules was 0.5–3 mm and the VSS content was 66% of the dry weight. This sludge originated from a full-scale UASB reactor [17, 18] treating wastewater from three paper mills (Eerbeek, The Netherlands).

Continuous UASB reactor experiments

Three reactor experiments were carried out in a lab-scale continuous-flow UASB reactor (1.6 L) as described previously [15] (chapter 2). The reactor temperature was controlled at 30°C using a thermostatic water bath. The pH of the reactor liquid was measured with a sulfide resistant pH electrode (Hamilton Flushtrode, Hilkomij B.V., Rijswijk, The Netherlands) and controlled between 7.2 and 7.5 by adding sodium hydroxide or hydrochloric acid from 0.1 M stock solutions. The produced biogas was led through a NaOH scrubber (3 M) and a column filled with soda lime pellets to remove H_2S and CO_2. The remaining biogas flow was measured with a Mariott's bottle.

The reactor was fed with an oxygen-free synthetic influent (pH 12), containing about 80 mM MT (stock solution). It was stored in a sealed glass bottle in which the volume of the consumed liquid was replaced by nitrogen gas to prevent autooxidation of MT into dimethyl disulfide (DMDS). Dilution water (pH 4), containing all micro and macronutrients, was added using peristaltic pumps (Watson Marlow, Falmouth, Cornwall, UK). It contained the following macronutrients (in mg/L): NaCl (28000), NH_4Cl (480), K_2HPO_4 (200), $MgCl_2{\cdot}6H_2O$ (160), KCl (770), $CaCl_2{\cdot}2H_2O$ (160) and yeast extract (100). Micronutrients (1 ml/L) were added according to Paulo *et al*. [19].

During the first experiment the combined influent flow contained 2-7 mM MT. The total influent flow was about 0.75 L/day at the start of the continuous operation resulting in a hydraulic retention time (HRT) of 50 h, and was stepwise increased to 4.3 L/day (HRT of 9 h, Table 4.1).

Table 4.1. Experimental conditions of the three reactor experiments.

Reactor experiment	Period no.	Period (day)	Main sodium salt (M)	HRT (h)	pH reactor
1	I	0-12	NaCl (0.48)	50	7.2-7.5
	II	12-124	NaCl (0.48)	24	7.2-7.5
	III	124-145	NaCl (0.48)	12	7.2-7.5
	IV	145-154	NaCl (0.48)	9	7.2-7.5
2	I	0-10	NaCl (0.48)	24	7.3-7.5
	II	10-31	NaCl (0.48)	12	7.3-7.5
	III	31-60	NaCl (0.24) + $NaHCO_3$ (0.24)	12	7.9-8.1
	IV	60-77	NaCl (0.24) + $NaHCO_3$ (0.24)	9	7.9-8.1
	V	77-96	$NaHCO_3$ (0.48)	9	8.2-8.4
3	I	0-360	$NaHCO_3$ (0.48)	9	8.1-8.4

In the second reactor experiment, NaCl (28 g/L) in the medium was first replaced by 14 g/L NaCl and 20 g/L $NaHCO_3$ and finally by 40 g/L $NaHCO_3$. When $NaHCO_3$ was included in the medium sufficient alkalinity was present and pH control was not needed. The complete replacement of NaCl by $NaHCO_3$ resulted in a pH increase from about 7.5 to 8.3. In the third experiment, MT loading rates were further increased. The pH of the reactor medium was kept between 8.1 and 8.4 and the reactor medium contained 40 g/L $NaHCO_3$ (Table 4.1).

Batch incubations: effect of salt and sulfide concentrations on MT degradation

To study the effect of salt on MT degradation, reactor sludge samples, collected on day 117 of reactor experiment 1, were incubated in 120-ml serum flasks filled with 50 ml sodium bicarbonate (4 g/L) buffered medium, and a headspace composed of 1.7 bar N_2/CO_2 (80:20) [20]. In every flask, 3 ml of the sludge suspension was added which corresponds to 6.2 mg VSS per flask. MT was added from a stock solution to a concentration of 2–3 mM, and additional NaCl and $NaHCO_3$ were added in concentrations of 15, 30, 50 and 70 g/L.

The MT degradation capacity of the reactor sludge at high salinities was compared with the MT degradation capacity of granular sludge from an anaerobic MT-degrading lab-scale bioreactor (see *Inoculum characteristics* section) after 126 days of operation [15] (chapter 2). Incubations were done with 0.2 g VSS granular sludge and additional NaCl and $NaHCO_3$ was added in concentrations of 10 and 25 g/L. MT degradation was monitored by measuring the sulfide concentration in the liquid phase. Specific degradation rates were calculated from the linear part of the degradation curves.

The effect of sulfide on MT degradation was studied using reactor sludge samples of day 96 of experiment 2 (21 mg VSS per flask). MT (2 mM) and sulfide (0.5-13.6 mM) were added to the flasks from concentrated stock solutions. The incubation procedure was similar

to the method described above. The sodium bicarbonate concentration in these flasks was 40 g/L, as in reactor experiments 2 and 3, resulting in a medium with pH 8.3. MT was measured in the headspace of the flasks. All experiments described were carried out in duplicate at 30°C.

Analytical techniques

Volatile organic sulfur compounds (VOSC) were analyzed together in a single measurement by high performance liquid chromatography (HPLC) (Separations, Hendrik Ido Ambracht, The Netherlands) as described by van Leerdam *et al.* [8] (chapter 3). Total VOSC concentrations have been calculated, *i.e.* the sum of individual MT, ethanethiol, DMS (dimethyl sulfide) and DMDS concentrations. These values were used in our analyses rather than only MT for expressing the MT degradation, as MT is readily autooxidized to DMDS and other VOSC might be formed as well. Autooxidation may take place in the concentrated stock solution or during the sampling procedure. For the sulfide toxicity experiment, the MT content in the headspace of the flasks was monitored using a Hewlett Packard CP9000 gas chromatograph according to van Leerdam *et al.* [8] (chapter 3). Propane (150 ml) was included in every flask as an internal standard.

Sulfate was measured on a DX-600 ion chromatograph system (Dionex Corporation, Salt Lake City, USA) according to Sipma *et al.* [21]. Sulfide [22] and polysulfide [23] were measured photometrically. Biogas composition (CH_4, CO_2, N_2, and H_2S) was analyzed on a Packard Becker gas chromatograph, model 433 (Delft, The Netherlands) according to Paulo *et al.* [19].

Total and volatile suspended solids were analyzed according to Standard Methods [24]. The particle size distribution of the sediment was determined by laser scattering image analysis (Coulter laser LS 230, Beckman Coulter, USA). Microscopic analysis of the biomass was performed with an Olympus BH2 epifluorescence microscope and an Olympus SZ40 stereo microscope.

Chemicals

All chemicals used were of analytical grade and supplied by Merck (Darmstad, Germany). A sodium mercaptide solution (3 M) of analytical grade was supplied by Arkema Group (Rotterdam, The Netherlands).

Results

Anaerobic MT degradation at high NaCl levels (experiment 1)

The UASB reactor was started up in batch mode operation by injecting MT directly into the reactor to obtain an initial concentration of 2 mM. MT disappeared completely within 4 days. The MT injection was repeated five times and sulfide was measured as the main reaction product. After 20 days of operation, the system was switched from batch to continuous mode at a MT influent concentration of 4 mM and a HRT of 50 h.

The superficial upward velocity was kept at 1 m/h to prevent excessive loss of suspended matter via the effluent. However, some biomass still washed out since the total suspended solids (TSS) content of the effluent amounted to 40-70 mg/L, corresponding to 4-7 mg/L VSS.

During the first 10 days of continuous operation, MT was only partially degraded and about 1 mM sulfide was measured in the effluent. In the second period, the HRT was decreased to 24 h and the MT influent concentration was adjusted to 2 mM (Fig. 4.1B) to maintain a volumetric loading rate of 2 mmol $MT \cdot L^{-1} \cdot day^{-1}$. After 14 days of operation MT degradation was nearly complete. The removal efficiency of the total VOSC compounds exceeded 95% and the total VOSC concentration in the effluent never exceeded 0.5 mM for the remainder of the experiment.

The volumetric loading rate was increased stepwise from 2 to 14 mmol $MT \cdot L^{-1} \cdot day^{-1}$ (Fig. 4.1A) by increasing the influent concentration from 2 to 7 mM and by decreasing the HRT to 9 h (Fig. 4.1B). Based on the initial amount of sludge, this corresponded to a specific sludge loading rate of 0.5 mmol $MT \cdot gVSS^{-1} \cdot day^{-1}$ at the start of the experiment up to 4 mmol $MT \cdot gVSS^{-1} \cdot day^{-1}$ at the end of the experimental run. Besides low concentrations of MT and DMDS, some trace amounts of DMS (< 0.1 mM) and incidentally also ethanethiol (< 0.05 mM) were detected in the effluent.

The biogas production increased from about 50 ml/day during the first 50 days, to around 110 ml/day till day 121 and reached values of up to 200 ml/day at the end of the experiment. Fifty to seventy-five percent of the methane produced at complete MT degradation was recovered. The methane concentration in the biogas varied between 60 and 70 vol.%. The balance was nitrogen gas, which was added to the headspace of the influent vials to keep the influent streams free of oxygen. The sulfide and the carbon dioxide concentration in the biogas never exceeded 0.2%.

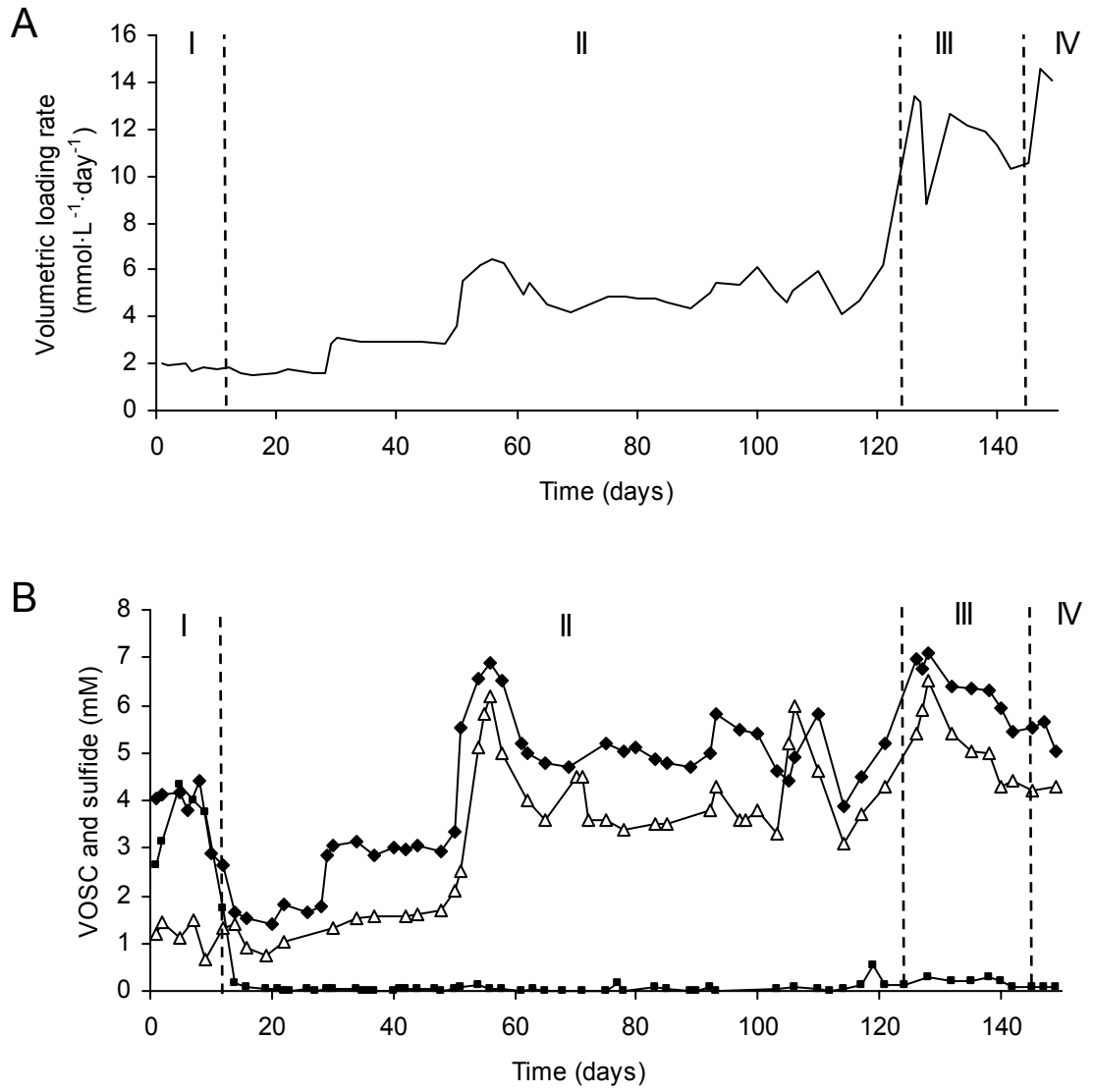

Fig. 4.1. Reactor experiment 1: the applied MT loading rate (A) and MT degradation (expressed as total volatile organic sulfur compounds, VOSC) at 30°C and pH 7.2-7.5 in a 28 g/L NaCl containing medium (B). ♦ Total VOSC influent; ■ total VOSC effluent; Δ sulfide effluent; HRT = 50 h (I), 24 h (II), 12 h (III) and 9 h (IV).

Anaerobic MT degradation at transient NaCl/$NaHCO_3$ concentrations (experiment 2)

The reactor was started up with the adapted sludge from the previous run after it had been stored unfed in the reactor for 2 months at room temperature. At a HRT of 24 h, the MT degrading activity restored within 5 days after startup (Fig. 4.2B). NaCl (28 g/L) that is present in seawater was stepwise replaced by $NaHCO_3$ (40 g/L) because $NaHCO_3$ contributes to the alkalinity of the aqueous solvent. The HRT was stepwise decreased to 9 h (Table 4.1). The applied loading rate varied between 4 and 16 mmol $MT \cdot L^{-1} \cdot day^{-1}$ (Fig. 4.2A), corresponding to specific loading rates of 1-4 mmol $MT \cdot gVSS^{-1} \cdot day^{-1}$ based on the initial VSS content of experiment 2.

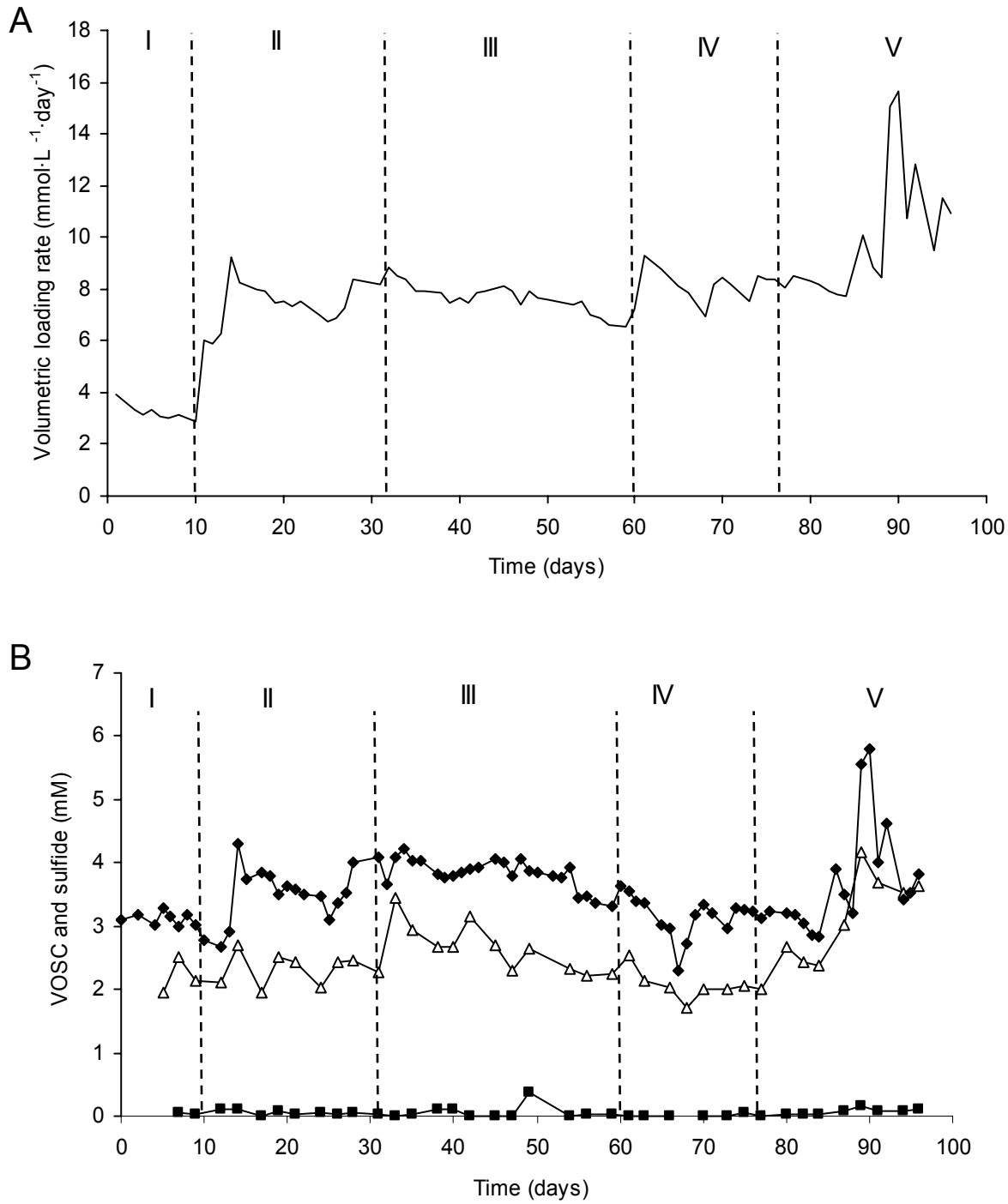

Fig. 4.2. Reactor experiment 2: the applied MT loading rate (A) and MT degradation (expressed as total volatile organic sulfur compounds, VOSC) at 30°C (B). ♦Total VOSC influent; ■ total VOSC effluent; Δ sulfide effluent. Periods are explained in Table 4.1.

Total VOSC concentrations in the effluent did not exceed 0.2 mM (Fig. 4.2B) and the VOSC removal efficiency always exceeded 95%. Besides trace amounts of MT and DMDS, low concentrations of DMS were found in the effluent (< 0.1 mM). Thus, the replacement of NaCl by $NaHCO_3$ in the medium and the simultaneous pH increase from 7.5 to 8.3 did not negatively affect the reactor performance. Washout of sludge via the effluent amounted to 100

mg/L total solids containing 10 mg/L VSS. In the reactor, the biomass was present in agglomerates of about 1-1,500 µm (Fig. 4.3).

The daily biogas production ranged from 50 ml/day in period 1 to 250 ml/day in period V. Fifty to eighty percent of the methane produced at complete MT degradation was recovered. The methane concentration in the biogas varied between 60 and 65%, whilst the remainder was mainly nitrogen gas. Only in period II, the sulfide concentration of the biogas was around 0.3%. Due to the gradual increase in pH of the reactor medium, no H_2S was stripped from the reactor medium into the biogas. The CO_2 concentration of the biogas remained below 1% in periods I and II and increased to 5-6% in periods III and IV and up to 8% in period V. This increase is probably due to the increased $NaHCO_3$ concentration of the influent.

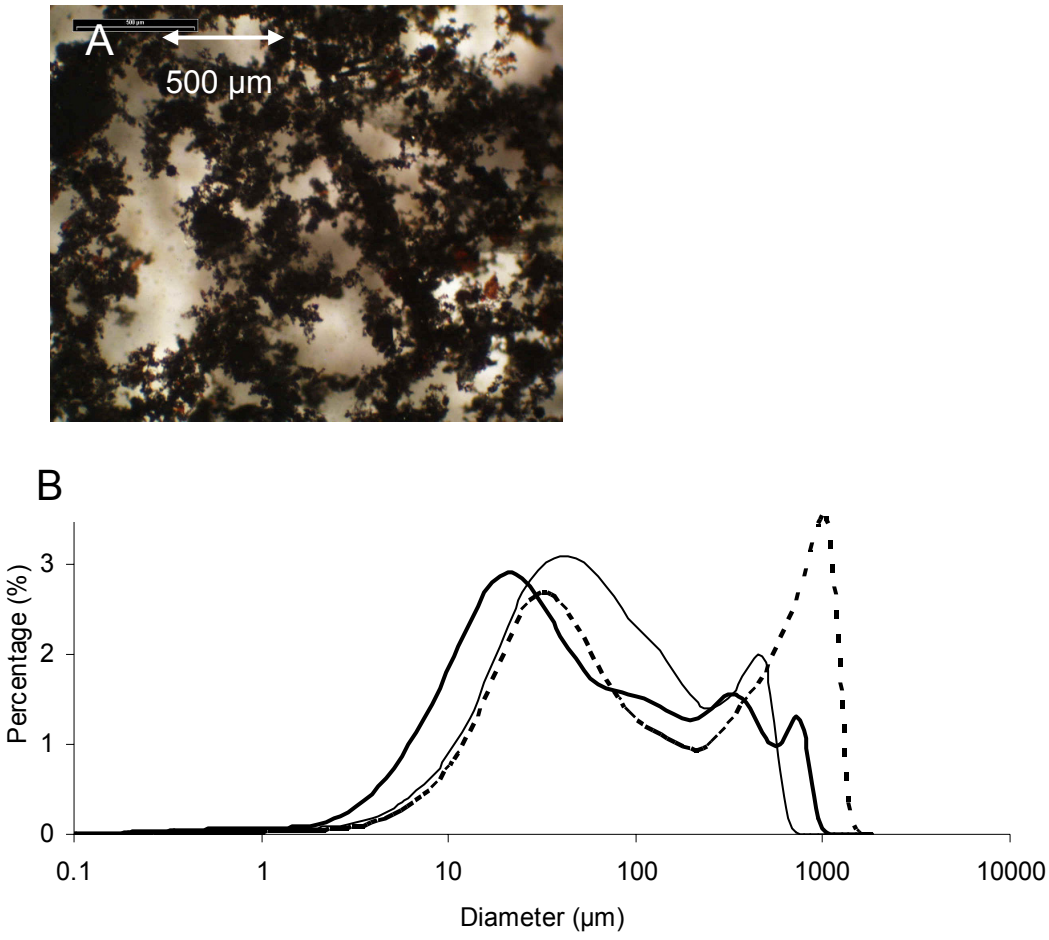

Fig. 4.3. Morphological characterization of the reactor biomass sampled from the reactor at t = 66 days, reactor experiment 2, arrow length = 500 mm (A) and particle size distribution of the reactor sludge at day 117 of experiment 1 (- - -), day 96 of experiment 2 (▬) and day 267 of experiment 3 (─) (B).

Anaerobic MT degradation at a high $NaHCO_3$ levels (experiment 3)

In experiment 3, the reactor was operated at volumetric loading rates between 5 and 20 mmol $MT \cdot L^{-1} \cdot day^{-1}$ for the first 300 days (pH 8.1–8.4), which was comparable to period V of reactor experiment 2. Hereafter, the loading rate was gradually increased to 37 mmol $MT \cdot L^{-1} \cdot day^{-1}$

(Fig. 4.4A). During the last 30 days of experiment 3, the sulfide concentration in the effluent ranged between 7 and 10 mM and the total VOSC concentration between 0 and 0.4 mM (Fig. 4.4B). The removal efficiency of MT approached 100% and more than 80% of the sulfur was recovered as sulfide. The sludge washout during this reactor experiment ranged between 7 and 60 mg TSS/L. The maximum specific degradation rate was 11 mmol MT·g VSS^{-1}·day^{-1} during the last 14 days (based on the VSS content of 5 g at the end of the experiment).

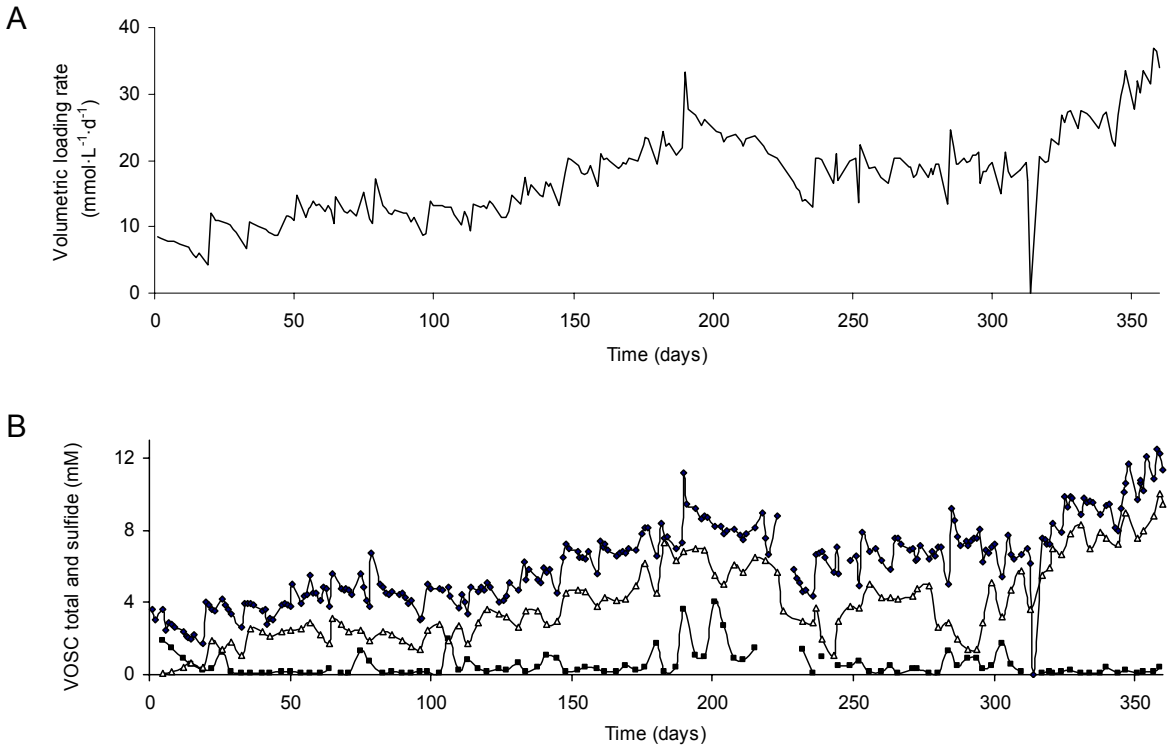

Fig. 4.4. Reactor experiment 3: the applied MT loading rate (A) and MT degradation (expressed as total volatile organic sulfur compounds, VOSC) at 30°C and pH 8.1-8.4 in a 40 g/L $NaHCO_3$ containing medium at a HRT of 9 h (B). ♦ Total VOSC influent; ■ total VOSC effluent; Δ sulfide effluent.

Effect of salinity on the MT degradation rate

The effect of the salt concentration on the specific MT degradation rate by the reactor sludge (batch incubations) was assessed by following the sulfide formation at different NaCl and $NaHCO_3$ concentrations (Fig. 4.5A). No lag phase was observed and complete MT degradation was reached between 6 and 13 days for nearly all salt concentrations tested. An apparent inhibiting effect of salinity was only observed for NaCl and $NaHCO_3$ concentrations above 0.8 M. In batches containing 1.2 M NaCl (70 g/L), only 30% of the MT was degraded within 13 days.

Lag phases for MT unadapted granular sludge varied between 36 and 78 days, depending on the salt concentration (data not shown). However, MT degradation started instantaneous with adapted granular sludge from a lab-scale reactor (chapter 2), except for the

incubations at 0.35 M $NaHCO_3$ (29 g/L), which had a lag phase of 7 days. Complete degradation was reached within 14 days, except for the highest NaCl and $NaHCO_3$ concentrations tested, which took 27-45 days. The degradation rate was inhibited by about 50% at the highest salt concentrations tested (Fig. 4.5B).

The specific sulfide production rate of the adapted granular sludge was 3-7 times higher than the rate of the unadapted granular sludge (Fig. 4.5B), but was still about two orders of magnitude lower than the rate of the sedimentary reactor sludge (Fig. 4.5A).

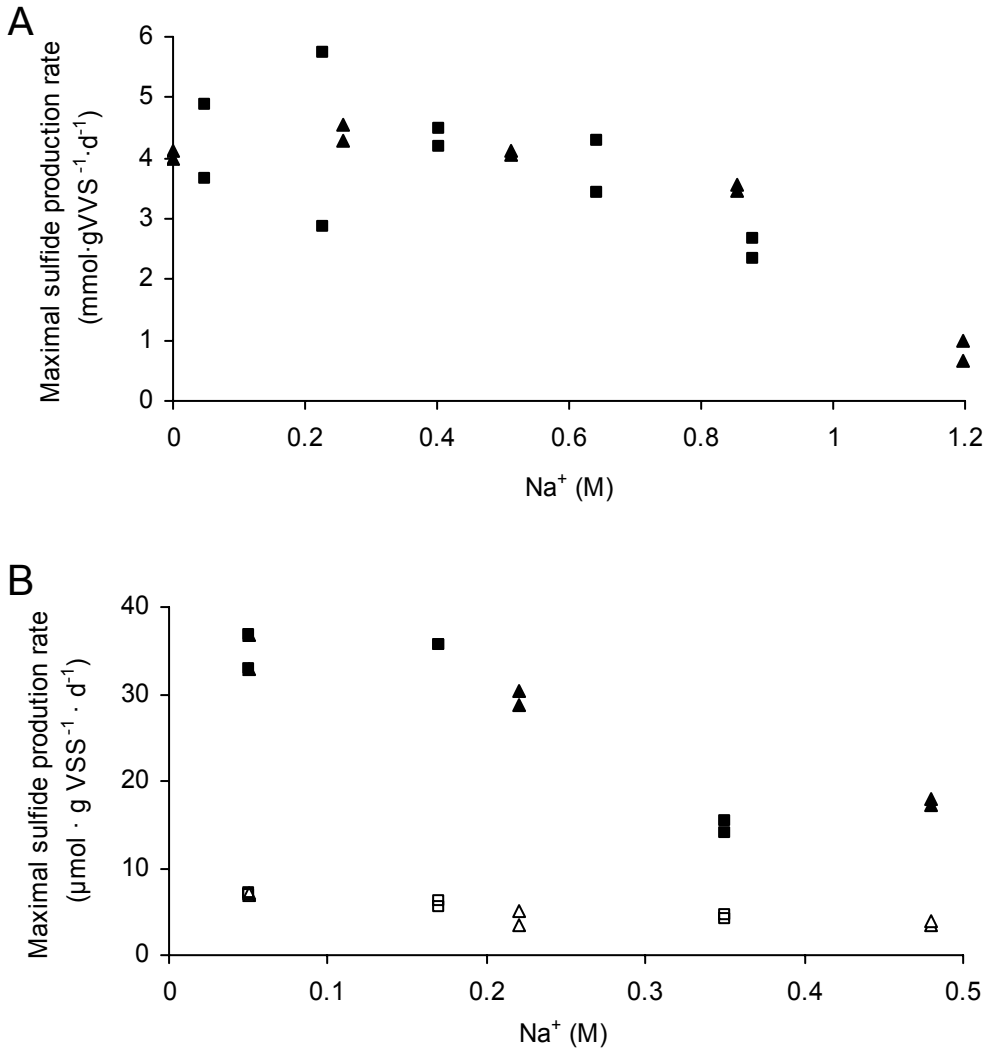

Fig. 4.5. Effect of sodium on the maximal sulfide production rate from MT (2-3 mM) at 30°C. (A) Reactor sludge (sampled at day 117 of the reactor experiment 1). (B) Granular sludge. Conditions: adapted (▲) and unadapted sludge (Δ) with NaCl (pH 7.2) and adapted (■) and unadapted sludge (□) with $NaHCO_3$ (pH 8).

Effect of sulfide on MT degradation

Depending on the initial sulfide concentration, MT was completely degraded between 6 and 15 days by the reactor sludge (Fig. 4.6). MT degradation did not start instantaneously but after a lag phase varying between 4 and 10 days. Therefore, the curves in Fig. 4.6 may represent the

effect of sulfide on the growth rather than on the activity. Inhibition was already observed around 3 mM sulfide and higher initial sulfide concentrations further inhibited the MT degradation rate.

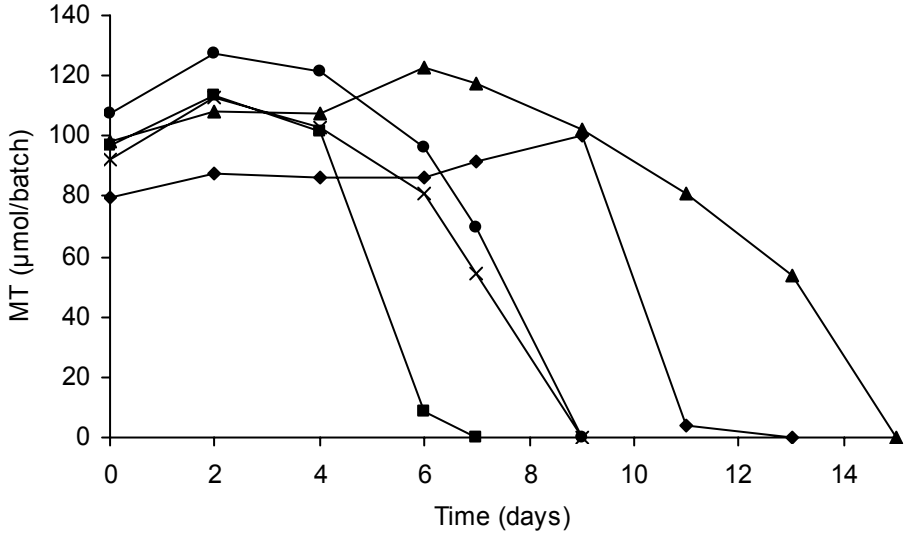

Fig. 4.6. Effect of the sulfide concentration on the lag phase and degradation rate of 2 mM MT (30°C, pH 8.3) by reactor sludge sampled at day 96 of experiment 2. The initial sulfide concentrations were (in mM): 0.5 (■), 2.5 (×), 3.7 (●), 8.8 (♦) and 13.6 (▲).

Discussion

MT degradation at high salinity

This study showed for the first time that anaerobic MT degradation can be applied for biotechnological purposes in a continuous anaerobic reactor operated at relatively high NaCl or $NaHCO_3$ concentrations (0.5 M). In addition, switching from NaCl to $NaHCO_3$ during operation, which coincided with an increase of the reactor pH from 7.5 to 8.3, did not affect the MT removal efficiency (Fig. 4.2). The critical factor enabling this MT degradation at high salt conditions was the inoculum selection. For the reactor sludge, the MT degradation rate at 30°C was fairly constant up to concentrations of 0.8 M NaCl or $NaHCO_3$ (Fig. 4.5A), showing that the Na^+ concentration applied in the reactor experiments (0.5 M) was not inhibiting. In contrast, 50% inhibition of the MT degradation rate by MT-adapted granular sludge, which was not adapted to high salt concentrations [15] (chapter 2) occurred between 0.35 and 0.50 M Na^+ (Fig. 4.5B).

Feijoo *et al.* [25] presented an overview on sodium inhibition in anaerobic digestion. For methanogenesis by reactor sludge from acetate and H_2/CO_2, IC_{50} values were 0.26-0.57 M Na^+ and 0.52 M Na^+, respectively. IC_{50} values reported for methanogenesis in marine sediments from acetate exceed 0.8 M Na^+. These values are in agreement with our findings for methanogenesis from MT by the granular sludge (Fig. 4.5B) and the sedimentary reactor sludge (Fig. 5A).

Continuous exposure to sodium results in an increased tolerance for sodium of the methanogens [25]. Sowers and Gunsalus [26] reported 50% inhibition of the growth rate of *Methanosarcina thermophila* pre-grown in low saline medium at 0.20 M NaCl, whereas the optimum for *M. thermophila* pre-grown in marine medium was 0.60 M NaCl. Adaptation of the granular sludge from the low salinity (~15 mM) MT-fed UASB reactor to higher salt concentrations than presented here (Fig. 4.5B) would be possible, but only after very long operation time.

MT degradation rates

During the consecutive reactor experiments, the MT loading was gradually increased from 2 mmol $MT \cdot L^{-1} \cdot day^{-1}$ (Fig. 4.1A) to 37 mmol $MT \cdot L^{-1} \cdot day^{-1}$ (Fig. 4.4A). The maximal specific sludge loading rate with complete MT degradation was 11 mmol $MT \cdot g\ VSS^{-1} \cdot day^{-1}$. This is higher than values reported for granular UASB sludge, which ranged from 0.33 to 2.8 mmol $MT \cdot g\ VSS^{-1} \cdot day^{-1}$ [5, 15, 16]. The complete degradation suggests that the highest possible MT degradation rate had not yet been reached. In addition, better mixing of substrate and biomass *e.g.* in a gas lift reactor or a sequencing batch reactor [27] could further improve the degradation rate.

The specific MT degradation rates of the reactor sludge in the batch experiments (Fig. 4.5A) were comparable to the specific activity in the reactor. The batch experiments confirmed that the specific MT degradation rate of the sedimentary reactor sludge (Fig. 4.5A) was much higher (two orders of magnitude) than the degradation rate of the granular sludge (Fig. 4.5B). This is because the fraction of *Methanosarcinacaea* in the reactor sludge, the family to which all known MT-degrading methanogens belong, is higher than in the granules (chapter 2 [15] and 5). Furthermore, the sediment particles are smaller than the granules, causing less diffusion limitation of the substrate. Thus, the estuarine sediment is a more promising inoculum source for full-scale applications than the granular UASB sludge, because of its higher specific MT degradation rate as well as its higher salt tolerance.

Characteristics of reactor biomass

Epifluorescence microscopy revealed that the active MT-degrading methanogenic archaea in the reactor sludge were present in aggregates of about 50 mm attached to sediment particles. In chapter 5 it is shown that the dominant MT-degrading methanogens in the reactor sludge were closely related to *Methanolobus taylorii* and *Methanosarcina mazei*, which grow well till 1.2 and 1.0 M NaCl, respectively [28-30].

In thermophilic cultures, adaptation of *M. thermophila* to high sodium concentrations occurs simultaneously with the loss of their capability to produce extracellular polysaccharides and/or disappearance by hydrolysis [26, 31]. As a consequence, the presence of high concentrations of salts stimulates the bacteria to grow individually or in little aggregates instead of forming granules [25]. This is in agreement with our observations (Fig.

4.3A), where no granulation or floc formation occurred during the reactor experiments, but only small aggregates/clusters on sediment particles were formed. The particle size distribution slightly changed during the consecutive reactor runs. Relatively more particles between 50 μm and 500 μm were formed and the big particles (~1,000 mm) disappeared (Fig. 4.3B). Thus, good biomass retention is of crucial importance for proper operation of MT-fed reactors at high salinity. At the MT loading rates adopted in this study, the UASB system was still able to retain the required biomass and biomass washout did not lead to reactor deterioration. The maximal biomass content in the effluent was 10 mg VSS/L. Since the effluent flow varied between 0.8 L/day and 4.3 L/day during the three reactor experiments, this equals a biomass washout of 8-43 mg VSS/d.

The biomass yield can be estimated from the data of Finster *et al.* [9], who found that the MT-degrading strain they isolated produced 3.06 g dry cell mass per mol of MT degraded. The loading rate of our reactors was between 2 and 37 mmol $MT \cdot L^{-1} \cdot day^{-1}$. Using the growth yield factor from Finster *et al.* [9], this equals to a biomass growth of 10-181 mg dry cell mass per day (wet reactor volume is 1.6 L). This indicates that sludge growth normally exceeded sludge washout in our experiments.

Sulfur balance

The anaerobic degradation of 1 mol MT by methylotrophic methanogens would yield 1 mol of sulfide (eqn 1). In general, between 65 and 100% (mol/mol) of the influent MT was recovered as sulfide in the effluent in our experiments and normally less than 10% was still present as VOSC (MT, ethanethiol, DMS, and DMDS), depending on the reactor performance (Fig. 4.1B, 4.2B and 4.4B). Comparable sulfide recoveries were obtained in an anaerobic lab-scale reactor treating a mixture of acetate, sucrose and MT [16]. Some elemental sulfur deposits were formed on the liquid-gas interface of the reactor, for example in period II of reactor experiment 1 (Fig. 4.1B). This may be due to the presence of residual oxygen in the dilution water and air diffusion into the reactor, which caused partial oxidation of sulfide. The produced elemental sulfur could not be measured quantitatively, because part of the sulfur particles adsorbed onto the glass reactor wall. Particularly at high hydraulic retention times and low MT loading rates oxygen ingress might have affected the sulfide recovery. Also other oxidized sulfur species such as sulfate (< 0.15 mM) and polysulfides (< 0.3 mM) were measured in the effluent. The contribution of these compounds to the total sulfur balance is for both estimated between 0 and 5%. Polysulfides can be formed by the reaction between elemental sulfur and sulfide [23]. Some of the sulfide and MT may have ended up in the biogas, due to their volatility. The measured sulfide concentrations in the biogas were below 0.3%. This amounts to a maximum of only 0.1% in the sulfur balance.

Sulfide toxicity

Figure 4.6 shows that sulfide inhibits the MT degradation by the reactor sludge already at concentrations around 3 mM. O'Flaherty *et al.* [32] reported IC_{50} values of total sulfide for the methanogenesis from acetate between 25 and 30 mM for different anaerobic sludge types at pH 8.0 and 8.5. This suggests that MT-degrading methanogens are more sensitive to sulfide toxicity than acetoclastic methanogens. In chapter 3, it is reported that 80% inhibition of the MT degradation rate by anaerobic granular sludge occurs at a total sulfide concentration of 4.3 mM (pH 7.2). The toxic effect of sulfide is believed to be caused by undissociated H_2S, which is uncharged and therefore membrane permeable [33]. This means that the pH plays an important role in sulfide toxicity.

Figure 4.6 suggests that at the sulfide concentration in the reactors (1-10 mM, Fig. 4.1, 4.2 and 4.4) inhibition of MT degradation could already occur. However, even in experiment 3 (Fig. 4.4B) where 10 mM sulfide was formed in the reactor, no inhibition of MT degradation was observed.

Sipma *et al.* [5] operated a UASB reactor (pH 7.5, 30°C) for anaerobic MT degradation, inoculated with granular sludge. At an influent concentration of 6 mM MT and 6 mM sulfide, MT was almost completely degraded, resulting in sulfide concentrations up to 12 mM in the reactor. However, an increase to 8 mM sulfide in the influent caused a drop in MT degradation efficiencies to 50% or lower in the presence of 10-12 mM sulfide in the reactor.

Conclusion

This research showed that complete anaerobic MT degradation is feasible in continuously operated reactors at sodium concentrations of 0.5 M at a volumetric loading rate of 37 mmol $MT \cdot L^{-1} \cdot day^{-1}$, whilst sulfide inhibition started already around 3 mM (both at pH 8.3). Sodium levels exceeding 0.8 M inhibited MT degradation by the sedimentary reactor sludge. In the developed LPG desulfurization process, sodium concentrations of at least 0.5 M are expected. Thus, the estuarine sediment investigated is a promising inoculum for full-scale application.

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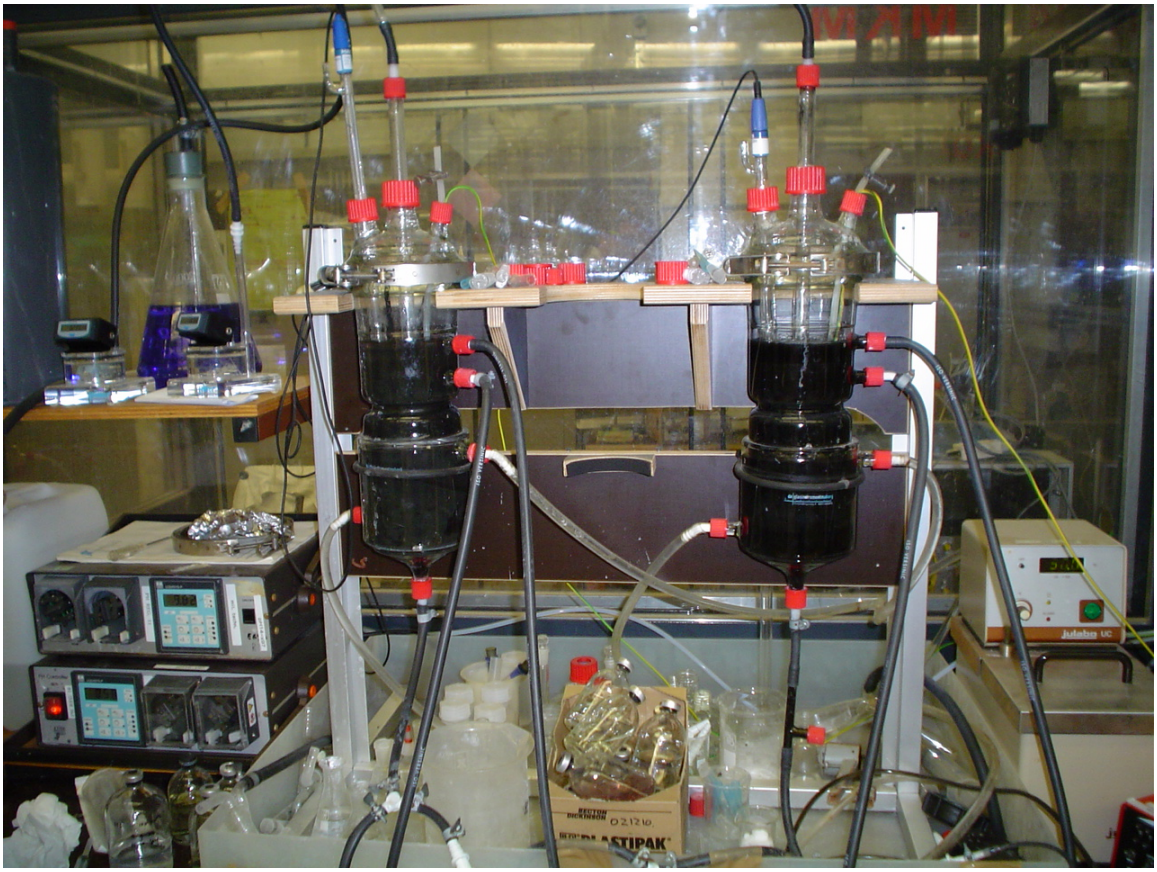

Photograph depicting the lab-scale UASB reactors used for the experiments as described in the chapters 2, 4, 5 and 6.

5

Methanethiol degradation in anaerobic bioreactors at elevated pH (≥ 8) : reactor performance and microbial community analysis*

Abstract

The degradation of methanethiol (MT) at 30°C under saline-alkaline (pH 8 - 10, 0.5 M Na^+) conditions was studied in a lab-scale Upflow Anaerobic Sludge Blanket (UASB) reactor inoculated with estuarine sediment from the Wadden Sea (The Netherlands). At a sodium concentration of 0.5 M and a pH between 8 and 9 complete MT degradation to sulfide, methane and carbon dioxide was possible at a maximum loading rate of 22 mmol MT · L^{-1} · day^{-1} and a hydraulic retention time of 6 hours. The presence of yeast extract (100 mg/L) in the medium was essential for complete MT degradation. 16S rRNA based DGGE and sequence analysis revealed that species related to the genera *Methanolobus* and *Methanosarcina* dominated the archaeal community in the reactor sludge. Their relative abundance fluctuated in time, possibly as a result of the changing operational conditions in the reactor. The most dominant MT-degrading archaeon was enriched from the reactor and obtained in pure culture. This strain WR1, which was most closely related to *Methanolobus taylorii,* degraded MT, dimethyl sulfide (DMS), methanol and trimethylamine. Its optimal growth conditions were 0.2 M NaCl, 30°C and pH 8.4. In batch and reactor experiments operated at pH 10, MT was not degraded.

* This chapter has been submitted for publication

Introduction

Liquefied petroleum gas (LPG) contains various volatile sulfur compounds of which H_2S, methanethiol (MT), ethanethiol (ET) and propanethiol (PT) are the most predominant ones. To desulfurize LPG a three-step integrated process was described [1] that involves: (i) extraction of thiols and H_2S from the LPG phase to a carbonate solution; (ii) anaerobic degradation of thiols to H_2S, CH_4 and CO_2 and (iii) partial oxidation of H_2S to elemental sulfur. The alkaline aqueous solvent is continuously recycled between the bioreactor and the extraction column.

This research focuses on the anaerobic degradation step of MT under alkaline conditions, because the oxidation of H_2S to elemental sulfur at saline-alkaline conditions has already been studied extensively [2, 3]. Since we found in preliminary experiments that ET and PT are not degraded under anaerobic conditions [4] (chapter 3), this compound was not considered in the present study.

Anaerobic MT degradation has been reported for marine and freshwater sediments, and has always been associated with dimethyl sulfide (DMS) degradation. Both methanogenic archaea and sulfate reducers may be responsible for MT degradation [5]. Methanogens appeared to be the most important MT-degrading anaerobes. Finster *et al.* [6] described a marine methanogen capable of growing on MT as the sole energy source and proposed the following stoichiometry for the reaction:

$$4\ CH_3SH + 3\ H_2O \rightarrow 3\ CH_4 + HCO_3^- + 4\ HS^- + 5\ H^+ \qquad \Delta G^{0'} = -51.0\ kJ{\cdot}mol^{-1}\ CH_4 \qquad (1)$$

Several methanogens capable of MT and DMS degradation are available in pure culture. They all belong to the *Methanosarcinaceae* genera *Methanosarcina, Methanolobus, Methanosalsus* or *Methanomethylovorans* [6-10].

Previous research showed that it is possible to degrade MT in a mesophilic (30°C) continuously operated anaerobic lab-scale reactor inoculated with Wadden Sea sediment (The Netherlands) (chapter 4) [11]. The goal of the present research is to assess the anaerobic degradation of MT at pH values of at least 8.0 and at 0.5 M sodium. The MT-degrading microbial community in the reactor sludge is studied as well.

Materials and methods

Continuous UASB reactor

Two reactor experiments were carried out using a continuously operated mesophilic (30°C) lab-scale UASB reactor (1.6 L) as described in chapter 4. The reactor was fed with an oxygen-free synthetic influent containing 2-7 mM MT by diluting an 80 mM MT stock solution with a solution containing micro and macro nutrients (chapter 4). The influent flow was approximately 4.3 L/day and the superficial liquid upward velocity was controlled at 1.5 m/h by external recirculation. The pH of the reactors was maintained between 8.2 and 8.4 by using a 40 g/L $NaHCO_3$ buffer. Sufficient alkalinity was present making pH control unnecessary.

Experiment 1 was divided in six periods. Period I (0-65 days) can be characterized as the startup period; in period II (65-108 days) the MT loading rate was gradually increased; in period III (108-125 days) yeast extract was excluded from the reactor medium, whereas it was supplied again from period IV (125-142 days) onwards till the end of the experiment. In period V (142-175 days) the hydraulic retention time (HRT) was decreased from 9 to 6 hours and in period VI (175-185 days) the reactor pH was increased from 8.2-8.4 to 8.9-9.1 by partially replacing $NaHCO_3$ by Na_2CO_3.

In experiment 2, the reactor was started up at pH 8.3 and a HRT of 9 hours (period I). The pH was increased to 10 by changing the buffer composition to 7 g/L $NaHCO_3$ and 23 g/L Na_2CO_3 between day 15 and 25 (period II) and was reduced to pH 8.3 again for the remainder of the experiment (period III).

Sediment characteristics

The reactor was originally inoculated with an estuarine sediment (Wadden Sea, Texel, The Netherlands) in a series of previous experiments (chapter 4). At the start of the current experiments, this reactor sludge had a MT-degrading capacity as it was exposed to MT for a prolonged period (> 200 days) (chapter 4). Since the reactor sludge mainly consisted of sand, the VSS (volatile suspended solids) content of the dry material was only 10% (5 grams). The particle size distribution of the inoculum sludge ranged from 1 µm to 1500 µm. At several time points samples were taken from the reactor sludge for molecular characterization (Table 5.2). Samples from previous reactor experiments (chapter 4), in which MT was degraded between pH 7.2 and 8.4, were also included.

Batch incubations

Batch incubations were performed to study MT degradation at pH 8 and 10, as well as to enrich, isolate and physiologically characterize the predominant MT-degrading microorganisms (see following three paragraphs).

MT degradation at pH 8 and 10

Serum flasks (120 ml) were filled with 50 ml (bi)carbonate buffered medium and trace elements according to De Bok *et al.* [12] and subsequently incubated with reactor sludge samples (0.4 g wet sediment, resulting in 16 mg VSS per flask). The flasks were sealed with viton stoppers and pressured to 1.7 bars with N_2/CO_2 (80:20). The flasks at pH 8 contained 40 g/L $NaHCO_3$, while the composition of the buffer was changed to 23 g/L Na_2CO_3 and 7 g/L $NaHCO_3$ to obtain pH 10. Na_2S (1 mM) was added to reduce the medium and MT was added from a concentrated stock solution (0.5 M $NaSCH_3$). MT degradation (3 mM, duplicates at 30°C) was monitored by measuring the sulfide concentration in the liquid phase.

Enrichment and isolation

Enrichment of MT-degrading microorganisms was started by inoculating 10 ml reactor sludge (sampled at day 134 of reactor experiment 1) under a N_2 headspace (1.7 bars). Dilution series up to a dilution of 10^{-9} were prepared in fresh medium from this sludge material. The anaerobic medium (MW2) contained: 0.2 g/L K_2HPO_4, 0.3 g/L NaCl, 0.1 g/L $MgCl_2 \cdot 6H_2O$, 0.77 g/L KCl, 0.11 g/L $CaCl_2 \cdot 2H_2O$, 40 g/L $NaHCO_3$, 0.3 g/L NH_4Cl, 0.1 g/L yeast extract, $5 \cdot 10^{-3}$ g/L resazurin, 0.1 g/L biotrypcase peptone and trace elements [12]. Enrichment and dilution series in MW2, to which in addition to MT (2 mM) usually also DMS (1 mM) and methanol (10 mM) were added, yielded two pure cultures (strains WR1 and AWS1). Purity of the cultures was tested by growing the isolated strains in basal medium (with and without methanol) supplemented with yeast extract (1 g/L) and glucose (20 mM) and by checking for contaminants by microscopy.

Physiological characterization

Physiological characterization was performed in anaerobic medium (HA2) containing: 1.0 g/L K_2HPO_4, 28.9 g/L NaCl, 0.045 g/L $MgSO_4 \cdot 7H_2O$, 0.1 g/L $MgCl_2 \cdot 6H_2O$, 0.045 g/L Na_2SO_4, 0.01 g/L $CaCl_2 \cdot 2H_2O$, 8.4 g/L $NaHCO_3$, 0.27 g/L NH_4Cl, 0.1 g/L yeast extract, 0.1 g/L biotrypcase peptone and trace elements [12]. This medium, with 20 mM methanol as substrate and pressurized with N_2 (1.7 bars) was used to determine the optimal pH, temperature and NaCl concentration. Growth rates were determined by measuring the amount of methane formed for cultures that were adapted to the specific conditions. To obtain a pH range between 7 and 9, $NaHCO_3$ and Na_2CO_3 were added in different ratios. Growth of strain WR1 on MT (2 mM), DMS (3 mM), trimethylamine (10 mM), methylamine (10 mM), acetate (10 mM), formate (10 mM) and hydrogen (H_2/CO_2 80:20) was tested at 0.2 M NaCl and pH 8.4.

DNA extraction, 16S rRNA gene amplification and DGGE analysis

Sludge samples withdrawn from the reactor (Table 5.2) were fixed in 60% (v/v) ethanol containing 25 mM NaCl. DNA extraction, 16S rRNA gene amplification (Table 5.1) and DGGE analysis were done as described previously [19] (chapter 2).

Table 5.1. PCR-primers used for DGGE-analysis, ARDRA, cloning and sequence analysis (SA). Primers marked with an asterisk contained the 40bp GC-clamp '5-CGCCCGGGGCGCGCCCC GGGCGGGGCGGGGGCACGGGGGG-3' when used for DGGE analysis.

Primer	Sequence ('5 → '3)	Application(s)	Reference(s)
Arch-109(T)f	ACTGCTCAGTAACACGT	DGGE, SA	[13, 14]
Uni-515r*	ATCGTATTACCGCGGCTGCTGGCAC	DGGE, SA	[14, 15]
Bac-968f*	GAACGCGAAGAACCTTAC	DGGE, Cloning	[16]
Bac-1401r	CGGTGTGTACAAGACCC	DGGE, Cloning	[16]
Arch-109f	AC(G/T)TGCTCAGTAACACGT	Cloning	[13]
Uni-1492r	CGGCTACCTTGTTACGAC	Cloning	[17]
Pg1	TGGCGGCCGCGGGAATTC	ARDRA	Promega
Pg2	GGCCGCGAATTCACTAGTG	ARDRA	Promega
T7	AATACGACTCACTATAGG	SA	Promega
Sp6	ATTTAGGTGACACTATAG	SA	Promega
Uni-519f	CAGC(A/C)GCCGCGGTAA(G/A/T/C)(A/T)C	SA	[15]
Arch-915r	GTGCTCCCCCGCCAATTCCT	SA	[18]

Table 5.2. Sludge samples from the lab-scale UASB reactors for molecular characterization of the archaeal community during the consecutive reactor experiments.

Sampling time (day)	Sample number in Fig. 5.4	pH	Sodium (M)	Reference
0 117	2 3	7.3-7.5	0.5 NaCl	Chapter 4
96	4	7.3-8.3	0.5 NaCl or $NaHCO_3$	Chapter 4
38 134 171 185	5 6 7 8	8.2-9.1	0.5 $NaHCO_3$	This chapter: Reactor experiment 1 (Fig. 5.1)
51 118 176 204 267 317 353	9 10 11 12 13 14 15	8.1 - 8.4	0.5 $NaHCO_3$	Chapter 4
32	16	8.3-10.0	0.5 $NaHCO_3$/Na_2CO_3	This chapter: Reactor experiment 2 (Fig. 5.3)

Cloning and sequencing

Cloning and sequencing of 16S rRNA gene amplicons was done according to De Bok *et al.* [19] (chapter 2). The pGEM®-T Easy vector system (Promega, Madison, MI, USA) was used to transform the 16S rRNA gene amplicons into *E. coli* and select for positive clones using ampicillin selection and blue/white screening. Clones were screened by amplified ribosomal DNA restriction analysis (ARDRA), using restriction enzymes *Msp*I, *Cfo*I and *Alu*I (Promega, Madison, USA). Sequence analysis was performed using pGEM®-T vector-targeted sequencing primers Sp6 and T7 and 16S rRNA-gene-targeted internal primers Uni-519f and Arch-915r (Table 5.1).

Analytical techniques

Volatile organic sulfur compounds (VOSC) were analyzed by high performance liquid chromatography (HPLC) as described in chapter 3. Total VOSC concentrations, *i.e*. the sum of MT, ET, DMS, DMDS, were used instead of only MT for expressing the MT degradation, as MT readily autooxidizes to DMDS, and other VOSC might be formed from MT as well. Autooxidation may, to a small extent, already take place in the concentrated stock solution and during the sampling procedure. Total and volatile suspended solids were analyzed according to Standard Methods [20]. Sulfide was measured photometrically [21]. Biogas composition (CH_4, CO_2, N_2, H_2S) was analyzed by gas chromatography (chapter 4) [11]. Microscopic analysis of sediment samples was performed with an Olympus BH2 epifluorescence microscope and an Olympus SZ40 stereo microscope.

Chemicals

All chemicals used were of analytical grade and supplied by Merck (Darmstadt, Germany). A sodium mercaptide solution (3 M) of analytical grade was supplied by the Arkema Group (Rotterdam, The Netherlands).

Nucleotide sequence accession numbers

The nucleotide sequences obtained in this study have been deposited in GenBank (accession no. EF376986 to EF376991).

Results

Methanethiol degradation at a pH between 8 and 9

The continuously operated Upflow Anaerobic Sludge Bed (UASB) reactor (experiment 1) was loaded at rates between 5 to 22 mmol $MT \cdot L^{-1} \cdot day^{-1}$ throughout the 185 days of operation (Fig. 5.1A). The MT influent concentration varied between 2 and 7 mM. From day 2 onwards, sulfide was detected in the effluent, indicating that MT degradation had started (Fig. 5.1B). However, degradation was incomplete during the first 34 days of the experiment; the total concentration of volatile organic sulfur compounds (VOSC) in the effluent ranged from 0.02 to 1 mM. Besides MT also trace amounts of dimethyl disulfide (DMDS) and DMS were detected in the effluent.

At day 23, a sharp increase in the MT loading rate immediately caused an increase in sulfide formation. The increase in the loading rate at day 36, however, resulted in a higher total VOSC concentration in the effluent. At that day, the DMDS fraction in the concentrated MT stock solution had reached 40% (mol/mol) of the total sulfur concentration due to autooxidation of MT with oxygen as a result of some air ingress in the flask of the MT stock solution in the foregoing period. This resulted in 1 mM DMDS and 3 mM MT in the influent. It is likely that this DMDS inhibited the MT and total VOSC degradation, as DMDS was shown previously to be inhibitory to MT degradation (chapter 3) [4]. The reactor performance recovered as soon as the stock solution was refreshed on day 40.

From day 65 till day 108 (period II), the MT influent concentration was gradually increased from 2.0 to 6.3 mM, which was followed by increases in the effluent sulfide concentration. The total VOSC concentration in the effluent never exceeded 1 mM (Fig. 5.1B), and therefore the VOSC removal efficiency was normally 90% or higher (Fig. 5.1C).

At day 108, yeast extract was excluded from the dilution medium to study its role in the MT degradation (period IV). This immediately resulted in a rapid decrease in the MT removal efficiency from almost 100% to less than 30%, and high VOSC effluent concentrations were measured (1.7-4.3 mM) (Fig. 5.1B and C). Upon readdition of yeast extract to the influent at day 125, the MT degradation efficiency restored to values exceeding 90% within 3 days (Fig. 5.1B and C, period IV).

From day 142 onwards (period V), the hydraulic retention time (HRT) was decreased from 9 to 6 hours. The volumetric loading rate was kept between 14 and 22 mmol $MT \cdot L^{-1} \cdot day^{-1}$ (Fig. 5.1A). Under these conditions MT was almost completely removed (Fig. 5.1B and C). Residual oxygen in the dilution water caused the partial oxidation of some sulfide to elemental sulfur, which deposited on the liquid-gas interface of the reactor.

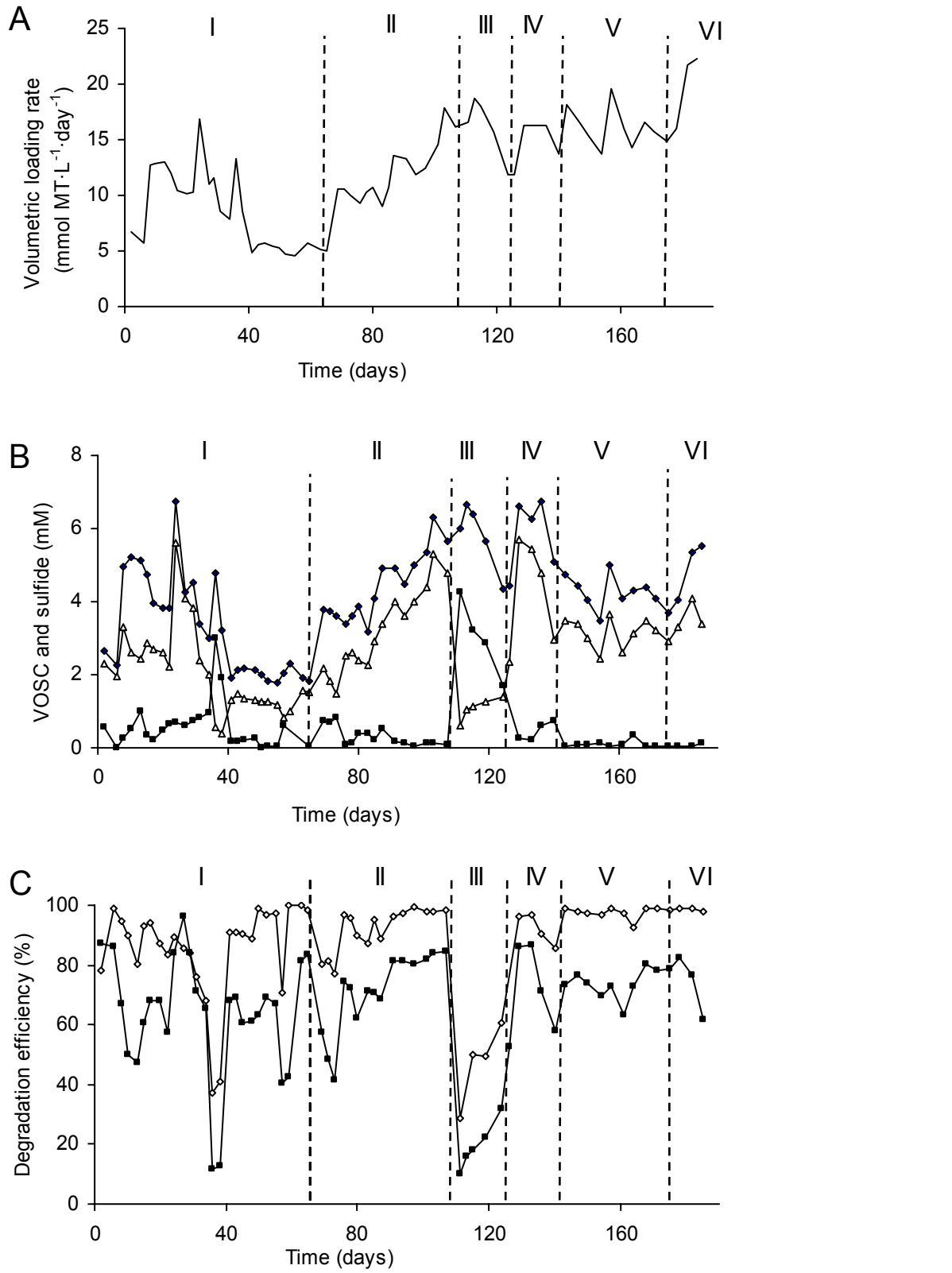

Fig. 5.1. Reactor experiment 1. Volumetric loading rate (A), anaerobic degradation: total VOSC (mainly MT) influent (♦); total VOSC effluent (■); sulfide effluent (Δ) (B) and degradation efficiency of methanethiol in the lab-scale UASB reactor (C) calculated based on total VOSC (◊) and sulfide (■) in the effluent.

From day 175 onwards (period VI), the reactor pH was increased to 8.9-9.1. Under these conditions, MT was degraded almost completely. The highest specific MT degradation rate measured in this experiment was 7 mmol MT·gVSS^{-1}·day^{-1} in period VI. During the entire experiment, the MT removal efficiency, in general, exceeded 90% (Fig. 5.1C) with a few exceptions. The degradation efficiency of MT, based on the measured sulfide concentration in the effluent, was normally between 70 and 80%.

The biogas production ranged from about 50 ml·day^{-1} at a loading rate of 5 mmol MT·L^{-1}·day^{-1} (period I) to 300 ml·day^{-1} during the highest loading rates (periods V and VI). The methane concentration in the biogas varied between 60 and 70%. The carbon dioxide concentration was around 5% till period V, but decreased to 1% during period VI, due to the increase of pH of the reactor medium (from 8.3 to 9.0). The remainder of the gas was nitrogen, as all the influent flows were kept anaerobically by flushing them with nitrogen gas. Sulfide could not be detected in the biogas by gas chromatography. However, traces of zinc sulfide accumulated in a wash bottle when the biogas was led through a zinc acetate solution, indicating that the biogas contained traces of sulfide.

Methanethiol degradation at pH 10

The possibility of anaerobic MT degradation at pH 10 was examined in batches inoculated with reactor sludge sampled at day 171 of experiment 1. As a control, the degradation was also tested at pH 8. No MT degradation occurred within 63 days at pH 10 (Fig. 5.2), while at pH 8 MT (3 mM) was stoichiometrically degraded to sulfide (eqn 1) between day 10 and 19.

The possibility to degrade MT at pH 10 was also studied in the UASB reactor. The reactor was started up at pH 8.3 at an MT influent concentration of 2.5 mM, which corresponds to a volumetric loading rate of 6 mmol MT·L^{-1}·day^{-1} (Fig. 5.3). The MT influent concentration was increased to 4 mM (11 mmol MT·L^{-1}·day^{-1}) when complete degradation was achieved. At day 15, the pH was subsequently increased to 10. MT degradation continued for 3 days, but was completely inhibited thereafter, as illustrated by the increased VOSC concentration and decreased sulfide concentrations in the effluent. At day 26, the pH was restored to 8.3. MT degradation resumed after 6 days, indicating that the pH increase did not inhibit the MT degradation irreversibly.

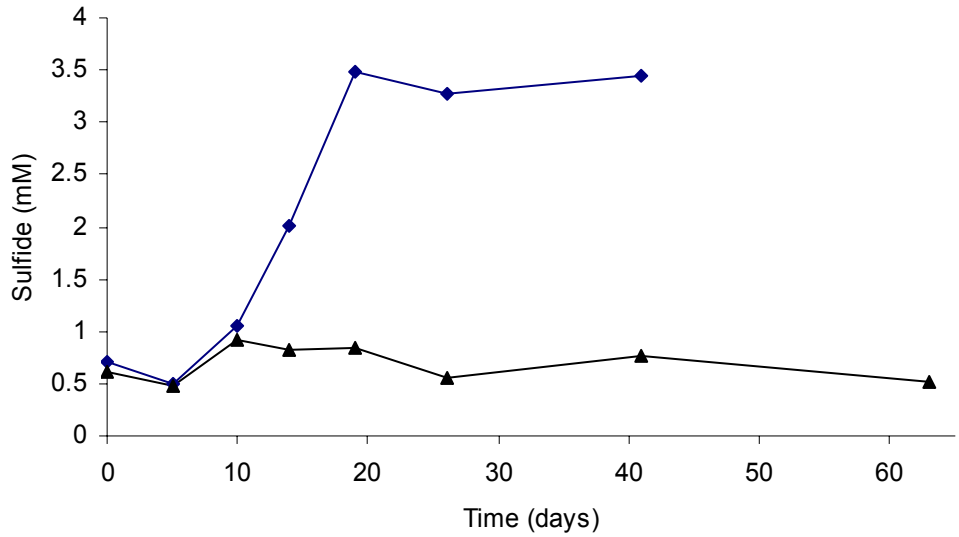

Fig. 5.2. Average sulfide formation from the degradation of 3 mM MT at pH 8 (♦) and 10 (▲) by Wadden Sea sediment (30°C) in duplicate incubations.

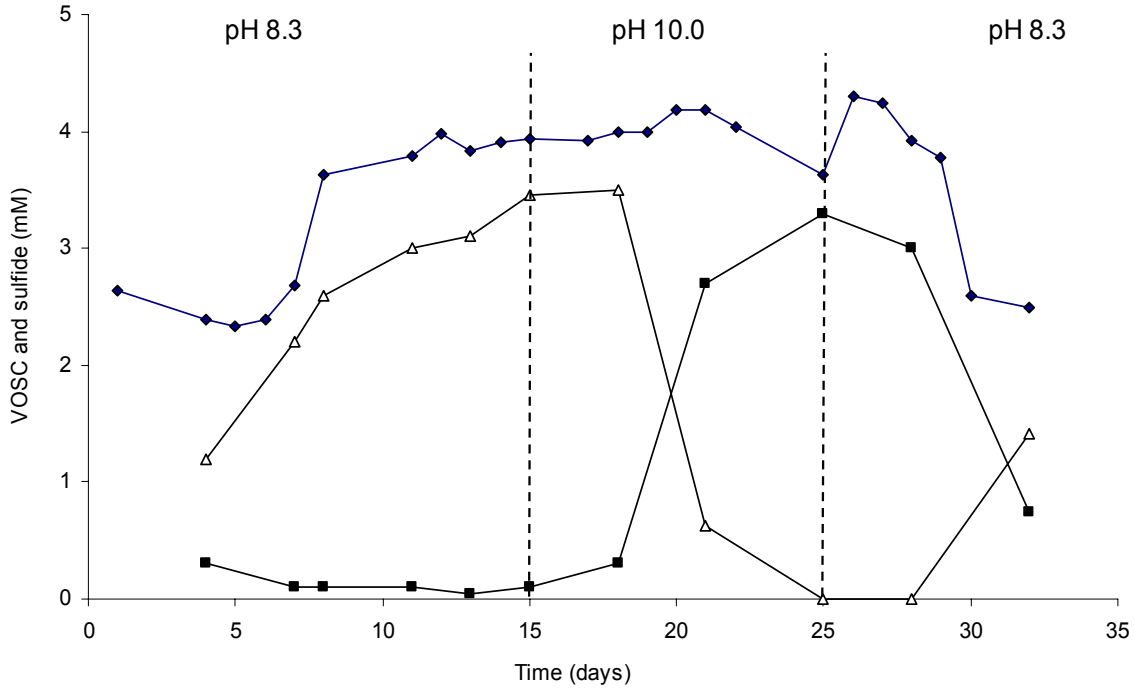

Fig. 5.3. Effect of the pH change from 8.3 to 10.0 and back to 8.3 on MT degradation in the reactor inoculated with Wadden Sea sediment at 30°C and a HRT of 9 hours. VOSC influent (♦); VOSC effluent (■); sulfide effluent (Δ).

Isolation and physiological characterization of dominant microorganisms

MT-degrading archaea were enriched from the reactor sludge as well as from the original estuarine sediment by serial dilutions. Strain AWS1 was isolated directly from the estuarine sediment in a medium at pH 8.8. Strain WR1 was isolated from the reactor sludge in pH 8.3 medium. The following criteria were used to determine whether the cultures were pure: i) all

cells observed in the cultures had the same morphology, ii) all cells produced autofluorescence when exposed to UV light, iii) PCR-DGGE using archaea specific primers yielded only one single band, iv) no product was obtained when DNA extracted from the culture was amplified with bacteria specific primers, v) points raised above were also true for cultures to which glucose (20 mM) or yeast extract (g/L) were added. Both strains were similar in morphology: sarcina-shaped cells that tended to aggregate. Since 16S rRNA sequences of the isolated strains were 100% similar, only strain WR1 was used for further characterization. Growth of strain WR1 on methanol occurred between 0 - 0.9 M NaCl, 10 - 40°C and pH 7-9. Optimal growth was found at 0.2 M NaCl, 30ºC and pH 8.4. Besides MT, DMS and methanol, this strain also grew on methylamine and trimethylamine, but no growth occurred on acetate, formate or H_2/CO_2. The strain required yeast extract for growth and the closest phylogenetic relative (cultured validated microorganism) was *Methanolobus taylorii*.

Community analysis of the sediment samples

The archaeal community of the reactor biomass from the current experiments (Fig. 5.1 and 5.3) and previous research (Table 5.2) (chapter 4) was analyzed by DGGE analysis, cloning and sequencing of 16S rRNA genes extracted from the samples (Fig. 5.4, Table 5.3). The methanogen that was isolated from the reactor (strain WR1) was present in the reactor throughout the course of the experiment, as was confirmed by sequencing of the corresponding amplicon from a DGGE gel. 16S rRNA gene clone libraries (V2-V8, Arch-109f and Uni-1492r) from reactor sludge samples 6, 9 and 16 (Table 5.3) showed that strain WR1 dominated the archaeal community of the reactor throughout the experiments. 16S rRNA genes related to other methanogenic archaea were detected in the reactor as well. Initially an archaeon related to *Methanosarcina mazei* (98.8%) was present in the reactor sediment (Fig. 5.4, band e), but eventually it was not detected anymore in the clone libraries and DGGE gels. At the end of the reactor experiments (Fig. 5.4, no 16; Table 5.3), 17% of the clones (4 out of 24) from the corresponding clone library contained 16S rRNA genes related to sequences of the hydrogenotrophic methanogens *Methanobrevibacter* and *Methanocalculus*.

Table 5.3. Identity of archaeal 16S rRNA genes retrieved from the lab-scale MT-fed reactor and percentage similarity to the closest related sequence in the NCBI database. a-j, positions of the amplicons in the DGGE-gel (Fig. 5.4); N, not identified in the DGGE-gel; **1.** Length of sequence used for homology search. Sequences were determined by cloning and sequencing of 16S rRNA genes, except for sequences less than 600 nucleotides in length. These were obtained by sequence analysis of amplicons excised from DGGE gels.
2. Number of base pairs used in alignment is indicated between brackets **3.** Abundance of 16S rRNA genes in clone libraries prepared from DNA isolated from the reactor sludge at three different time points (see Table 5.2 and Fig. 5.4) **4.** Out of 28 clones (time point 16) 4 were identified as chimeras using the option Chimera Check of the Ribosomal Database Project (http://35.8.164.52/html/index.html).

Position	Size[1]	Identity Genbank (Accession nr.)	Similarity[2] (%)	Abundance[3]		
				6	9	16
a	1364	*Methanolobus taylorii* (U20154)	98.9 (1329)	8/24	18/24	15/28
b	294	Uncultured archaeon clone WN-FWA-144 (DQ432527)	97.8 (279)			
		Methanolobus oregonensis (U20152)	97.5 (279)			
c	339	*Methanolobus taylorii* (U20154)	97.4 (309)			
d	311	Uncultured archaeon clone EV818FW051001BH4MD67 (DQ118545)	96.9 (286)			
		Methanolobus oregonensis (U20152)	94.7 (302)			
e	1368	Uncultured archaeon HDBW-WA05 (AB237738)	99.6 (1343)	7/24	1/24	-
		Methanosarcina mazei (AB065295)	98.8 (1363)			
f	1361	Uncultured euryarchaeote clone:KuA13 (AB077223)	98.5 (1354)			2/28
g	1367	*Methanobrevibacter arboriphilus* (AB065294)	100 (1364)			2/28
h	1364	*Methanolobus taylorii* (U20154)	98.9 (1330)			2/28
j	1362	*Methanolobus taylorii* (U20154)	95.0 (1293)			1/28
	888	*Methanocalculus chunshungensis* (AY23433)	99.3 (714)			2/28
i, j, N		Chimeras[4]				4/28

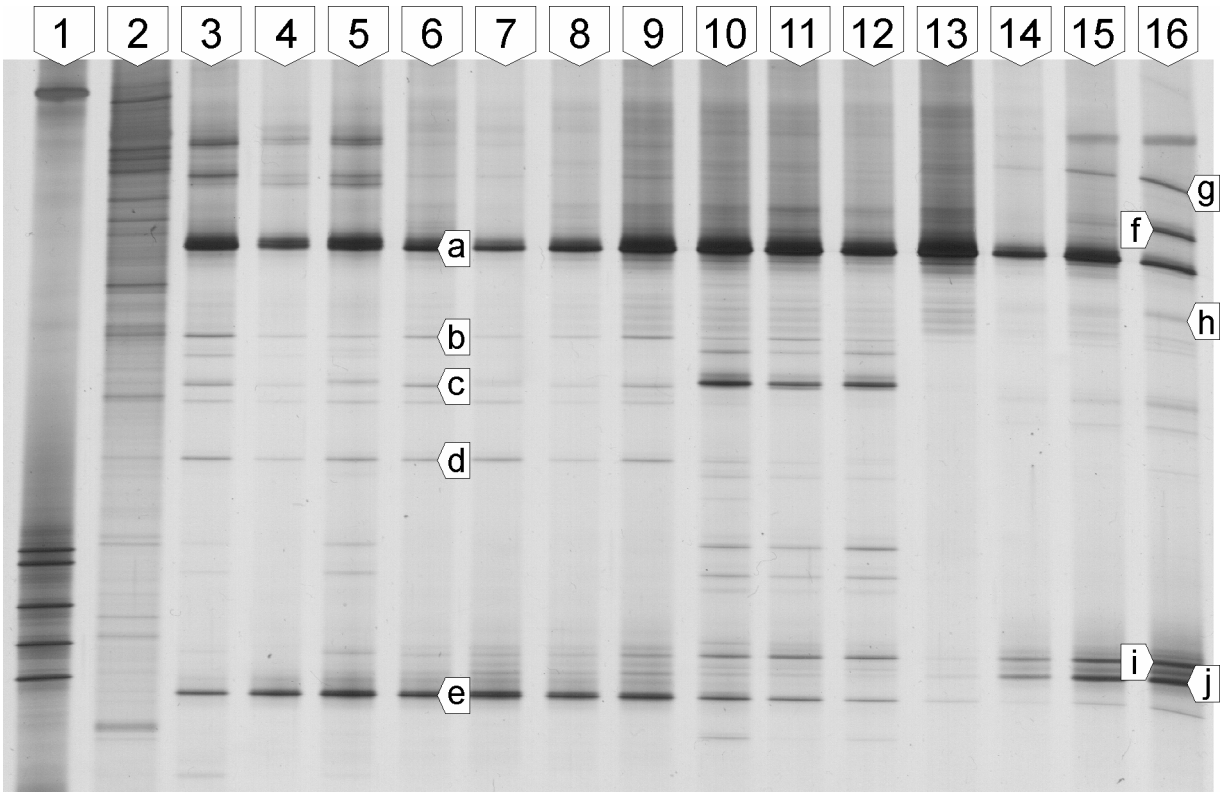

Fig 5.4. DGGE fingerprints of the 16S rRNA (V2-V3) from the archaeal communities in reactor sediment samples. DGGE marker (1); original sediment (2); sediment samples from the consecutive reactor experiments of chapter 4 and the current research as described in Table 5.2 (3-16). Letters are determined sequences, which are depicted in Table 5.3.

Discussion

Effect of pH and salt on MT degradation

This study shows that MT is degraded anaerobically in a continuously operated reactor inoculated with estuarine sediment from the Wadden Sea (The Netherlands) at 0.5 M Na^+ and pH values between 8 and 9 (Fig. 5.1), but not at pH 10 (Fig. 5.3). This is in agreement with the characteristics of the MT-degrading methanogen, strain WR1, isolated from the reactor, which had a pH optimum of 8.4, while it did not grow at pH 10. This is also consistent with the characteristics of the closest related organism, *Methanolobus taylorii* [22, 23]. Previous research showed that MT degradation in the same reactor also occurred at lower pH values, between 7.2 and 8.4 (chapter 4). Thus, the present research shows that the MT degradation range of the reactor sludge, based on Wadden Sea sediment, is between 7.2 and 9.1. MT degradation in a reactor inoculated with granular sludge was only successful between pH 7.2 and 7.5 (chapter 2). The pH increase from 8.3 to 10.0 for 10 days in reactor experiment 2 (Fig. 5.3) completely inhibited MT degradation and resulted in a shift in the microbial population. Part of the MT-degrading *Methanosarcinaceae* population disappeared from the reactor sludge (Table 5.3). Although growth of strain WR1 was optimal at 0.2 M NaCl (data not shown), growth was still possible at 0.9 M NaCl. This is in agreement with a previous study in which MT degradation by a sediment sample from the MT-degrading reactor was not inhibited by salt concentrations up to 0.8 M NaCl or $NaHCO_3$ (chapter 4).

Methanogenic conversion of MT and DMS in sediment slurries was first demonstrated by Zinder and Brock [24]. Since then, several methanogens have been isolated with DMS from marine, estuarine, salt marsh, salt lake and freshwater sediments [8]. All these methanogens belong to the genera *Methanosarcina, Methanolobus, Methanosalsus* and *Methanomethylovorans.* Some of the isolated strains were capable of degrading DMS at high pH and high salt, and most of them were shown to co-metabolize MT. *Methanohalophilus zhilinae*, isolated from a salt lake, grew on methylated substrates, including DMS, which was degraded via MT at pH 9.2 and 0.7 M NaCl [9, 25]. *Methanohalophilus oregonense* was isolated from the West Alkali Lake in Oregon (USA). It showed optimum growth at pH 8.1 - 9.1 and grew till 1.5 M NaCl. Growth was possible on DMS, methanol and trimethylamine [26]. *Methanolobus bombayensis* B-1^T, isolated from Arabian Sea sediments near Bombay (India) grows on DMS between pH 6.0 and 8.5 in the presence of 0.5 M NaCl [27]. Finster *et al.* [6] were the first to isolate a methanogen, strain MTP4, with MT as sole source of energy. It could also grow on DMS. Strain MTP4 grew optimally on MT between pH 6.9 and 7.6 and growth was possible at NaCl concentrations ranging from 35-400 mM. Some of the strains described in the literature show activity at pH 10. Hence, to be able to degrade MT in a reactor at pH 10 probably haloalkaliphilic methanogens from a soda lake sediments are required, e.g. from Mono Lake, California (10% salinity, pH 9.7) or Big Soda Lake, Nevada (8.9% salinity, pH 9.7) [7].

Microbial community dynamics

MT degradation was observed two days after the reactor startup (experiment 1) (Fig. 5.1B). This is a short period, compared to previous experiments where MT degradation started only after 6 to 25 days (chapter 2 and 4 and [28]). Clearly, the reactor sludge had adapted already to MT during previous experiments (chapter 4 and Table 5.2), and keeping the reactor unfed during the three weeks prior to the start of experiment 1 apparently did not negatively affect the reactor startup.

During the first two reactor experiments described previously (chapter 4) and reactor experiment 1 in this study (Table 5.2), species related to *Methanolobus taylorii* and *Methanosarcina mazei* were the dominant MT-degrading species in the reactor (Table 5.3, Fig. 5.4). *Methanolobus taylorii* is a moderately alkaliphilic methylotrophic, marine methanogen which can grow on MT and DMS over a pH range from 7.2 to 8.8 [23]. The genus *Methanosarcina* also includes a few species capable of degrading MT and DMS [29], although *Methanosarcina mazei* was never tested for MT degradation. It grows on acetate, methanol and methyl amines [30, 31]. A related archeaon, *Methanosarcina siciliae*, is known to grow on both MT and DMS [32, 33]. Thus, *Methanosarcina* species present in the reactor may have taken part in the MT degradation during the first three reactor experiments mentioned in Table 2. After that, *Methanosarcina mazei* was outcompeted and eventually not detected anymore in the clone libraries (Table 5.3) and DGGE analysis (Fig. 5.4), probably because the pH (8.9-9.1) in the reactor during the last period of experiment 2 in this research (Fig. 5.1B, period VI) exceeded the pH range of *Methanosarcina mazei* (pH 5.5-8.5) [34, 35].

After the last reactor experiment, in which the reactor was exposed to a 10-day pH shock at pH 10 (Fig. 5.3), about 17% (4 out of 24) of the clones from the corresponding clone library contained 16S rRNA genes related to hydrogenotrophic methanogens (Table 5.3). It is unknown whether or not these organisms played a role in MT degradation, as in addition to direct methanogenesis, syntrophic MT degradation by bacteria coupled to hydrogen utilization by methanogens might have occurred [5]. Anaerobic conversion of methanol, the non-sulfur containing C_1 analogue of MT, to H_2 and CO_2 is possible, although this reaction is thermodynamically unfavorable [36-38]. However, in syntrophic association with a hydrogenotrophic microorganism, a large fraction of the methanol is completely oxidized to H_2 and CO_2 by acetogens [37]. A similar mechanism for MT degradation might have occurred during reactor experiment 2.

Apart from methanogenic archaea, MT can be degraded by sulfate-reducing bacteria (SRB) as well ([29] and chapter 2). As no sulfate was present in the reactor medium, an active SRB population could not develop. Although MT was mainly degraded by methanogens, the bacterial DGGE (not shown) showed that a vital, dynamic bacterial population was present in the reactor during the consecutive reactor runs listed in Table 5.2. It should however be noted, that besides MT, also yeast extract might have provided bacterial substrates.

Conclusion

Complete anaerobic MT degradation in a lab-scale UASB reactor (30°C) inoculated with Wadden Sea sediment was possible at a sodium concentration of 0.5 M and a pH between 8 and 9 at a maximum loading rate of 22 mmol $MT \cdot L^{-1} \cdot day^{-1}$. Species related to the genera *Methanolobus* and *Methanosarcina* dominated the archaeal community in the reactor sludge. The most dominant MT-degrading archaeon in the reactor was obtained in pure culture. This strain WR1, which was most closely related to *Methanolobus taylorii,* degraded MT, dimethyl sulfide, methanol and trimethylamine. Optimal growth conditions were 0.2 M NaCl, 30°C and pH 8. In batch and reactor experiments operated at pH 10, MT was not degraded.

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6

Anaerobic methanethiol degradation and methanogenic community analysis in an alkaline (pH 10) biological process for Liquefied Petroleum Gas (LPG) desulfurization [*]

Abstract

Anaerobic methanethiol (MT) degradation by mesophilic (30°C) alkaliphilic (pH 10) communities was studied in a lab-scale Upflow Anaerobic Sludge Bed (UASB) reactor inoculated with a mixture of sediments from the Wadden Sea (The Netherlands), Soap Lake (Central Washington) and Russian soda lakes. MT degradation started after 32 days of incubation. During the first 252 days complete degradation was achieved till a volumetric loading rate of 7.5 mmol $MT \cdot L^{-1} \cdot day^{-1}$, and sulfide, methane and carbon dioxide were the main reaction products. Temporary inhibition of MT degradation occurred after MT peak loads and in the presence of dimethyl disulfide (DMDS), which is the autooxidation product of MT. From day 252 onwards, methanol was dosed to the reactor as co-substrate at a loading rate of 3 to 6 $mmol \cdot L^{-1} \cdot day^{-1}$ to stimulate growth of methylotrophic methanogens. Methanol was completely degraded and also a complete MT degradation was achieved till a volumetric loading rate of 13 mmol $MT \cdot L^{-1} \cdot day^{-1}$ (0.77 mmol $MT \cdot gVSS^{-1} \cdot day^{-1}$). However, from day 354 till the end of the experimental run (day 365), acetate was formed and MT was not completely degraded anymore, indicating that methanol-degrading homoacetogenic bacteria had partially outcompeted the methanogenic MT-degrading archaea. The archaeal community in the reactor sludge was analyzed by DGGE and sequencing of 16S rRNA genes. The methanogenic archaea responsible for the degradation of MT in the reactor were related to *Methanolobus oregonensis*. A pure culture, named strain SODA, was obtained by serial dilutions in medium containing both trimethyl amine and dimethyl sulfide (DMS). Strain SODA degraded MT, DMS, trimethyl amine and methanol. Flowsheet simulations revealed that for sufficient MT removal from Liquefied Petroleum Gas, the extraction and biological degradation process should be operated above pH 9.

[*] This chapter has been submitted for publication.

Introduction

Liquefied Petroleum Gas (LPG) is one of the top products of the crude distillation at refineries and consists mainly of light hydrocarbon compounds (C_3 and C_4). Light sulfur-containing compounds, such as H_2S, methanethiol (MT) and ethanethiol (ET), are present as sulfur impurities [1, 2]. Even though European sulfur norms for LPG do not yet exist, it is normally desulfurized to levels below 10 ppm. This study presents a new haloalkaliphilic biological process for LPG desulfurization. This novel process is an alternative for currently used physical-chemical methods like the Merox process, in which thiols are extracted from the LPG and oxidized to their corresponding disulfides that can be removed from the liquid phase [1, 3].

In the new biological desulfurization process, H_2S and MT are removed from LPG in three steps [4]. The process involves (Fig. 6.1): (i) extraction of the sulfur compounds from the LPG phase into a (bi)carbonate-containing solution (eqns 1-2); (ii) anaerobic degradation of MT to H_2S, CO_2 and CH_4 (eqn 3) and (iii) partial oxidation of H_2S to elemental sulfur (eqn 4). In a settler, the sulfur particles are removed by gravity separation and the cleaned alkaline process water is reused in the extraction process. A concentrated Na_2CO_3 stream is needed to maintain a constant pH and a make-up water stream replenishes the bleed stream for discharge of the sulfate ions (eqn 5).

In the extractor column, H_2S and MT are transferred from the LPG to the alkaline water phase, where partial dissociation of the acidic compounds takes place (eqn 1 and 2):

$$H_2S\ (LPG) \leftrightarrows H_2S\ (aq) \leftrightarrows HS^-\ (aq) + H^+\ (aq) \quad (1)$$

$$CH_3SH\ (LPG) \leftrightarrows CH_3SH\ (aq) \leftrightarrows CH_3S^-\ (aq) + H^+\ (aq) \quad (2)$$

Subsequently, MT is biologically degraded in the anaerobic bioreactor:

$$4\ CH_3SH + 3\ H_2O \rightarrow 3\ CH_4 + HCO_3^- + 4\ HS^- + 5\ H^+ \quad (3)$$

In the aerobic bioreactor, H_2S is biologically oxidized according to the following two equations:

$$H_2S + ½\ O_2 \rightarrow S^0 + H_2O \quad (4)$$

$$H_2S + 2\ O_2 \rightarrow SO_4^{2-} + 2\ H^+ \quad (5)$$

Although the formation of sulfate yields more energy than the formation of elemental sulfur, it is possible to reach a selectivity between 90 and 95% for the formation of elemental sulfur by applying oxygen-limiting conditions [5, 6]. Any MT that is not biologically degraded in

the anaerobic reactor is chemically oxidized to dimethyl disulfide (DMDS) in the aerobic reactor:

$$2\ CH_3SH + ½\ O_2 \rightarrow CH_3SSCH_3 + H_2O \qquad (6)$$

The recirculation flow rate through the consecutive process steps is determined by the amount of MT and H_2S that can be extracted into the aqueous phase. Obviously, more MT and H_2S are absorbed at increasing alkalinity, which in turn leads to a reduction of the recirculation flow. This results in smaller process equipment and less energy costs. An increase in alkalinity, *i.e.* higher Na_2CO_3 concentrations, will automatically lead to increased sodium levels. Levels of at least 1 M Na^+ are expected. As these Na^+ concentrations impose high osmotic stress to microorganisms [7], salt tolerant microorganisms are needed to apply this new biotechnological process. Because the oxidation of H_2S to elemental sulfur at increased sodium levels has already been studied extensively [8, 9], the present research focuses on the anaerobic degradation of MT at elevated alkalinity (pH 10). Since we found that ethanethiol and propanethiol are not degraded under anaerobic conditions [10] (chapter 3), these compounds are not considered in the present study.

Anaerobic biological MT degradation has been reported in marine, estuarine, salt marsh, salt lake and freshwater sediments. Methanogenesis is the main process to degrade MT anaerobically [11]. All known methanogens that are able to grow on MT belong to the genera *Methanosarcina, Methanolobus, Methanosalsus* and *Methanomethylovorans* [12-17]. In sediments from some saline and alkaline lakes, methanogenic MT degradation was observed at pH values exceeding 9 and sodium concentrations up to 0.7 M NaCl [14, 18, 19]. Finster *et al.* [13] described a marine methanogen capable of growing on MT as the sole energy source and proposed equation 3 as stoichiometry for the reaction.

Previous research showed the feasibility of anaerobic MT degradation in a mesophilic (30°C) lab-scale UASB (Upflow Anaerobic Sludge Bed) reactor at pH values up to pH 9 at 0.5 M sodium (chapter 5). The inoculum was an estuarine sediment from the Wadden Sea (The Netherlands). The current research focuses on anaerobic MT degradation at elevated pH (10) and salt (Na_2CO_3, Na_2SO_4, 0.8 M total Na^+) concentrations in defined media using estuarine and salt lake sediments as inoculum. The methanogenic community that developed in the anaerobic reactor was characterized as well. Furthermore, the laboratory experiments are compared with a flowsheet simulation of the LPG desulfurization process (Fig. 6.1) to determine if LPG is sufficiently desulfurized (< 10 ppm S-total) at the applied process conditions.

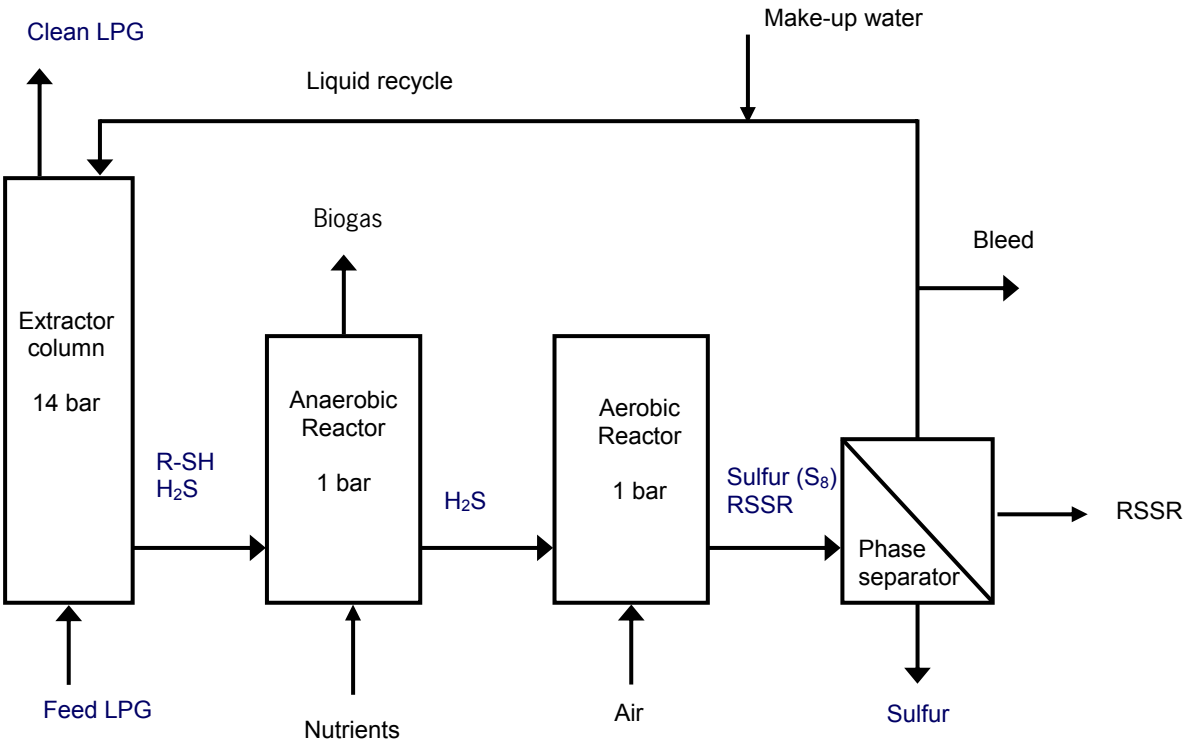

Fig. 6.1. Schematic representation of the novel process for desulfurization of LPG [4, 20].

Materials and methods

Continuous UASB reactor

Reactor experiments were carried out in a continuously operated mesophilic (30°C) lab-scale Upflow Anaerobic Sludge Bed (UASB) reactor (1.6 L) as described in chapter 4. The produced biogas was measured with a MilliGascounter (type MGC-1, model PMMA (Plexiglas)/Polycarbonate, Ritter GMBH, Bochum, Germany) with a measurement range between 1 ml/h and 1.2 L/h.

The reactor was fed with an oxygen-free synthetic influent (pH 12), containing about 80 mM MT (stock solution), which was freshly prepared every 10 to 14 days and stored under a N_2 blanket. The medium contained the following macronutrients (g/L): Na_2CO_3 (23), $NaHCO_3$ (7), Na_2SO_4 (14.8), NaCl (1.7) and (in mg/L) NH_4Cl (500) K_2HPO_4 (700) $MgCl_2{\cdot}6H_2O$ (60), $CaCl_2.2H_2O$ (100) and yeast extract (100). Micronutrients (1 ml/L) were added according to Paulo *et al.* [22]. This resulted in a total sodium concentration of 0.8 M and a pH varying between 9.9 and 10.1 in the reactor medium. Sufficient alkalinity was present to make pH control unnecessary.

Initially, the reactor was inoculated with Wadden Sea sediment (see *Sediment characteristics*) only and operated for 32 days at a volumetric loading rate of 2.2 mmol MT · $L^{-1}{\cdot}day^{-1}$ (experiment 1). After that, the reactor was kept at an ambient temperature for three months without MT feeding and was subsequently inoculated with additional biomass originating from the Soap Lake (Table 6.1). The reactor was operated for 21 days at a

volumetric loading rate of 4.0 mmol $MT \cdot L^{-1} \cdot day^{-1}$ (experiment 2). Eight weeks after finishing the second experiment, the reactor was inoculated with additional biomass from the Kalunda Steppe (Table 6.1) and operated for a year at pH 10 at varying MT loading rates (experiment 3). During experiment 3, the influent flow contained 2-8 mM MT and amounted to 3.2 L/d, resulting in a hydraulic retention time (HRT) of 12 hours. The superficial liquid upward velocity was controlled at 2 m/h by external recirculation. From day 252 onwards, methanol was dosed to the reactor as additional substrate at volumetric loading rates between 3 and 6 $mmol \cdot L^{-1} \cdot day^{-1}$. This was done to stimulate biomass growth, as all known MT-degrading methanogens also utilize methanol [11].

Sediment characteristics

The reactor was inoculated with sediments from the Wadden Sea (Den Oever, The Netherlands), Soap Lake (Central Washington) and Russian soda lakes (Table 6.1). Wadden Sea sediment (pH 8, 0.5 M total Na^+) was collected at the marina of Den Oever (The Netherlands). Two Soap Lake samples (pH 10) were used. A mixolimnion sample originated from the upper portion of the lake, which contains about 15 g/L salinity, 100 mM sulfate and 3 mM sulfide. A monolimnion sample originated from the bottom of the lake, which contained about 140 g/L salinity, 100 mM sulfate and about 100 mM sulfide. The third sediment mixture (pH 10, about 70 g/L total salinity) originated from the Tanatar lake and the Cock Soda Lake (Kalunda Steppe, Russia).

Table 6.1. Composition of the inoculum, based on the initial amount of VSS (volatile suspended solids) in the UASB reactor.

Sediment	Dry matter (g)	VSS (g)	VSS content (% dm)
Wadden Sea	216	18	8.3
Soap Lake	25.8	3.7	14
Mix Russian soda lakes	87	5.1	5.9
Total	329	26.8	8.6

Media and cultivation conditions

Reactor sludge samples were taken for isolation and molecular characterization. Isolation was performed in a medium containing (in g/L): K_2HPO_4 (1), NaCl (10), $Na_2S \cdot 9H_2O$ (0.25), $MgCl_2 \cdot 6H_2O$ (0.1), $CaCl_2 \cdot 2H_2O$ (0.01), $NaHCO_3$ (7), Na_2CO_3 (19), NH_4Cl (0.27), yeast extract (0.1), biotrypticase peptone (0.1), vitamins and trace elements according to Stams *et al.* [23] and resazurin (0.5 mg/L). Serum bottles (120 ml) containing 50 ml medium were sealed with viton stoppers, the gas in the headspace was exchanged and pressurized with N_2 (1.7 bar). The medium was autoclaved for 20 minutes at 121°C. $MgCl_2$, $CaCl_2$, NH_4Cl, Na_2S, vitamins and resazurin were added aseptically from filter sterilized (0.22 μM) stock

solutions. Substrates were added from the following concentrated sterile stock solutions: methanol and sodium sulfate (1 M), MT sodium salt (0.1 M), trimethylamine (0.5 M) and DMS (0.1 M). The methanogenic inhibitor 2-bromoethanesulfonic acid (BrES) was added from a 1 M sterile stock solution.

The presence of sulfate-reducing bacteria (SRB) in the reactor was assessed in duplicate batch cultures in medium containing: (I) methanol (20 mM) or (II) methanol (20 mM) plus MT (2 mM) together with yeast extract (1 g/l), sulfate (20 mM) and 2-bromoethanesulfonic acid (0.4 mM).

Enrichment and isolation of MT-degrading haloalkaliphilic methanogenic archaea

A bicarbonate-buffered medium (50 ml) supplemented with 20 mM methanol and 2 mM MT was inoculated with a 2.5 ml sludge sample and incubated for 8 weeks at 30°C. Serial dilutions in media with three different substrate combinations were made: (I) methanol (20 mM), (II) MT (2 mM) plus DMS (3 mM) and (III) methanol (20 mM) plus MT (2 mM) and DMS (3 mM). The highest dilution of each series in which one or more of the substrates were degraded, was transferred to fresh medium containing one of the following substrates or combination of substrates: trimethylamine (TMA, 5 mM), TMA (5 mM) plus DMS (1.5 mM), TMA (5 mM) plus MT (2 mM) and methanol (20 mM). Each of these four conditions were also tested for dilutions of interest in medium in which the yeast extract and peptones were replaced with fermented yeast extract (0.1 g/L).

Purity of the enrichments was checked by microscopy after growth in medium containing yeast extract (1 g/l) and trypticase peptone (1 g/l). Growth was tested at pH values of 8.0, 8.8, 9.4 and 10.0 and at 30°C and 37°C. Besides methanol, MT, DMS and TMA also hydrogen (1.7 bar H_2/CO_2, 80:20) was tested as substrate.

DNA extraction and 16S rRNA gene amplification

To monitor the microbial population dynamics in the sludge, thirteen sludge samples from the reactor, sampled during the entire period of operation, were fixed in 60% (vol/vol) ethanol containing 25 mM NaCl. DNA extraction and 16S rRNA gene amplification of these samples and of enrichment cultures was carried out according to De Bok *et al.* [12] (chapter 2).

DGGE, cloning and sequencing

Denaturing gradient gel electrophoresis, cloning and sequencing were carried out according to De Bok *et al.* [12] (chapter 2).

Analytical techniques and microscopy

Volatile organic sulfur compounds (VOSC) were analyzed by high performance liquid chromatography (HPLC) as described by De Bok *et al.* [12] (chapter 2). For expressing the MT degradation, the VOSC concentration *(i.e.* the sum of MT, DMS and DMDS) was used

instead of MT alone because MT is readily autooxidized to DMDS. In addition, DMS can be formed as an intermediate during anaerobic MT degradation. Autooxidation may to a small extent already take place in the concentrated stock solution as well as during the sampling procedure. Total and volatile suspended solids were analyzed according to Standard Methods [24]. Sulfide was measured photometrically [25]. Biogas composition (CH_4, CO_2, N_2, H_2S) was analyzed on a gas chromatograph as described by Van Leerdam *et al.* [21] (chapter 4). Microscopic analysis of sediment samples was performed with an Olympus BH2 epifluorescence microscope.

Chemicals

All chemicals used were of analytical grade and supplied by Merck (Darmstad, Germany). A concentrated sodium mercaptide solution ($NaSCH_3$, 3 M) of analytical grade was supplied by Arkema Group (Rotterdam, The Netherlands).

Flow sheet simulation

Flow sheet simulations of the novel process for LPG desulfurization (Fig. 6.1) were carried out with OLI-ESP (Environmental Simulation Program) version 6.7 (2004). OLI is a commercially available software package that uses a thermodynamic and mathematical framework for predicting the equilibrium properties of aqueous chemical systems (www.olisystems.com). The main goal of the simulations was to get insight into MT removal from LPG in the extractor unit at varying pH and sodium carbonate concentrations. The required pH and salt concentrations, which follows from this simulation, define the boundary conditions for the biological reactors as well.

The process was simulated at different pH values in the extraction liquid (recycle stream). Table 6.2 presents the main parameters for the untreated LPG stream and the recycle stream. The number of theoretical equilibrium steps applied in the extractor was set on 6, which is a common number in many petroleum refining operations [26]. MT degradation (eqn 3) was set on 99%, an efficiency that was reached during laboratory experiments [4, 21, 27]. Equations 4 and 5 were set on 95% and 5% efficiency, respectively, as it is possible to oxidize sulfide to elemental sulfur with a selectivity of 95% [6]. The remaining MT will be oxidized to DMDS in the aerobic reactor. Therefore, equation 6 was set on a 95% efficiency. The make-up water was composed of a concentrated sodium (bi)carbonate stream and a water stream of 48 L/h in total. The bleed stream was set on 1% of the recycle stream to prevent accumulation of sulfate.

Table 6.2. Process parameters and flows of the ingoing LPG and the recycle stream just before entering the extraction column.

	Unit	LPG in	Recycle stream (extraction liquid)
T	°C	20	30
P	atm.	14	14[a]
pH	-	-	9.07, 9.23, 9.41, 9.79, 10.07
total Na^+ ($\cdot 10^3$)	mol/h	0	11.6-36.0[b]
	mol/L	0	1.1-3.6[b]
n-C_4H_{10}	mol/h	4829	0
C_3H_8	mol/h	9875	0
i-C_4H_{10}	mol/h	4829	0
H_2S	mol/h	79.31	[c]
	ppmw	2704	[c]
CH_3SH	mol/h	9.89	[c]
	ppmw	476	[c]
Total flow	m^3/h	1.85	9.9-10.2
Density	kg/L	0.539	1.06-1.17

a: The pressure of the recycle stream is increased from 1 to 14 atmosphere before it enters the extraction unit.
b: Main sodium species are: Na^+, $NaHCO_3$, $NaCO_3^-$, $NaSO_4^-$. Total mass flow and speciation depend on pH.
c: Present in low concentrations (1-6 μM or < 0.06 mol/h), depending on the process conditions

Results

Methanethiol degradation at pH 10 in bioreactors

In the first experiment, the UASB reactor was inoculated with Wadden Sea sediment only and started up at pH 10 at a volumetric loading rate of 2.2 mmol $MT \cdot L^{-1} \cdot day^{-1}$. MT was not biodegraded during the 32 days of operation, but was partially (up to 70 %) chemically oxidized to DMDS (data not shown). Subsequently, the reactor was inoculated with additional biomass originating from the Soap Lake (experiment 2). The applied volumetric loading rate was 4.0 mmol $MT \cdot L^{-1} \cdot day^{-1}$. During the 21 days period of operation, no MT degradation was observed (data not shown). Considering the startup time of reactor experiment 3, this incubation time was probably too short to achieve MT degradation (see below).

The reactor was inoculated with additional biomass from the Kalunda Steppe and restarted at a volumetric loading rate of 4.0 mmol $MT \cdot L^{-1} \cdot day^{-1}$ (experiment 3). Only small amounts of sulfide were detected in the effluent (~ 0.3 mM) during the first 30 days of operation (Fig. 6.2A), but after 46 days complete MT degradation was obtained. The reactor loading rate was subsequently increased to 7 mmol $MT \cdot L^{-1} \cdot day^{-1}$. This resulted in a decrease in reactor performance and MT accumulated in the reactor. Therefore, the loading rate was reduced back to 4.0 mmol $MT \cdot L^{-1} \cdot day^{-1}$ at day 60. Four days later, a complete MT degradation was established (Fig. 6.2A).

The presence of DMDS in the reactor affected the removal efficiency of the total volatile organic sulfur compounds (VOSC). Although the concentrated MT influent stock solution was stored anaerobically, some DMDS was formed in the storage bottle. The accumulation of

VOSC (MT and DMDS) in the effluent at day 98, 241, 279 and 338 (Fig. 6.2A) can be explained by the presence of 0.9-1.7 mM DMDS in the influent on these days. The reactor performance immediately improved after feeding the reactor with a freshly prepared influent solution without DMDS.

At day 109, the volumetric loading rate was increased from 4 to 6 mmol $MT \cdot L^{-1} \cdot day^{-1}$. This resulted in a temporary inhibition of the MT bioconversion but on day 130 the reactor performance had fully recovered. Unfortunately, a shock load of 11 mmol $MT \cdot L^{-1} \cdot day^{-1}$ overnight at day 139 resulted in an incomplete MT degradation for a week. At day 197, the increase in volumetric loading rate to 11 mmol $MT \cdot L^{-1} \cdot day^{-1}$ initially caused a rapid increase in the sulfide concentration to 4.3 mM in the effluent, but eventually MT accumulated in the reactor (Fig. 6.2A). Thus, either sulfide or MT inhibited MT degradation.

Effect of methanol on methanethiol degradation

Complete MT degradation at a volumetric loading rate of 7.5 mmol $MT \cdot L^{-1} \cdot day^{-1}$ was reached on day 250. From day 252 onwards, methanol was dosed to the reactor as a co-substrate at a volumetric loading rate between 3 and 6 $mmol \cdot L^{-1} \cdot day^{-1}$ to stimulate growth of the methylotrophic methanogenic archaea responsible for the degradation of MT. Indeed, from day 252 till day 313, MT was almost always completely degraded up to a volumetric loading rate of 13 mmol $MT \cdot L^{-1} \cdot day^{-1}$ (0.77 mmol $MT \cdot gVSS^{-1} \cdot day^{-1}$). Methanol in the effluent rarely exceeded 0.1 mM (Fig. 6.2B).

At day 319, MT accumulated in the reactor due to a malfunctioning of the dilution water pump, but the reactor recovered within 5 days (Fig. 6.2A). The last 11 days of the experiment (day 354-365), the MT degradation was far from complete and the total VOSC concentration in the effluent varied between 2 and 4 mM. Methanol was still almost completely degraded (< 0.1 mM) while acetate (0.5 to 0.6 mM) accumulated in the effluent (Fig. 6.2B). This indicates that homoacetogenic bacteria started to outcompete methanogenic archaea for methanol.

During reactor experiment 3, the sludge washout amounted to 50 to 125 mg/L dry matter. This means a loss of 4 to 10 mg biomass per liter (13-32 mg/day) since approximately 10% of the sediment was organic matter. The biogas production varied between 50 and 100 $ml \cdot day^{-1}$ depending on the MT loading rate. After methanol addition, this increased from 100 upto 200 $ml \cdot day^{-1}$. This means that up to 80% of the theoretically expected amount of methane was recovered. Sometimes the gas-liquid-solid device was clogged by the sediment, which caused a pressure build-up in the reactor and an apparent lower gas production. The methane content of the biogas reached 78%. The balance was nitrogen gas, as the influents were kept anaerobically under a nitrogen gas blanket. Less than 0.1% of the biogas consisted of carbon dioxide and hydrogen sulfide was not detected at all.

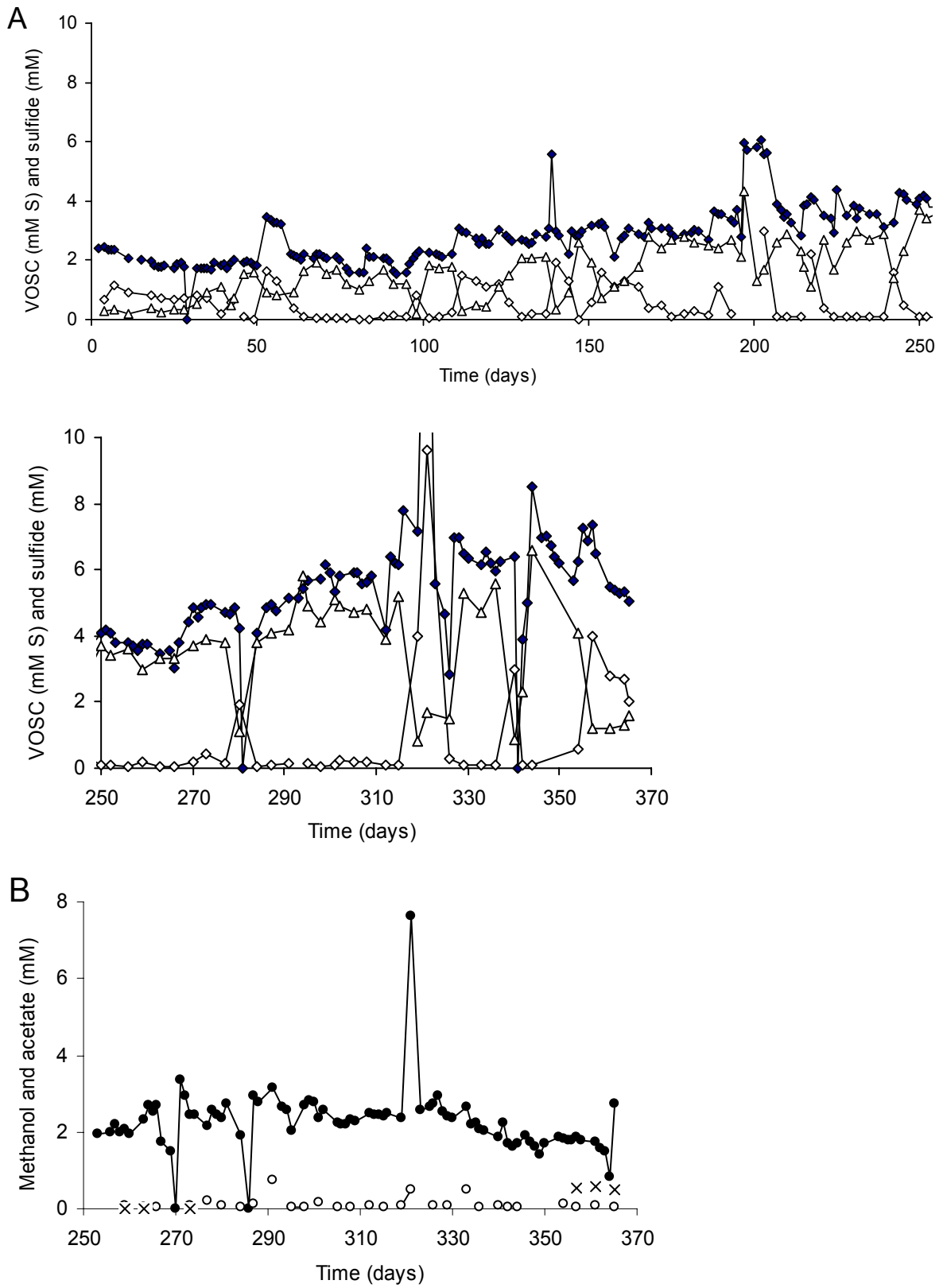

Fig. 6.2. Methanethiol degradation (A) and simultaneous methanol degradation (B) in the reactor inoculated with three different sediments (experiment 3, 30°C, pH 10). -♦- methanethiol influent, -◊- VOSC total (MT, DMS, DMDS) effluent, -Δ- sulfide effluent, -●- methanol influent, -○- methanol effluent, × acetate effluent.

Enrichment and isolation of MT-degrading haloalkaliphilic methanogenic archaea

In batches inoculated with reactor sludge, methane was produced while methanol and MT were completely degraded by methanogens. No growth was observed in batches supplemented with sulfate and 2-bromoethanesulfonic acid, while batches supplemented with sulfate also yielded methanogenic archaea. After 8 weeks of incubation at 30°C, methane was detected in the 10^{-9} dilution of the series containing trimethyl amine and DMS. Microscopic examination revealed a homogeneous culture of coccoid cells that produced fluorescence upon radiation with ultraviolet light. This culture was designated as strain SODA. Addition of yeast extract (1 g/l) and peptones (1 g/l) as extra substrate to the media did not result in growth of other (non-autofluorescing) organisms. Growth occurred at all pH values tested (*i.e.* 8.0, 8.8, 9.4 and 10) and pH 8.8 appeared to be optimum. Strain SODA grew on MT, DMS, trimethyl amine and methanol, but could not utilize H_2. Yeast extract supported growth and was required for complete degradation of substrates.

Molecular characterization of the archaeal population

The archaeal community of the reactor biomass and enrichment cultures were analyzed by DGGE and sequence analysis of 16S rRNA genes. DGGE analysis revealed that the methanogenic archaeon isolated by serial dilutions (Fig. 6.3, lane I) represented the dominant MT-degrading organism in the reactor. Sequence analysis revealed that the closest phylogenetic relative of the strain is *Methanolobus oregonensis*. The other amplicons detected in the DGGE gels also represented archaea closely related to *Methanolobus oregonensis*. Other methanogenic archaea were not detected in the reactor.

During reactor experiment 1, MT was not degraded and as a consequence no dominant archaeal species could be identified with DGGE (Fig. 6.3, lane 1-2). After the Soap Lake sediment was included as inoculum (experiment 2, Fig. 6.3., lane 4), MT degradation was not observed either. Nevertheless, after 21 days of operation a clear band was detected in the DGGE gel (Fig. 6.3, lane 5), which was also detected in all samples from the reactor during experiment 3 (Fig. 6.3, lane 6-13) after Kalunda Steppe sediment was added as inoculum. This suggests that an alkaliphilic MT-degrading methanogen already accumulated in the reactor during experiment 2. From day 250 of experiment 3 onwards, methanol was dosed to the reactor (Fig. 6.2B; Fig. 6.3, between lane 10 and 11). This did not change the DGGE pattern significantly (Fig 6.3, lane 10-13), which suggests that methanol and MT were degraded by the same species.

To construct a clone library of archaeal 16S rRNA genes present in the reactor, DNA extracted from sample 7 was amplified using primers specific for the V2-V8 (~ 1360 bp) region of the archaeal 16S rRNA genes [12] (chapter 2). Sixty clones were analyzed by amplified rDNA restriction analysis (ARDRA) using restriction enzymes *Msp*I, *Cfo*I and *Alu*I. Three clones that showed a deviating restriction pattern, and one of the clones representative for the other 57 clones were analyzed by DGGE analysis and compared to the 16S rRNA gene

content of each of the reactor samples (Fig. 6.3, A-D). The 16S rRNA gene sequences were determined and showed that the organism responsible for MT degradation at pH 10 is closely related (97% similarity) to *Methanolobus oregonensis*.

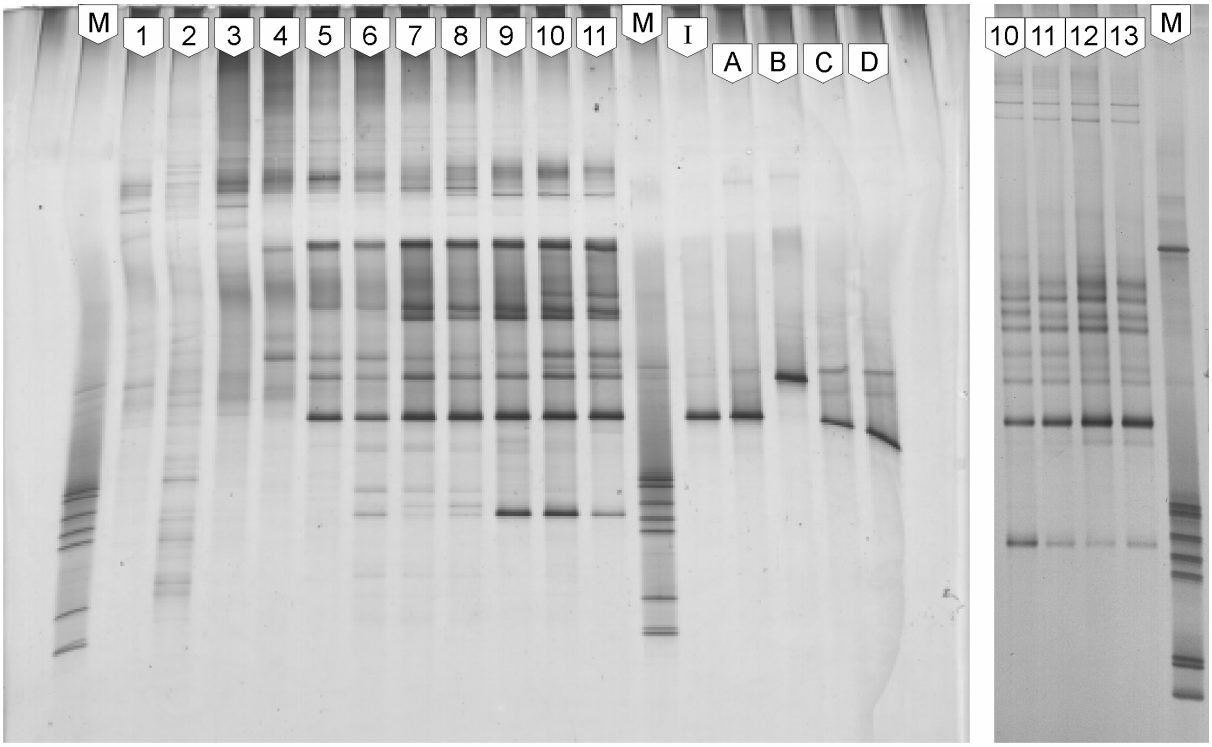

Fig. 6.3. DGGE fingerprints of the 16S rRNA (V2-V3) from the archaeal community in a reactor inoculated with a mixture of estuarine and soda lake sediments. M. DGGE marker; 1-2. Wadden Sea sediment (first reactor experiment) t = 0 d (1) and 32 d (2); 3-4. Wadden Sea sediment (3) and Soap Lake sediment (4), t = 0 d (second reactor experiment); 5. Mixture Wadden Sea and Soap Lake sediment, t = 21 d (end second reactor experiment); 6-13. Mixture Wadden Sea, Soap Lake, Kalunda Steppe sediments (third reactor experiment, Fig. 6.2). 6. t = 29 d, 7. t = 96 d, 8. t = 154 d, 9. t = 182 d, 10. t = 246 d, 11. t = 298 d, 12. t = 336 d, 13. t = 365 d; I. Methanogenic enrichment culture on methanol plus MT; A-D. Clones.

Simulation of the process

The simulated MT removal efficiencies in the extractor at the prevailing pH values amounted to at least 98% (Table 6.3). The corresponding total sulfur concentrations in the treated LPG were ≤ 10 ppmw (Table 6.3) and existed mainly of MT. H_2S is completely removed from the LPG phase (< 1 ppbw). Table 6.3 shows that under the conditions tested (Table 6.2), the pH in the extractor and thus through the entire system, must be at least 9 to desulfurize LPG to levels below 10 ppmw.

The pH and ionic strength were constant throughout the process: maximal deviations of ± 0.02 of the reported values (Table 6.3) for both pH and ionic strength were found in the simulations. The sodium (bi)carbonate aqueous medium sufficiently buffers the entire process. Obviously, at the highest pH values simulated (9.79 and 10.07), the highest MT removal efficiencies were obtained. It seems that an increase in the ionic strength has an

adverse effect on the MT removal efficiency. This so-called 'salting out effect' [28, 29] is the reason for the slightly higher MT removal efficiency at pH 9.07 compared to the values found at pH 9.23 and 9.41 (Table 6.3).

The extraction of the sulfur compounds resulted in a loaded aqueous solvent containing 7.8 mM sulfide and 0.95 mM MT, which was subsequently led to the anaerobic reactor. Here, MT was degraded to H_2S that in turn was oxidized to elemental sulfur in the aerobic reactor according to equations 3-6 at the efficiencies indicated in the *Material and methods* section.

Table 6.3. Effect of the pH on the performance of the extractor (OLI simulation)

pH liquid recycle	S-total LPG-out (ppmw)	MT removal efficiency (wt %)	Ionic strength (M)
9.07	7.54	98.4	1.18
9.23	9.22	98.1	1.75
9.41	8.12	98.3	2.05
9.79	2.55	99.5	1.96
10.07	2.26	99.5	4.46

Discussion

Anaerobic MT degradation at halo-alkaline conditions

This research shows that anaerobic MT degradation in a continuously operated UASB reactor is feasible at pH 10, 30°C and 0.8 M Na^+. A methanogenic archaeon that was enriched from the reactor appeared to be closely related to *Methanolobus oregonensis*, and was most likely introduced in the reactor by inoculation with either Soap Lake sediment or the mixture of Kalunda Steppe sediments. Methanogenic conversion of MT and DMS in sediment slurries was first demonstrated by Zinder and Brock [30]. Since then, various methanogens have been isolated with DMS, which is degraded via MT to methane, carbon dioxide and sulfide, from marine, estuarine, salt marsh, salt lake and freshwater sediments [11]. These methanogens belong to the genera *Methanosarcina, Methanolobus, Methanosalsus* and *Methanomethylovorans*. *Methanolobus* species developed in the reactor of the present study (Fig. 6.3).

In the current research, the reactor was inoculated with sediments from saline-alkaline environments. The sediments from the Soap Lake and from the Kalunda Steppe originate from areas with salt concentrations (Na_2CO_3) approaching saturation levels, highly reduced conditions (till 100 mM sulfide) and high pH values (~10). Methanogenic archaea so far identified in soda lakes are obligately methylotrophic organisms and do not use acetate or

H_2/CO_2 as energy yielding substrates, but use methanol, methylamines, DMS and MT. In those environments, halo-alkaliphilic MT-degrading organisms will thrive [31].

MT and DMS were degraded in batch experiments inoculated with this mixture of the Kalunda steppe sediments at pH 10 (data not shown). After the reactor was inoculated with additional biomass from the Kalunda steppe soda lakes (experiment 3), the startup period was still about 32 days (Fig. 6.2A). This is a longer startup time than observed during previous reactor experiments at milder process conditions (pH 7-9, ≤ 0.5 M Na^+), where it took less than 15 days before MT degradation started [4, 27] (and chapter 2, 4 and 5). This might be due to slower growth at the experimental conditions or a slower adaptation of the microbiota in the reactor sludge to MT.

It should be noted that a different methanogenic culture developed in UASB reactors inoculated with solely Wadden Sea sediment and operated at lower pH (chapter 4 and 5). At lower pH, methanogens closely related to *Methanosarcina mazei* and *Methanolobus taylorii* were the dominant MT-degrading species (pH 7-9, 30°C, 0.5 M Na^+). With this sludge, operation at pH 10 was not possible (experiment 1 this study and chapter 5).

Methanolobus oregonensis (= *Methanohalophilus oregonense*) was first isolated from an anoxic aquifer (pH 10) from an alkaline desert lake in south central Oregon [18]. Strain WAL1^T grew on trimethylamine and slowly on DMS and methanol at 0.1-1.5 M Na^+, 20-40°C and a pH range of 7.6 to 9.4. Yeast extract stimulated growth [18, 32]. In our research, growth of the *Ml. oregonensis* related methanogen (strain SODA) on MT and methanol was optimal at pH 8.8. In the reactor (pH 10, 0.8 M Na^+, 30°C) MT and methanol were degraded simultaneously by methanogens, although the growth conditions were sub-optimal.

The salt tolerance for the reactor sludge mixture in experiment 3 was not tested in batch experiments. In incubations with reactor sludge originating from Wadden Sea sediment only (pH 8) [21] (chapter 4), no salt inhibition effect on MT degradation was found till 0.8 M Na^+. Taking into account the salt range of *Ml. oregonensis* (0.1-1.5 M Na^+), the salt tolerance for the reactor sludge is expected to be higher.

MT degradation rates

An almost complete MT degradation was achieved (Fig. 6.2A) till a volumetric loading rate of 13 mmol $MT \cdot L^{-1} \cdot day^{-1}$. Sipma *et al.* [4, 27] reported volumetric MT degradation rates of 10-50 $mmol \cdot L^{-1} \cdot day^{-1}$ in mesophilic (30°C) lab-scale UASB reactors inoculated with anaerobic granular sludge, but this was at pH values of 7.3-7.6 and freshwater conditions (about 20 mM total salt concentration). Van Leerdam *et al.* [21] (chapter 4) reported a maximal volumetric MT degradation rate of 37 $mmol \cdot L^{-1} \cdot day^{-1}$ in a lab-scale UASB reactor inoculated with Wadden Sea sediment at pH 8.1-8.4 and 0.5 M Na^+.

The maximum specific sulfide production rates in the batch incubations inoculated with unadapted Soap Lake sediment were 0.50 mmol $sulfide \cdot gVSS^{-1} \cdot day^{-1}$ at pH 8.7 and 0.24 mmol $sulfide \cdot gVSS^{-1} \cdot day^{-1}$ at pH 10.1 (data not shown). In the reactor, the maximal specific

degradation rate was 0.77 mmol·gVSS^{-1}·day^{-1}. The biomass thus clearly adapted to MT during the course of the experiment. However, the biomass from the Wadden Sea sediment was probably inactivated or lysed due to the high pH. Only taking into account the initial VSS content from the Soap Lake and Kalunda Steppe sediment (Table 6.1), the specific degradation rate would be about a factor 3 higher. It was however not possible to determine the exact VSS content during the experiments on a regular basis due to practical limitation, *i.e.* too much biomass is required for VSS analysis. The VSS content in the reactor decreased during its operation due to sediment sampling, washout, and lysis and was approximately 10 g at the end of reactor experiment 3. This shows that the maximal specific MT degradation rate could be a factor 2-3 higher than the 0.77 mmol·gVSS^{-1}·day^{-1} mentioned above. In previous research, MT was degraded in an anaerobic reactor (pH 8.5, 30°C, 0.5 Na^+) inoculated with Wadden Sea sediment at specific loading rates up to 11 mmol MT·gVSS^{-1}·day^{-1} [21] (chapter 4).

Effect of co-substrate and intermediates on MT degradation

The reactor was fed with methanol from day 252 onwards to stimulate methanogenic activity and biomass growth. The increased methane production rate and absence of methanol and acetate in the effluent suggested that methanol was degraded to methane and carbon dioxide. However, during the last 11 days of the experiment acetate was formed in concentrations up to 0.6 mM in the effluent. The pH, methanol, bicarbonate and cobalt concentration play a role in the competition between methanogens and homoacetogens for methanol [33]. Accumulation of MT in the reactor during the last 11 days of the experiment (Fig. 6.2) may have inhibited the methanogens in the reactor. However, the presence of methanol may also gradually have favored growth of homoacetogens.

The presence of DMDS in the reactor negatively affected the degradation of MT and the total volatile organic sulfur compounds. Concentrations of 0.9 to 1.7 mM DMDS already upset the reactor (Fig. 6.2). However, the reactor performance immediately improved upon feeding the reactor with a freshly prepared MT influent without DMDS. DMDS conversion to MT is catalyzed by a DMDS reductase that uses NADH as reductant [34]. This reduction might be a rate limiting step in the degradation of DMDS to methane, carbon dioxide and sulfide. In chapter 5, 1 mM DMDS was found to strongly inhibit MT degradation in an anaerobic MT degrading bioreactor (30°C, pH 8.5) inoculated with Wadden Sea sediment, which is in agreement with the current findings. Thus, DMDS formation is undesired and must be prevented to enable proper anaerobic MT degradation.

Application

The Merox process is an established LPG desulfurization method. In this process, the majority of the H_2S is removed in a prewash step with a low strength caustic solution whereafter a high strength caustic soda solution is used to extract low molecular weight thiols

from the hydrocarbon feedstock. The thiols are subsequently catalytically oxidized to water insoluble disulfide oils that are skimmed from the water phase and collected for disposal [3].

The main advantages of the novel biological process (Fig. 6.1), compared to the Merox process, are (i) the absence of an expensive catalyst, (ii) absence of waste materials (*i.e.* spent sulfidic caustics and disulfides) and (iii) the production of reusable elemental sulfur. The process simulation (Table 6.3) shows that the new desulfurization process must be operated at elevated pH levels (9-10) to enable a sufficient MT and H_2S extraction from LPG. The current research showed that MT degradation at these pH values is possible (Fig. 6.2.), provided that an appropriate inoculum is used and the influent DMDS concentration is kept low.

From previous research, it is known that H_2S inhibits anaerobic MT degradation already below 10 mM [10, 21] (chapters 3 and 4). If more than 10 mM H_2S is expected in the anaerobic reactor due to high H_2S concentrations in the LPG and the applied aqueous recycle/LPG ratio, a H_2S prewash like in the Merox process can be an option. The H_2S loaded solvent is then directly led into the aerobic reactor.

MT is a toxic compound for microorganisms as well. In chapter 3 it was shown that 50% inhibition of the methanogenic activity of anaerobic granular sludge occurs between 7-10 mM MT. Also in the current reactor experiment, it was shown that short duration MT peak loadings inhibit the MT-degrading activity almost completely, *e.g.* on day 139 (Fig. 6.2). Therefore, MT concentrations exceeding 10 mM in the anaerobic reactor are not recommended. MT that is not degraded in the anaerobic reactor will be led to the aerobic reactor, where it is rapidly oxidized to DMDS or to (dimethyl) polysulfides (chapter 7). A further oxidation to sulfate is not likely because of the preferential conversion of sulfide compared to DMDS [35] and the oxygen-limiting conditions in the aerobic reactor [36]. To prevent DMDS from entering the extractor column, the recycle stream should pass a filter (*e.g.* an active carbon filter), which removes the sparingly soluble DMDS. Also, solid elemental sulfur particles that are not removed in the settler shall be removed by this filtration step as both DMDS and elemental sulfur would redissolve in the LPG and thereby increase its total sulfur content.

Conclusion

MT degradation at pH 10 at a maximal volumetric degradation rate up to 13 mmol MT · L^{-1}· day^{-1} was feasible in a UASB reactor (T = 30°C; 0.8 M Na^{+}) inoculated with a mixture of soda lake sediments. The species responsible for MT degradation were isolated on trimethyl amine and DMS (strain SODA) and degraded MT, DMS, trimethyl amine and methanol. The closest phylogenetic relative of the isolate is *Methanolobus oregonensis* (97% similarity). Flowsheet simulations revealed that for sufficient MT removal from liquefied petroleum gas (< 10 ppmw in the treated LPG), the extraction and biological degradation process should be operated at pH 9 or higher. This is well possible if an appropriate inoculum is used.

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7

Reactions between methanethiol and biologically produced sulfur*

Abstract
Methanethiol (MT) reacts with biologically produced sulfur (both 1-16 mM; pH 8.7 and 10.3; 30-60ºC) to form a mixture of (poly-)sulfur compounds. The first reaction step is a S_8 ring opening by nucleophilic attack to form $CH_3S_9^-$. The reaction rate depends on the MT and bio-sulfur concentrations, pH and temperature. The activation energy of this reaction was determined to be 70 kJ·mol^{-1} at pH 8.7 and 16 kJ·mol^{-1} at pH 10.3. The $CH_3S_9^-$ ion is unstable and subject to further nucleophilic attacks to form shorter-chain sulfur compounds. The main end-products are polysulfides (S_3^{2-}, S_4^{2-}, S_5^{2-}), dimethyl polysulfides [$(CH_3)_2S_2$, $(CH_3)_2S_3$] and dissociated H_2S. Also longer-chain dimethyl polysulfides [$(CH_3)_2S_4$-$_7$] were formed at μM levels. Excess MT results in complete methylation of the initially formed polysulfides. An increased molar MT/S^0 ratio results in the formation of relatively more $(CH_3)_2S_2$ over $(CH_3)_2S_3$. The reaction between MT and biologically produced sulfur enables an enhanced MT removal from various liquid and gaseous hydrocarbon streams.

* A modified version of this chapter has been submitted for publication.

Introduction

Volatile sulfur compounds like H_2S and short-chain thiols (methanethiol, ethanethiol and propanethiol) have to be removed from various hydrocarbon streams, such as natural gas and liquefied petroleum gas (LPG) for reasons of toxicity, environmental protection and corrosivity. As an alternative for the currently used physical-chemical treatment methods, Van Leerdam *et al.* (chapter 6) and Sipma *et al.* [1] describe a new biological process for LPG desulfurization that consists of three integrated process steps. In the first step, the volatile sulfur compounds are extracted from the LPG into an aqueous phase. Subsequently, in an anaerobic bioreactor, MT is degraded to H_2S, CH_4 and CO_2. In a second aerobic bioreactor, H_2S is partially oxidized to elemental sulfur, which is removed from the process. In chapter 3 [2] it was found that ethanethiol and propanethiol are not degraded under anaerobic conditions. If thiol degradation in the anaerobic reactor is incomplete, thiols reach the aerobic reactor, where they may spontaneously react with biologically produced sulfur (bio-sulfur) to form polysulfides (S_x^{2-}) and organic polysulfides (RS_xR, R is an alkyl group). This oxidation of thiols by elemental orthorhombic sulfur (S_8) to (organic) polysulfides has been studied in the presence of a base-catalyst [3, 4]. Bio-sulfur has different colloidal properties than standard S_8 particles as it has an overall hydrophilic character [5, 6]. Because for bio-sulfur no information is available concerning its reaction with thiols and the bioprocess described above does not rely on a catalyst the presented research focuses on the reaction between bio-sulfur particles and thiols.

Thiolates (RS^-) are strong nucleophiles, which are able to open any sulfur-sulfur single bond in a polysulfane [7]. MT reacts with elemental sulfur to form methyl polysulfide (eqn 1):

$$CH_3SH + S_8 \rightarrow CH_3S_9^- + H^+ \quad (1)$$

Methyl polysulfides are however instable. Evidence was only found for the existence of RS^- and RS_2^- [7, 8]. The opening of the ring is the rate determining step [8]. In a second step, shorter dimethyl polysulfides are formed along with the formation of polysulfides (eqn 2):

$$CH_3SH + CH_3S_9^- \rightarrow CH_3S_xCH_3 + S_y^{2-} + H^+ \ (x + y = 10) \quad (2)$$

According to Jocelyn [9], H_2S is formed if the thiol present is in excess (eqn 3) and an polysulfide if sulfur is present in excess amounts (eqn 4):

$$2\ RSH + 1/8\ S_8 \rightarrow H_2S + RSSR \quad (3)$$

$$2\ RSH + n/8\ S_8 \rightarrow S_{n-x}^{2-} + 2\ H^+ + RS_xR \text{ (sum of reactions 1 and 2)} \quad (4)$$

Reactions 1-4 already proceed conveniently at temperatures of 20-60° C. The dimethyl polysulfide with ten S atoms is very unstable [10]. Organic polysulfides tend to decompose by equilibrium with other chain lengths or by the formation of elemental sulfur, even after pure organic polysulfides have been obtained. Species with a sulfur chain length between 2 and 8 can be expected [7] (eqn 5 and 6):

$$2\ R_2S_n \leftrightarrows R_2S_{n+x} + R_2S_{n-x} \quad (5)$$

$$R_2S_{n+x} \leftrightarrows R_2S_n + x/8\ S_8\ (x = 6\text{-}8) \quad (6)$$

Reactions 5 and 6 are accelerated by light, heat, and numerous catalysts, of which strong nucleophiles (*e.g.* RS^-, HS^-, S_x^{2-}) are the most effective. They can occur through ionic species or at a catalyst surface as well as by a radical mechanism [11]. Obviously, after the reaction between MT and sulfur has proceeded for some time, a very complex mixture of (di)methyl polysulfides, polysulfides and nucleophilic species will be formed.

The goal of this paper is to study the chemical reaction between MT and bio-sulfur to gain more insight in the formation of (dimethyl) polysulfides during biological desulfurization of natural gas and LPG. A novel approach is followed to directly measure polysulfids and organic polysulfides by high-pressure liquid chromatography (HPLC), as described by Kamyshny *et al.* [12]. This paper reports on the effects of environmental parameters (MT and bio-sulfur concentration, pH and temperature) on the initial reaction rate and on the distribution of the (dimethyl) polysulfides formed in the reaction mixture.

Material and methods

Elemental sulfur

Bio-sulfur was obtained from a full-scale bioreactor of the wastewater treatment plant of Industriewater Eerbeek (Eerbeek, The Netherlands) that removes H_2S from anaerobically produced biogas, prior to its incineration in a gas-engine for electricity production. The bio-sulfur particles are excreted by chemotrophic *Thiobacillus* species [13] and most likely consist of S_8 rings [14]. The bio-sulfur suspension (30 g/L) was dialyzed in demineralized water to remove salts (mainly sodium bicarbonate and sodium sulfate) until the conductivity of the sulfur suspension was below 40 $\mu S \cdot cm^{-1}$. The bio-sulfur suspension has a particle size distribution between 0.01 and 100 μm (Fig. 7.1), the average diameter of the sulfur particles was 18.7 μm and the density of the bio-sulfur was estimated to be 1.7 g/cm^3 [14]. The specific surface area (A_s) of the sulfur particle was A_s = 0.19 m^2/g. In the experiments, bio-sulfur concentrations varied between 1 and 16 mM. Hence, the initial concentration of the surface (A_c) varied between 6 and 97 m^2/m^3.

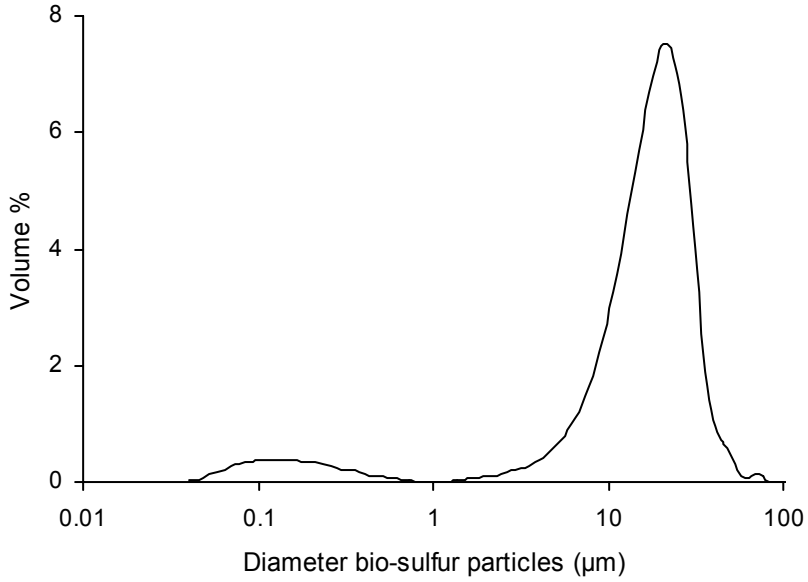

Fig. 7.1. Particle size distribution of the dialyzed bio-sulfur particles.

Experimental set-up

In a thermostated 500-ml round bottom flask (30-60°C), bio-sulfur was mixed with a (bi)carbonate buffer solution and was nitrogen flushed. Deaerated solutions of 20 g/L $NaHCO_3$ (ionic strength I = 0.24 M) and 23 g/L Na_2CO_3 plus 7 g/L $NaHCO_3$ (I = 0.73 M) were used as buffer solutions of pH 8.7 and 10.3, respectively. The MT solution was supplied to the flask through a septum to prevent oxygen intrusion. The contents of the glass flask was pumped through a flow-through quartz cuvette and the UV absorbance (λ = 285 nm) was continuously measured during the course of the experiments (15-25 minutes). The total liquid volume for each experiment was 200 ml. The MT and bio-sulfur concentration both varied between 1 and 16 mM (bio-sulfur concentration in moles of sulfur atoms per liter). Compared to the total reaction time, the mixing time is considered to be negligible. The total pump tube volume was 8.0 mL, and the pump speed was 7 ml·min^{-1}; the retention time of the liquid outside the flask was approximately 70 s.

To identify the reaction products after increased reaction time (2-3 hours), separate anaerobic batch tests were performed in 120- and 250-ml flasks. Bio-sulfur was dissolved in deaerated buffer (20 g/L $NaHCO_3$), after which the flasks were sealed with viton stoppers. Before MT or DMDS (dimethyl disulfide) was added, the headspace of the flasks was flushed with nitrogen gas to remove all oxygen. Liquid samples were taken with a syringe through the stopper. During the experimental period, the flasks were continuously shaken (120 rpm, 30°C).

Analysis

The total of all produced polysulfides and (di)methyl polysulfides was determined indirectly by spectrophotometry (Perkin-Elmer, Lambda 2, UV/VIS-spectrophotometer) at a wavelength of 285 nm [14]. At this wavelength, MT and bio-sulfur solutions did not show any significant absorbance at all concentrations tested (*i.e.* $A_{285} < 0.1$) compared to the medium solution (data not shown). A linear relationship between the measured absorbance (A_{285}) and the concentration of the products was found till $A_{285} \approx 2.5$.

In addition, MT and dimethyl polysulfides were detected in undiluted samples by HPLC (Separations, Hendrik Ido Ambracht, The Netherlands) with a Chrompack (Varian Chrompack International, Bergen op Zoom, The Netherlands) C-18 column of 20 cm length. The oven temperature was 30°C. The eluent flow rate was 0.6 ml/min. The injection volume of the samples was 20 µl, and an ultraviolet detector (Gynotek, Germering, Germany) was used to monitor MT and dimethyl polysulfides simultaneously at wavelengths of 220, 230, 265 and 285 nm. The eluent consisted of 40% methanol and 60% water (method A) or 70% methanol and 30% water (method B). Only with method A, MT could be detected. With method B the MT peak overlapped with the peak of thiosulfate formed from (poly)sulfide oxidation during sampling/analysis. With method B, dimethyl polysulfides till CH_3-S_8-CH_3 could be identified within a retention time of 50 minutes. The composition of the reaction mixtures was confirmed by solutions of pure reference compounds (MT, DMDS, DMTS [dimethyl trisulfide]) and by the dependence of the retention time of the dimethyl polysulfides on the number of sulfur atoms in the molecule. The correlation of the logarithm of the chromatographic retention time, ln(Rt), with the number n of sulfur atoms in the molecule is very strong for homological R-S_n-R lines, where R is an alkyl group [12, 15]. The dimethyl polysulfides could also be identified in the HPLC chromatogram by their characteristic UV spectra between 200 and 400 nm and by the ratio of the peak areas at the four wavelengths measured. Concentrations of DMDS and DMTS were calculated after calibration with standard solutions. More complex dimethyl polysulfides with four or more sulfur atoms are less stable and not commercially available. Therefore, concentrations of dimethyl polysulfides from $CH_3S_4CH_3$ up to $CH_3S_8CH_3$ were calculated from calibration curves of a K_2S_5 solution. The K_2S_5 solution disproportionates to an equilibrium mixture with a known distribution of S_3^{2-} up to S_8^{2-} polysulfide ions. After methylation of these polysulfides with methyl trifluoromethanesulfonate (methyl triflate) to dimethyl polysulfides the individual concentrations were related to the peak areas on a HPLC chromatogram as shown by Kamyshny *et al.* and Rizkov *et al.* [12, 15]. This rapid chemical methylation of polysulfides is a novel approach to determine polysulfide concentrations. The K_2S_5 solution and reaction samples (0.1 ml) were dissolved in 0.8 ml methanol together with 0.1 ml methyl triflate. The polysulfides are rapidly methylated by the methyl triflate and measured by HPLC as dimethyl polysulfides. Unfortunately, this method is not suitable to distinguish between S_x^{2-} and $CH_3S_x^-$ as both are methylated to $CH_3S_xCH_3$ by the excess amount of methyl triflate.

The total sulfide concentration includes free sulfide and polysulfides [16]. At the experimental pH of 8.7 and 10.3, the main sulfide species are HS^- and S_x^{2-}. The method used was based on a modified methylene blue method as described by Trüper and Schlegel [17]. Immediately after sampling, 1 ml zinc acetate (20 g/L) per ml of sample was added to prevent oxidation of sulfide. The formed precipitate was diluted with demineralised water. The total sulfide concentration in the diluted zinc sulfide precipitate was measured with the Dr Lange cuvette test LCK653 (Hach Lange, Germany). Bio-sulfur was determined by measuring the dry weight after filtration over a 0.45 μm filter and drying at 50°C for 24 hours. The particle size distribution of the obtained bio-sulfur suspension was determined by laser scattering image analysis (Coulter laser LS 230, Beckman Coulter USA).

Calculation of reaction rate

The slope of the absorbance curve ($\Delta A_{285}/\Delta t$) is used to determine the reaction rate of the initial reaction between bio-sulfur and MT. The initial slope is calculated in the linear part of the curve, *i.e.* after passing the dead time slot of about 30 seconds. Because of the poor solubility of S_8 rings, the reaction will essentially take place at the surface of the sulfur particles [14]. In this heterogeneous reaction, the reaction kinetics depend on the available surface area of the sulfur particles and thus on the particle size distribution.. Therefore, the surface concentration, A_c (m^2/m^3) was used as a key parameter to calculate the reaction order and the reaction rate constants.

Chemicals

A 3.0 M sodium methylmercaptide ($NaCH_3S$) solution supplied by Arkema (Rotterdam, The Netherlands) was used as the methanethiol-containing reagent. The solution was made from gaseous methanethiol, dissolved in a high-purity NaOH solution. The gas contained only a few impurities, *i.e.* < 0.7% methanol, < 0.3% DMS and some trace amounts of DMDS. $NaHCO_3$ and Na_2CO_3 (>99.7% pure) and liquid DMDS, DMTS, ethanethiol and propanethiol (all > 98.5% pure) were purchased from Merck (Darmstadt, Germany). Methyl trifluoromethanesulfonate (methyl triflate) was purchased from Acros Organics, Pittsburgh, PA.

Results

To study the formation rate and selectivity of (dimethyl) polysulfides formation from the reaction between bio-sulfur and MT, experiments were carried out with varying MT and bio-sulfur concentrations at temperatures between 30°C and 60°C and at pH 8.7 and 10.3.

Direct determination of reaction products

Batch experiments were performed (30°C, pH 8.7) to determine the concentration and sulfur chain length of the products of the reaction between bio-sulfur and MT. Different MT/S^0 ratios were tested (Table 7.1). In all three experiments, MT was completely removed within 45 minutes, after which an equilibrium was reached between the various reaction products (Fig. 7.2). The main reaction products were sulfides (HS^- and S_x^{2-}), DMDS ($CH_3S_2CH_3$) and DMTS ($CH_3S_3CH_3$). Also some long-chain dimethyl polysulfides ($CH_3S_{4\text{-}7}CH_3$) were formed at trace amounts levels (Table 7.1).

In experiment A, the solution colored yellow during the first 5-10 minutes of the experiment, but after 20 minutes the solution became colorless again. As monomethyl polysulfides ($CH_3S_x^-$) and polysulfides (S_x^{2-}) are known for their yellow color [4, 18] and dimethyl polysulfides ($CH_3S_xCH_3$) are colorless, this indicates that during the start of the reaction first (monomethyl) polysulfides were formed (eqn 1 and 2), which were further methylated within the next 20 minutes, *e.g.* (eqn 7):

$$S_x^{2-} + 2\ CH_3SH \leftrightarrows CH_3S_xCH_3 + 2\ HS^- \qquad (7)$$

The total sulfide in the equilibrium mixture at the end of experiment A must be mainly attributed to HS^-, as the presence of polysulfides would color the solution yellow till concentrations as low as 0.04 mM S_5^{2-} (data not shown).

In contrast to experiment A, the yellow color formed during the first 5 minutes of experiment B and C, remained till the end of the experiment. This indicates that polysulfides remained present during the entire experimental period (120-150 minutes). The two times higher bio-sulfur concentration in experiment B as compared to A, also resulted in a higher dimethyl trisulfide concentration (Table 7.1). In experiment C, only half of the initial MT was added compared to experiments A and B and about two times less total sulfide was formed (Table 7.1, Fig. 7.2).

Treatment of diluted samples with methyl triflate at the end of experiments B and C to distinguish between (methyl) polysulfides and dimethyl polysulfides, showed that S_3^{2-}, S_4^{2-}, S_5^{2-} and S_6^{2-} or their methyl derivates ($CH_3S_x^-$) were present in the reaction mixture (Table 7.1).

Table 7.1 Maximum product concentration in batch experiments (pH 8.7, 30ºC).

Reagents	Exp. A	Exp. B	Exp. C
Bio-sulfur (mM S^0)	8.0	16	16
Methanethiol (mM)	8.0	8.0	4.0
Products			
MeS_2Me (mM)	2.53	1.96	0.98
MeS_3Me (mM)	1.18	3.04	2.1
MeS_4Me (mM)	0.06	0.16	0.16
MeS_5Me (mM)	0.01	0.04	0.04
MeS_6Me (mM)	0.002	0.006	0.01
MeS_7Me (mM)	n.d.	0.001	0.001
Total sulfide (mM)	3.50	3.34	1.59
$CH_3S_2^-$ or S_2^{2-}	n.a.	n.d.	n.d.
$CH_3S_3^-$ or S_3^{2-}	n.a.	+	+
$CH_3S_4^-$ or S_4^{2-}	n.a.	+	+
$CH_3S_5^-$ or S_5^{2-}	n.a.	+	+
$CH_3S_6^-$ or S_6^{2-}	n.a.	n.d.	+

n.d. not detected; n.a. not analysed; + detected, only qualitative analysis possible

Effect of the bio-sulfur concentration on the initial reaction rate

Figure 7.3A shows an example of an absorbance curve recorded from the reaction of bio-sulfur with MT at 50°C and pH 8.7. After passing a dead time period of about 30 seconds, the absorbance rapidly increased due to the formation of (methyl) polysulfides. Higher bio-sulfur concentrations resulted in increased reaction rates and higher plateau values, indicating higher concentrations of (methyl) polysulfides. As the initial S_8 ring opening (eqn 1) is the rate limiting step, the increase in absorbance is very likely the combined effect of equation 1 and the subsequent reactions (eqn 2-4).

At the highest bio-sulfur concentration tested (16 mM), the A_{285} plateau value is outside the linear region of the calibration curve ($A_{285} > 2.5$). The results can however still be used as the kinetic calculations are based on the values below $A_{285} = 2.5$ At lower concentrations, the absorbance decreased to a lower plateau value after a reaction time of about 12 minutes (Fig. 7.3A). Lower initial bio-sulfur concentrations result in relatively more short-chain (dimethyl) polysulfides (*e.g.* DMDS) in the reaction mixture (Table 7.1, Fig. 7.2). These short-chain (dimethyl) polysulfides have a lower specific relative UV response [12].

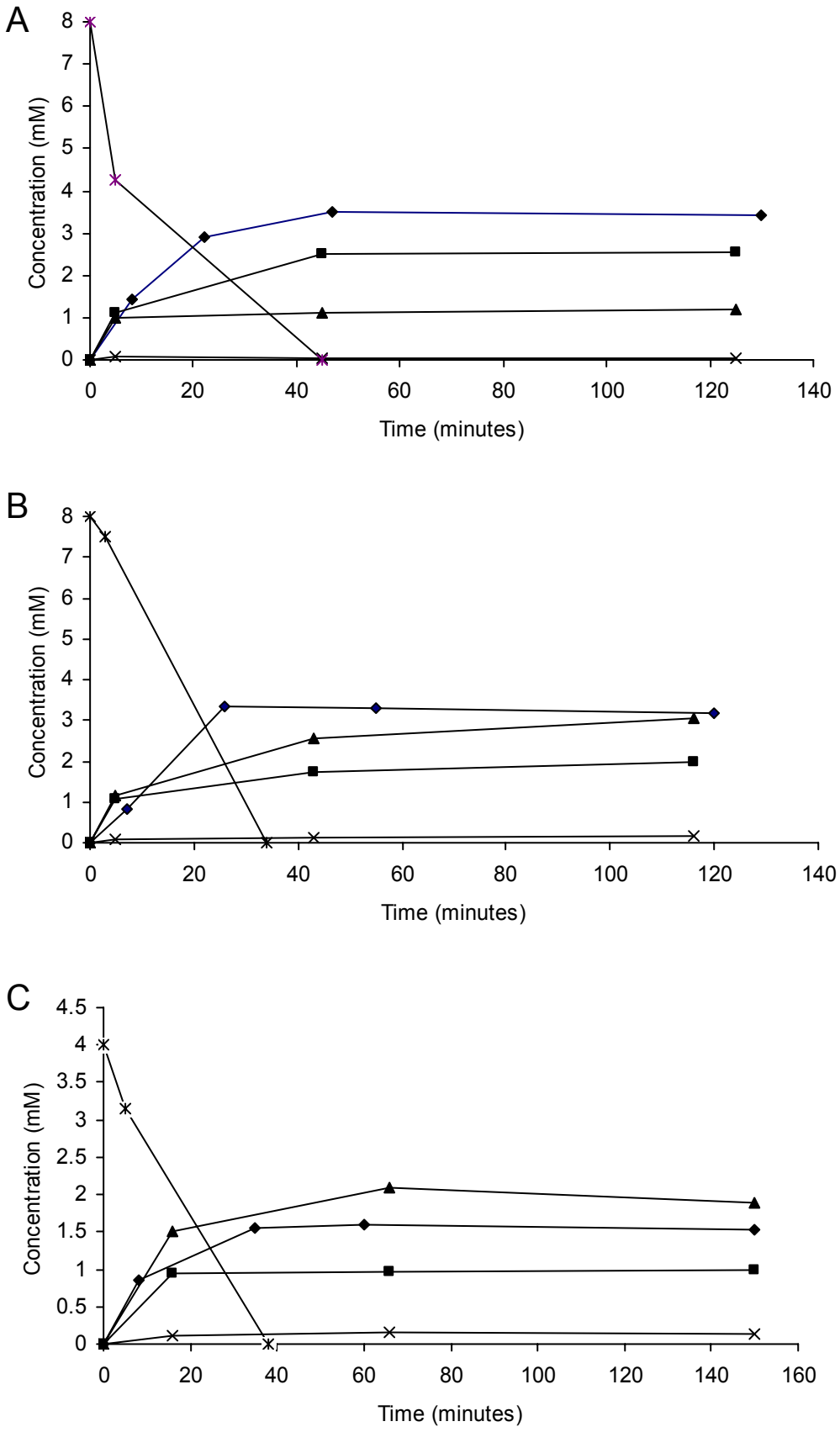

Fig. 7.2. Formation of dimethyl polysulfides (30°C, pH 8.7) in a mixture with 8 mM S^0 and 8 mM MT (A), 16 mM S^0 and 8 mM MT (B) and 16 mM S^0 and 4 mM MT (C); -*- MT, -♦- H_2S total, -■- DMDS, -▲- DMTS, -×- DMTeS ($CH_3S_4CH_3$).

Table 7.2 shows the effect of the bio-sulfur concentration on the initial reaction rate for all temperatures tested (30-60°C) at pH 8.7 and 10.3. An increase in both bio-sulfur concentration and reaction temperature enhance the initial reaction rate. As a result, no bio-sulfur particles could be observed at pH 8.7 after the 20 minutes reaction time at 50°C and 60°C. At the lower temperature experiments (30°C and 40°C) in combination with the highest bio-sulfur concentrations (8 and 16 mM), bio-sulfur particles were still present as could be seen from the residual turbidity in the reaction flask. At pH 10.3, no residual sulfur was observed after the course of the 15 minutes experimental period. Mixtures containing 4 and 8 mM bio-sulfur colored yellow during the experiment indicating that polysulfides were present.

Figure 7.3B shows the effect of the bio-sulfur concentration on the total amount of formed sulfide after a 20 minutes reaction time (pH 8.7, 50ºC). After this period, the reaction mixtures became clear and colorless (2 and 4 mM bio-sulfur) or clear and yellow (8 and 16 mM bio-sulfur). It is known that the intensity of the color depends on the chain length and the concentration of the polysulfide. Up to about 6-7 mM bio-sulfur MT is in excess and equation 3 applies, *i.e.* no formation of polysulfides and hence no discoloration is observed . At bio-sulfur concentrations exceeding 6-7 mM, the total sulfide concentration ($[HS^-] + [S_x^{2-}]$) in the reaction mixture did not increase anymore and equation 4 applies. At 16 mM bio-sulfur the yellow color was more intense than at 8 mM bio-sulfur, indicating that the average chain-length of the polysulfides formed was higher at 16 mM bio-sulfur. The polysulfides are formed through reactions 2 and 4 or by the reaction of H_2S with residual bio-sulfur (eqn 8) [14]:

$$HS^- + x/8\ S_8 \leftrightarrows S_{(x+1)}^{2-} + H^+ \qquad (8)$$

At excess bio-sulfur (> 6-7 mM), [total sulfide] ≈ ½ [MT] (Fig. 7.3B), which is in agreement with equation 4. Also in Figure 7.2, the $MT/Sulfide_{tot.,max}$ ratios were close to 2. Theoretically, MT should already be in excess from 4 mM bio-sulfur onwards instead of 6-7 mM (eqn 3 and 4). Although not measured, we assume that not all MT had reacted at bio-sulfur concentrations of 2 and 4 mM within the course of the 20 minutes experimental period. Hence, the total sulfide concentration in the mixture was still increasing. Figure 7.2 confirms that the maximum total sulfide concentration was only reached after 20-45 minutes, although this was measured at slightly different conditions (30ºC and different bio-sulfur- and MT-concentrations).

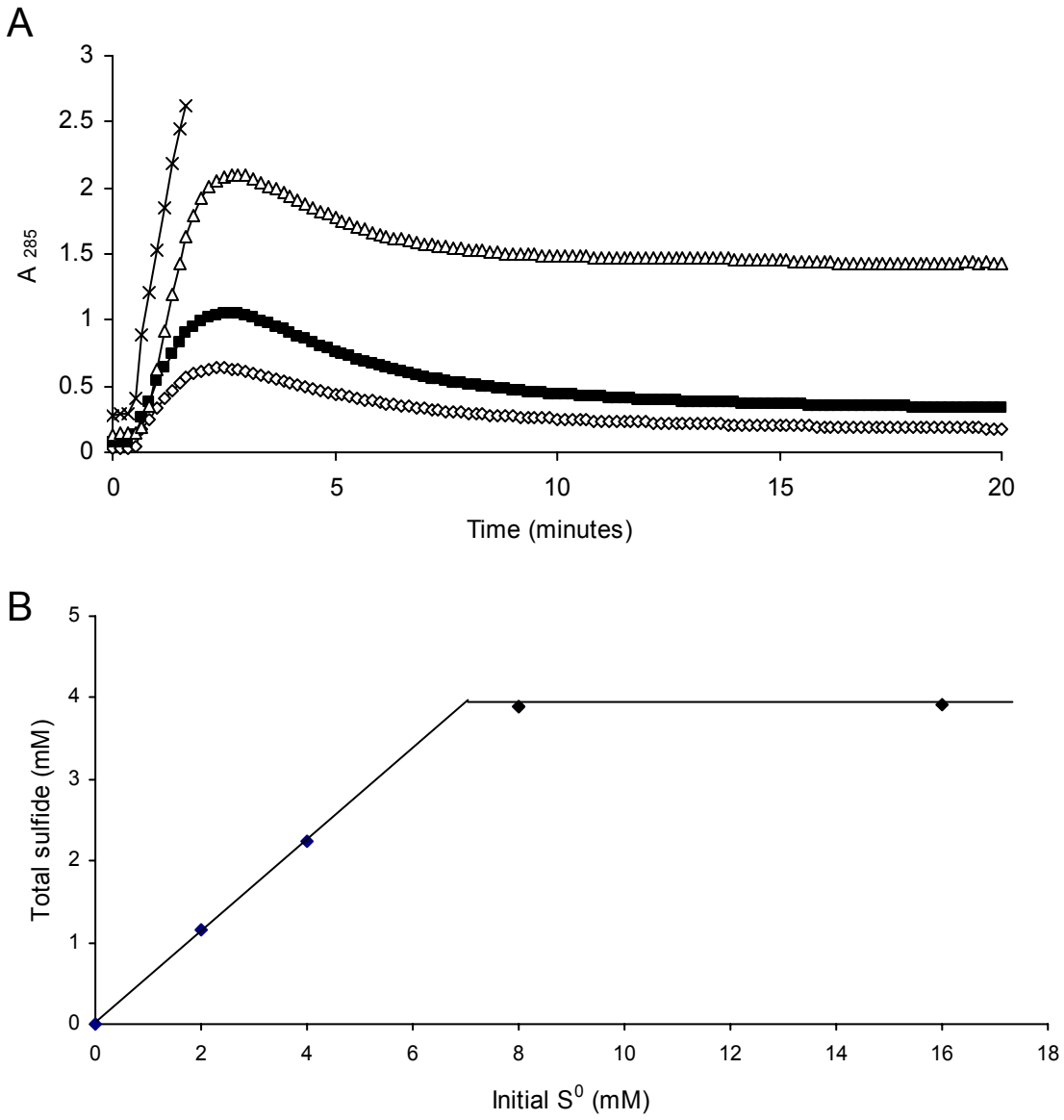

Fig. 7.3. The effect of the bio-sulfur concentration [in mM:16 (×), 8 (Δ), 4 (■) and 2 (◊)] on the absorbance curves (MT_0 = 8.0 mM, pH 8.7, 50°C) (A); the effect of the initial bio-sulfur concentration on the total sulfide formation after 20 minutes reaction time (50°C, pH 8.7, MT_0 = 8 mM) (B).

Table 7.2. Overview of the effect of the MT and bio-sulfur concentration, pH and temperature on the initial reaction rate, R_x ($\Delta A_{285}/\Delta t$ [min^{-1}]).

MT (mM)	bio-sulfur (mM)	R_x (30ºC)	R_x (40ºC)	R_x (50ºC)	R_x (60ºC)
pH 8.7					
4	8	0.068	-	0.26	-
6	8	0.23	-	0.75	-
16	8	1.94	-	3.29	-
8	2	0.089	0.25	0.42	0.81
8	4	0.18	0.47	0.68	1.06
8	8	0.38	0.73	1.50	2.22
8	16	0.48	1.09	2.17	3.19
pH 10.3					
1	4	-	0.27	-	0.54
2	4	-	0.57	-	0.81
8	4	-	-	-	2.84
4	1	0.38	0.52	0.61	0.66
4	2	0.65	0.68	0.81	0.91
4	4	1.01	1.06	1.33	1.53
4	8	1.51	1.73	2.36	2.56

-: not measured

Effect of the MT concentration on the initial reaction rate

The effect of the MT concentration on the initial reaction rate was investigated in the 30°C-60°C temperature range (Fig. 7.4, Table 7.2). As expected from non-zero order reaction kinetics, higher MT concentrations resulted in increased initial reaction rates. The maximum recorded values for A_{285} are almost about similar for all MT concentrations (Fig. 7.4), which is in contrast to the experiments with varying initial bio-sulfur concentrations (Fig. 7.3A). Therefore, it seems that the bio-sulfur particles concentration determines the maximum A_{285}, *i.e.* more bio-sulfur results in more product (higher absorbance). Increased MT concentrations enhance the initial reaction rate and the subsequent reaction steps. At the highest MT concentration the absorbance curve rapidly levels off to a lower equilibrium value (Fig. 7.4). This behavior can be explained by the formation of short-chain (dimethyl) polysulfides (S_2, S_3) from the long-chain (dimethyl) polysulfides, viz. short-chain compounds have a lower specific relative UV response, since the absorbance is proportional to the concentration of polysulfide excess sulfur atoms in the polysulfide molecule (x-1 mol S^0 in S_x^{2-}) [12, 19].

The mixture containing 16 mM MT (Fig. 7.4) was clear and colorless after the 20 minutes experiment. This shows that all bio-sulfur has reacted and dimethyl polysulfides were formed. The mixtures containing 4 and 6 mM MT became yellow and still contained some

bio-sulfur particles in suspension after the 20 minutes reaction time. This indicates that polysulfides were present in the 4 and 6 mM MT mixtures after 20 minutes.

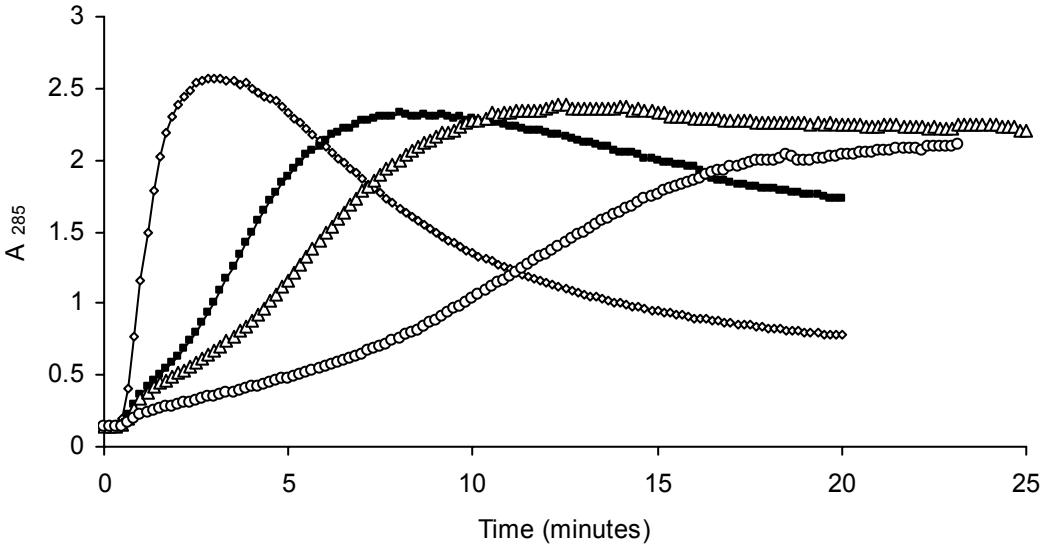

Fig. 7.4. The effect of the MT concentration [in mM:16 (◊), 8 (■), 6 (Δ) and 4 (○)] on the absorbance curve (S^0 = 8.0 mM, pH 8.7, 30°C).

Long term stability

To test the long term A_{285} stability of reaction mixtures with MT and bio-sulfur, the absorbance of two mixtures containing 5 or 10 mM MT in the presence of 2 mM bio-sulfur was measured overnight (20°C, pH 8.7). In case of 5 mM MT, the maximum absorbance (A_{285} = 1.2) was reached after 33 minutes and this value did not decrease during the experimental time of 1065 minutes (data not shown). In case of 10 mM MT, the maximal absorbance (A_{285} = 1.2) was reached after 12 minutes and subsequently dropped down to 0.47 after 196 minutes. After 1100 minutes the absorbance had only slightly decreased to 0.44, showing that the mixture was already in close equilibrium after 196 minutes. The lower equilibrium absorbance at 10 mM MT can be explained by the formation of higher DMDS and H_2S concentrations, according to equation 3, which have a lower absorbance at 285 nm than the longer chain (dimethyl) polysulfides.

Reaction between DMDS and sulfur

DMDS (7.3 mM) hardly reacted with bio-sulfur (8 mM) during a 103 minutes test (pH 8.5, 30°C; data not shown). Afterwards, 6.5 mM DMDS and 80% of the initial bio-sulfur was recovered. The A_{285} value was constant during the experimental time and no discoloration was observed. From literature it is known that the reaction between DMDS and elemental sulfur without the presence of a catalyst is very slow [11, 20]. In the presence of H_2S and a catalyst

(*e.g.* amines), DMDS dissolves elemental sulfur by formation of a mixture of dimethyl polysulfides [8, 11, 20, 21].

Determination of the reaction order and activation energy

The initial reaction rate (R_x) of the reaction between bio-sulfur an MT (eqn 1) is assumed to follow the general rate law:

$$R_x = -\left(\frac{d[CH_3SH]}{dt}\right)_{t=0} = -\left(\frac{d[S_8]}{dt}\right)_{t=0} = k[CH_3SH]^{\alpha}[S_8]^{\beta} \quad (9)$$

Reaction orders α and β were determined at pH 8.7 and 10.3, using the differential method. The data from Table 7.2 were plotted on a log-log scale (Fig. 7.5). The reaction order with respect to MT and bio-sulfur at pH 8.7 are α = 2.1 ± 0.4 and β = 0.76 ± 0.07. The bio-sulfur concentration is expressed as active surface area (Ac). The reaction order with respect to MT is stronger affected by pH than the reaction order with respect to the bio-sulfur concentration (Table 7.3). The specific reaction constants (k) at pH 8.7 were determined from Fig. 7.5A and 7.5B, where the y-axis cut off is log k + β log $[S]_0$ and log k + α log $[MT]_0$, respectively. The Arrhenius equation (eqn 10) was used to calculate the activation energy for the initial reaction at pH 8.7 and pH 10.3 by plotting the reciprocal temperature (1/T) against ln(k) (Fig. 7.6, Table 7.3).

$$\ln(k) = A - E_{act}/RT \quad (10)$$

It follows that the activation energy at pH 8.7 is 4-5 times higher than at pH 10.3. The reason for this is that at pH 10.3 about 50% of the MT is present in the dissociated form (CH_3S^-), whilst at pH 8.7 this is only 2%. CH_3S^- is a stronger nucleophile than molecular MT, which results in higher reaction rate constants (k) and a lower activation energy (E_{act}).

Table 7.3. Summary of kinetic data of the initial reaction of MT with bio-sulfur.

pH	Reaction order in MT, α	Reaction order in bio-sulfur, β	E_{act} (kJ·mol^{-1})
8.7	2.1 ± 0.4	0.76 ± 0.07	70
10.3	0.89 ± 0.12	0.64 ± 0.04	16

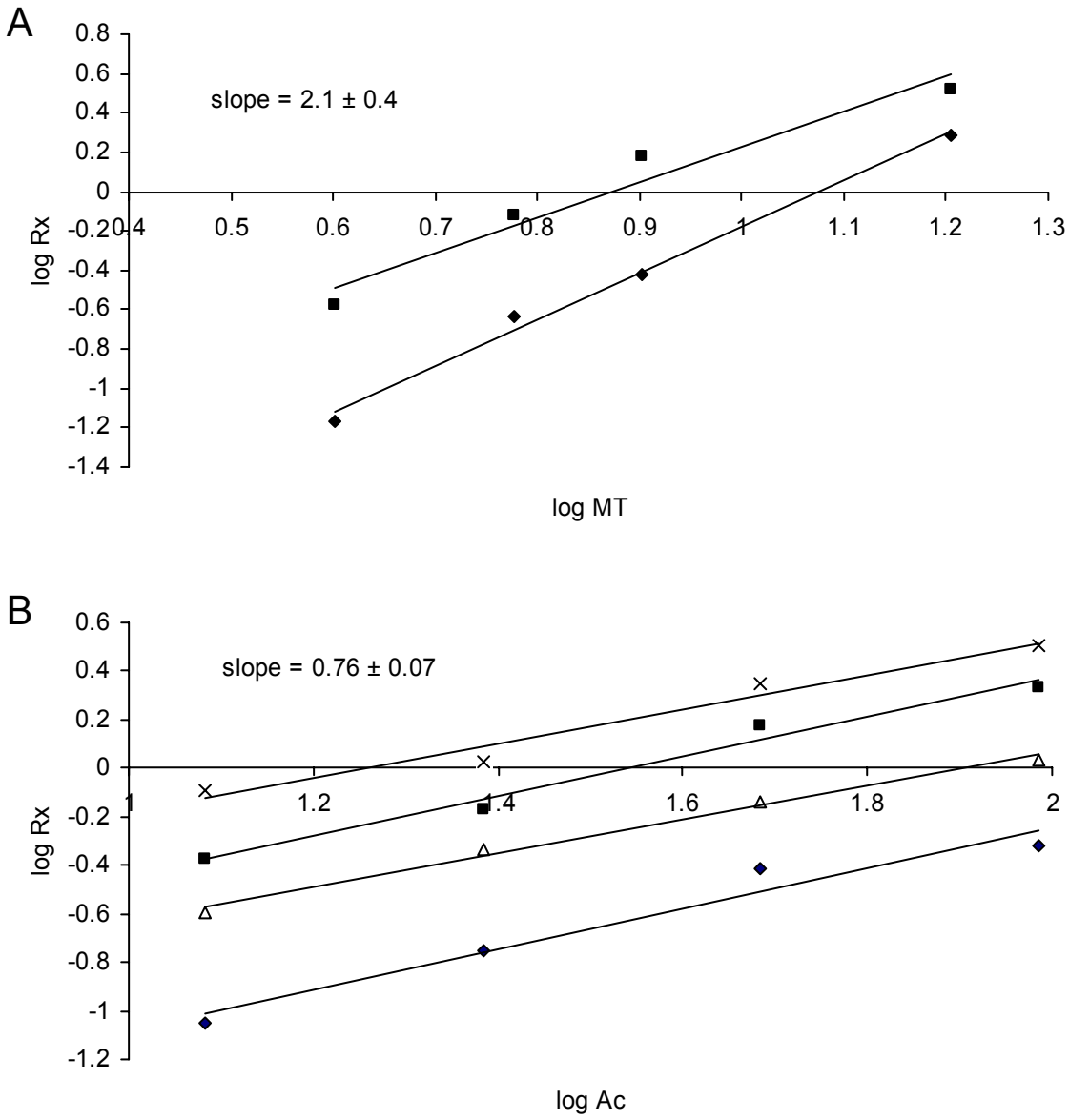

Fig. 7.5 Determination of the reaction order α in MT (pH = 8.7, S_0 = 8.0 mM [48.4 m^2/m^3]) (A) and β in bio-sulfur (pH 8.7, MT_0 = 8.0 mM) (B); α = 2.1±0.4 and β = 0.76 ± 0.07; ♦ 30°C, Δ 40°C, ■ 50°C, × 60°C.

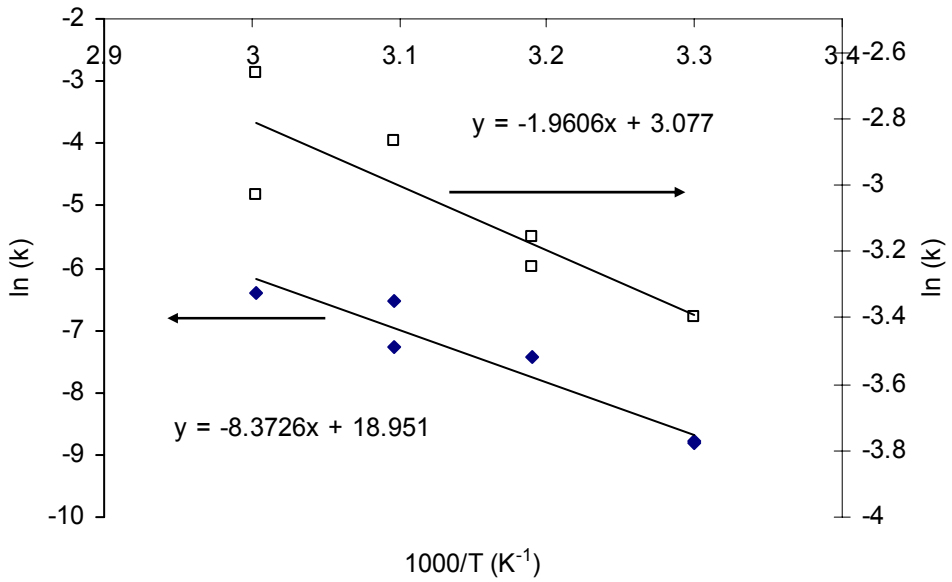

Fig. 7.6 Arrhenius plot for the determination of the activation energy. From the slope ($-E_{act}/R$), the activation energy is calculated at 70 kJ·mol^{-1} (pH 8.7, □) and 16 kJ·mol^{-1} (pH 10.3, ♦).

Discussion

Reaction mechanism

In this study, the reaction kinetics and formed products of the reaction between methanethiol and bio-sulfur were investigated in a (bi)carbonate buffered aqueous solution. This study shows the effect of the initial MT and bio-sulfur concentration, pH and temperature on the reaction rate and the formed products from this reaction.

The overall reactions (eqn 3 and 4) between thiols and elemental sulfur were already described some decades ago [4, 9]. Alkyl tri- and alkyl tetrasulfides can be conveniently prepared from thiols and elemental sulfur in the presence of catalytic amounts of n-butylamine at 25-63°C [8]. However, the process described in this thesis is not based on an amine catalyst. The molar thiol/S^0 ratio, the polarity of the solvent, the reaction time, the temperature, as well as the nature of the rest group of the thiol determine the products formed [4, 22]. In addition, also the pH plays a role in the product formation.

In our experiments, at a molar MT/S^0 ratio of 1.0, the DMDS concentration was higher than the DMTS concentration (Fig 7.2A), while at a MT/S^0 ratio of 0.50 (Fig. 7.2B) and 0.25 (Fig. 7.2C) DMTS was formed in higher concentrations than DMDS. According to Vineyard [4] high DMDS yields can be obtained at a molar thiol/S^0 ratio of 2.5 and DMTS at molar thiol/S^0 ratios of at least 1.25. If the molar thiol/S^0 ratio is appreciably less than 1.25, a mixture of (dimethyl) polysulfides is obtained [4].

In our experiments, dimethyl tetrasulfide was a minor reaction end-product (Fig. 7.2) in a reaction with the sterically unhindered methanethiol. According to Vineyard [4, 22] only considerable amounts of alkyl tetrasulfide can be formed with a sterically hindered thiol (*e.g.*

t-butyl thiol) at a molar thiol/S^0 ratio greater than stoichiometric (2-3) for alkyl tetrasulfide formation.

After the reaction between MT and bio-sulfur has proceeded for some time, a complex mixture of (dimethyl) polysulfides and nucleophilic species is formed. The relative strength of various nucleophiles ($S_x^{2-} > CH_3S^- > HS^-$), which determines the position of the equilibrium, may be given by their thiophilicity [7]. Taking into account the preceding discussion and the measured end-products in our experiments (Table 7.2), the following reaction mechanism is proposed for our experiments:

$$CH_3SH + S_8 \leftrightarrows CH_3S_9^- + H^+ \quad (1)$$
$$CH_3SH + CH_3S_9^- \leftrightarrows CH_3S_5CH_3 + S_5^{2-} + H^+ \quad (11)$$
$$CH_3SH + CH_3S_9^- \leftrightarrows 2\ CH_3S_5^- + H^+ \quad (12)$$

$CH_3S_5CH_3$ and S_5^{2-} (eqn 11) are more stable end-products than $CH_3S_5^-$. There is no evidence for the existence of methyl polysulfides longer than $CH_3S_2^-$ [8, 23], however $CH_3S_5^-$ can be formed as an intermediate and reaction 12 might also occur to a minor extent. The nucleophile CH_3SH can also break the sulfur chain at another place resulting in products with a different chain length. In addition, the formed nucleophiles – (methyl) polysulfides – can react with the residual bio-sulfur to form intermediates temporary exceeding a chain length of S_9.

An excess amount of MT results in higher DMDS yields and shorter-chain (dimethyl) polysulfides [4, 22] (Fig. 7.2). Also hydrogen sulfide (H_2S) is formed in subsequent reactions:

$$CH_3S_5CH_3 + CH_3SH \leftrightarrows CH_3S_3^{2-} + CH_3SSSCH_3 + H^+ \quad (13)$$
$$S_5^{2-} + CH_3SH \leftrightarrows CH_3S_3^{2-} + S_3^{2-} + H^+ \quad (14)$$
$$S_5^{2-} + CH_3SH \leftrightarrows CH_3S_5^{2-} + HS^- + H^+ \quad (15)$$
$$CH_3S_5^- + CH_3SH \leftrightarrows HS^- + CH_3S_5CH_3 \quad (16)$$
$$CH_3S_5^- + CH_3SH \leftrightarrows CH_3SSCH_3 + S_4^{2-} \quad (17)$$
$$CH_3S_3^- + CH_3SH \leftrightarrows CH_3SSCH_3 + S_2^{2-} + H^+ \quad (18)$$
$$CH_3S_3^- + CH_3SH \leftrightarrows CH_3SSSCH_3 + HS^- \quad (19)$$

It should be noted that in addition to the proposed reaction mechanism (eqn 11-19) some DMDS might be formed from the direct autooxidation of MT during sampling and HPLC measurement, where traces of oxygen can be present:

$$2\ CH_3SH + ½\ O_2 \rightarrow CH_3SSCH_3 + H_2O \quad (20)$$

This means that the reported DMDS concentrations may be slightly overestimated. Reaction 18 is not likely to occur because no S_2^{2-} could be detected in batch experiment B and C after additions of methyl triflate (Table 7.1). The short chain S_2^{2-} and S_3^{2-} are the strongest

polysulfide nucleophiles and thus the most reactive ones [24]. However, S_2^{2-} and S_3^{2-} have been observed only at very high alkalinities (pH > 14) [14, 25]. In contrast to this, $CH_3S_3^{2-}$ or S_3^{2-} was formed at pH 8.7 in our experiments (Table 7.1).

If individual dimethyl polysulfides are isolated, they disproportionate to smaller dimethyl polysulfides and elemental sulfur. The formed dimethyl polysulfides can exchange sulfur atoms between molecules. This is called interconversion [7], *e.g.*:

$$R_2S_3 + R_2S_5 \leftrightarrows 2\ R_2S_4 \leftrightarrows R_2S_2 + R_2S_6 \qquad (21)$$

Such reactions are reversible and proceed at moderate temperatures (0-120 °C) when compounds with cumulated S-S bonds ([dimethyl)polysulfides, elemental sulfur) are considered. Interconversion reactions are promoted by UV radiation as well as by cationic, anionic, and nucleophilic catalysts, which may be present as impurities. At higher temperatures (50-150°C) dimethyl polysulfides decompose to CH_3SH, CS_2 and H_2S via a number of reaction steps with CH_3SSH and $CH_3SCH_2SSCH_3$ in trace quantities [11, 20]. Interconversion reactions at high temperatures (>150 °C) will probably proceed via a radical chain reaction mechanism [8]. Radical reactions were not likely to occur during our experiments as they were performed at moderate temperature (20-60°C).

Reaction kinetics

The activation energies were determined at 70 kJ·mol^{-1} and 16 kJ·mol^{-1} for the initial reaction of MT with bio-sulfur (eqn 1) at pH 8.7 and 10.3, respectively (Table 7.3). No reference was found for the activation energy of this reaction in literature. To compare: the S-S bond dissociation energy is 150 kJ·mol^{-1} and rotation around S-S bonds in acyclic organic disulfides requires an activation energy of 25-40 kJ·mol^{-1} [7]. For the uncatalyzed reaction between sulfide and elemental sulfur (eqn 8) Kleinjan *et al.* [14] and Hartler *et al.* [26] found an activation energy of 29.6 kJ·mol^{-1} (pH 8.0) and 31.4 kJ·mol^{-1} (pH ≥ 13), respectively. The rate of the reaction at pH > 8 was only to a minor extent influenced by the hydroxide ion concentration. The activation energy for diethyl trisulfide exchange is 121 kJ·mol^{-1}, where only 13 kJ·mol^{-1} is reported for the exchange of linear polythiyl radicals and linear dialkyl polysulfides [27]:

$$R1\text{-}SSS\text{-}R1 + R2\text{-}SSS\text{-}R2 \leftrightarrows 2\ R1\text{-}SSS\text{-}R2 \qquad (22)$$

$$R'S\cdot + R\text{-}S_xR \rightarrow R\text{-}S_xR' + RS\cdot \qquad (23)$$

Obviously, the size of the bio-sulfur particles plays an important role in the observed reaction rates. Large particles have a lower specific surface, and therefore, the reaction rate will be lower than for small particles [14]. This means that subsequent reactions (eqn 11-19) already take place before all bio-sulfur has reacted away. Also other thiophiles ($CH_3S_x^-$, S_x^{2-})

will attack the sulfur rings. The relatively high ionic strength, required for the buffer is not expected to have a large effect on the reaction rate constants, because the rate-determining reaction step (eqn 1) is a reaction between an ion and an uncharged molecule. This type of reaction is generally unaffected by the ionic strength of the medium [14, 28].

Ethanethiol and propanethiol

Batch experiments with bio-sulfur (2-8 mM) and ET (4 mM) or PT (4 mM) (data not shown) gave similar results as experiments with MT. This is in agreement with our expectations, as these are all simple primary thiols. The bio-sulfur dissolved and the solution colored yellow at increased initial bio-sulfur concentrations (8 mM). Reaction products were probably polysulfide, diethyl or dipropyl polysulfide and H_2S. The initial reaction rate increased with pH (8.3 versus 10.3), bio-sulfur concentration and temperature (30°C versus 50°C). In general, one would expect a lower reaction rate of the initial ring opening (eqn 1) and the subsequent reactions due to steric hindrance of longer alkyl groups. According to Vineyard [4], dialkyl disulfides and trisulfides are the main products formed from the reaction between elemental sulfur and any alkyl thiol.

Application

The reaction between thiols and bio-sulfur may enable an enhanced thiol removal from various gaseous and liquid hydrocarbon streams. Some natural gas desulfurization processes are based on the absorption of sulfur compounds (thiols, H_2S) in an aqueous phase and subsequent partial (biological) oxidation of H_2S to elemental sulfur. Part of the sulfur is recycled over the absorber column [16] and (organic) polysulfide formation occurs in the absorber column, which might enhance the removal rate of thiols and H_2S.

Enhanced thiol removal might also occur in the extractor of the biological LPG desulfurization process as previously described. When less than stoichiometric amounts of bio-sulfur are recycled all sulfur might react with H_2S and thiols (*e.g.* eqn 4 and 8) and enhance the extraction to a certain extent. However, while (poly)sulfide dissolves well in water, dimethyl polysulfides have a higher affinity for LPG and the presence of elemental sulfur in the LPG is undesired. More research should therefore be done to investigate whether recycling of some elemental sulfur is beneficial for the extraction process. In contrast to MT, ethanethiol and propanethiol are not degraded anaerobically [2]. Dimethyl polysulfides, which are formed in the extractor or in the aerobic biological reactor of the biological LPG desulfurization process will probably be oxidized to elemental sulfur, (thio)sulfate and CO_2 or less oxidized C_1 compounds, depending on the amount of oxygen in the aerobic bioreactor.

Acronym	Full name	Chemical formula
MT	methanethiol	CH_3SH
ET	ethaenethiol	CH_3CH_2SH
PT	propanethiol	$CH_3CH_2CH_2SH$
-	methyl thiolate	CH_3S^-
DMDS	dimethyl disulfide	CH_3SSCH_3
DMTS	dimethyl trisulfide	$CH_3S_3CH_3$
DMTeS	dimethyl tetrasulfide	$CH_3S_4CH_3$
-	dimethyl polysulfide	$CH_3S_xCH_3$
-	organic polysulfide	RS_xR
-	methyl polysulfide	$CH_3S_x^-$
-	polysulfide	S_x^{2-}
LPG	liquefied petroleum gas	C_3H_8 and C_4H_{10}

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8

Summary and general discussion

8.1. Introduction

The research presented in this thesis focuses on the removal and biological degradation of volatile organic sulfur compounds present in LPG (liquefied petroleum gas). LPG mainly consists of propane and butane and the main sulfur impurities are H_2S, methanethiol (MT), ethanethiol and propanethiol [1, 2]. The total sulfur content of LPG can reach 5000 ppm, but after desulfurization this is generally less than 10 ppm.

Currently, LPG is mainly desulfurized by physical-chemical methods. A caustic extraction step transfers thiols from the LPG phase to an aqueous phase. Subsequently, the thiols are chemically oxidized to water insoluble disulfides, which are skimmed from the aqueous phase and further processed [3].

This thesis describes research on a novel, but already patented [4] LPG desulfurization process, based on anaerobic and aerobic biological degradation of the sulfur compounds with elemental sulfur as the end-product (Fig. 8.1). The research mainly focuses on anaerobic biological degradation of methanethiol to H_2S, CH_4 en CO_2 (chapter 2-6). The extraction step is not studied by laboratory experiments in this thesis, but only by flowsheet simulations. The second biological step (sulfide oxidation) has already been described by Sorokin and Kuenen [5] and the reactor application by Van den Bosch *et al.* [6]. In this chapter, the following topics will be summarized and discussed:

- Ability of different inocula to anaerobically degrade volatile organic sulfur compounds
- Toxicity of volatile organic sulfur compounds for methanogenesis
- Performance of continuously operated reactors for anaerobic methanethiol degradation
- Microbial characterization of the sludge from the MT-degrading reactors
- Chemical reactions between biologically formed sulfur and thiols
- Application of the novel biological process for LPG biodesulfurization

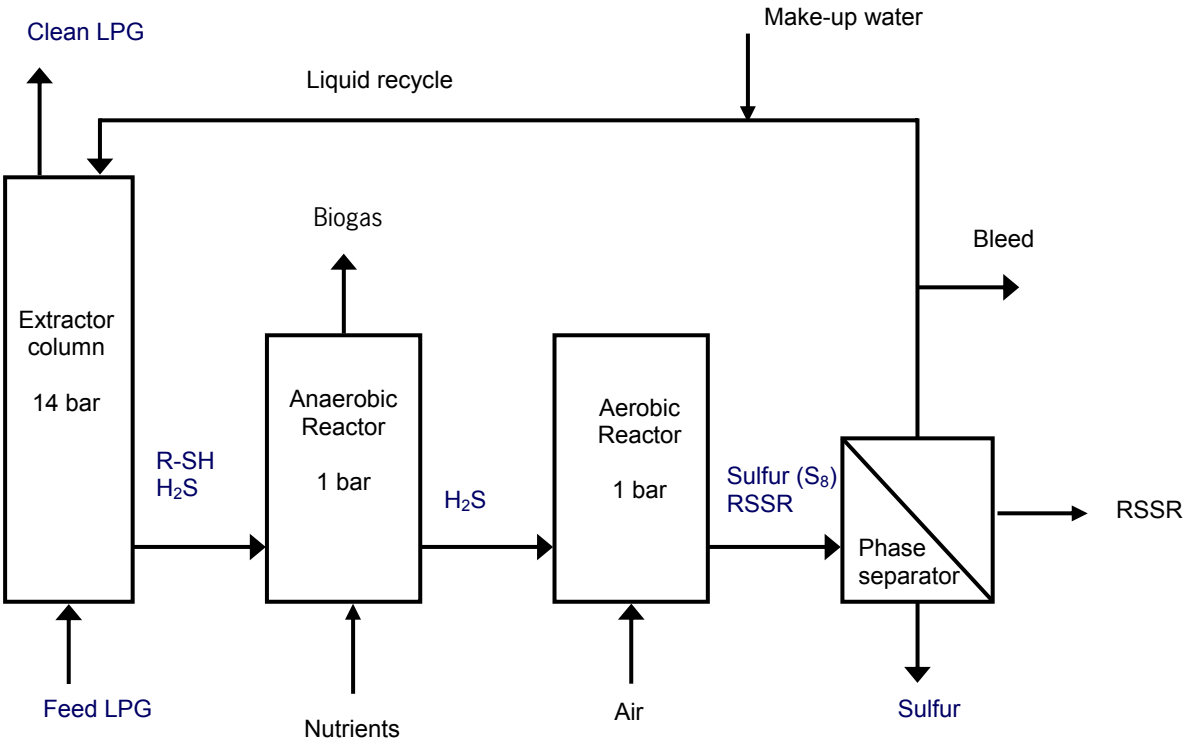

Fig. 8.1. Biological process for LPG desulfurization [4, 7].

8.2. Ability of different inocula to anaerobically degrade volatile organic sulfur compounds

A variety of sludges and sediments samples show anaerobic MT-degrading capacity. These samples include anaerobic (granular) sludge from full-scale reactors, (polluted) harbor sediment and freshwater, salt lake and estuarine sediments. The MT related compounds dimethyl sulfide (DMS) and dimethyl disulfide (DMDS) are degraded by the MT-degrading samples as well. Methanogenic archaea are mainly responsible for the degradation, but in a few cases also sulfate-reducing bacteria are involved as well (chapter 3). Anaerobic degradation of higher thiols (ethanethiol, propanethiol) has not been observed with any of the sludge or sediment samples investigated.

Until now, MT- and DMS-degrading methanogens have been isolated from marine systems and salt lakes (chapter 1) and only one from a freshwater sediment [8, 9]. This thesis (chapter 2 and 3) and recent research by Sipma *et al.* [7] show that anaerobic granular sludge from methanogenic bioreactors degrade MT and DMS as well. MT degradation coupled to sulfate reduction is slower than methanogenic conversion. Complete degradation of 4 mM MT (pH 7.2) by anaerobic granular sludge (Eerbeek) took 4 times longer under sulfate-reducing conditions than under methanogenic conditions. Only three sulfate-reducing bacteria, which can grow on MT or DMS, have been isolated. They have all been isolated from a thermophilic anaerobic digester [10]. Lomans *et al.* [11] suggested that MT degradation under sulfate-reducing conditions occurs via interspecies hydrogen transfer. Sulfate-reducing

bacteria thus need a syntrophic partner for MT-degradation, which would imply that no pure culture can be obtained.

8.3. Toxicity of volatile organic sulfur compounds for methanogenesis

H_2S toxicity for methanethiol degradation

H_2S inhibits methanogenic activity [12]. An initial concentration of 4.3 mM total H_2S (H_2S + HS^-) (pH 7.2) results in an 80% decrease of the methanogenic degradation rate of MT (2 mM) by anaerobic MT adapted granular sludge (chapter 3). Also with MT adapted sediment samples as inoculum (pH 8.3), originating from the Wadden Sea, inhibition of the methanogenic MT degradation occurs at initial H_2S concentration around 3 mM (chapter 4).

Fifty percent inhibition of the methanogenic degradation of acetate (pH 8.0-8.5) occurs at 25 to 30 mM total H_2S [13]. From this, it can be concluded that methanogenic MT degradation is stronger inhibited by H_2S than the methanogenic degradation of acetate. The toxic effect of H_2S is pH dependent [12], because the toxic effect is mainly caused by the non-dissociated H_2S. In order to reduce the H_2S inhibition it is beneficial to operate the anaerobic MT-degrading reactor at pH values exceeding pH 8, as at these pH values, more than 90% of the H_2S will be present in the dissociated form (HS^-).

Toxicity of DMDS on methanethiol degradation

The first step in DMDS degradation is the reduction to 2 molecules of MT. This reduction is probably the rate limiting step in the complete degradation of DMDS to H_2S, CH_4 and CO_2. The complete degradation of DMDS by anaerobic granular sludge (pH 7.2, 30°C) proceeds 2 times slower than complete MT and DMS degradation at concentrations till 2 mM and even about 10 times slower at concentrations between 2 and 8 mM (chapter 3). DMDS inhibits the anaerobic degradation of MT. In a reactor experiment with Wadden Sea sediment as inoculum (pH 8.3, 30°C), the presence of 1 mM DMDS causes incomplete MT degradation. The degradation efficiency decreases from 70% to 40%. During a reactor experiment with salt lake sediments as inoculum (pH 10.0, 30°C), 0.9-1.7 mM DMDS causes a decrease in the MT degradation efficiency of > 90% to below 50%. After the removal of DMDS from the influent, the MT degradation capacity recovers within 3 days (chapter 6). Also Kiene *et al.* [14] find that high DMDS concentrations inhibit methanogenesis. Endogenous methanogenesis of sediment samples from (halo) alkaliphilic environments is stimulated by 1-2 mM DMDS compared to controls without substrate addition. However, at concentrations of 8 mM and higher, DMDS inhibits the endogenous methane formation by at least 80% (22°C, pH 9.7) [14]. The presence of DMDS, because of MT autooxidation, is thus undesired and must be prevented by proper reactor operation and design.

Toxicity of thiols for methanogenesis

Thiols inhibit methanogenic activity above a certain concentration, possibly due to an inactivation of certain enzymes. A thiol-disulfide exchange reaction, in which a thiolate group (RS^-) attacks a sulfur atom of a disulfide bond, is a well known biochemical reaction by which disulfide bonds are formed and broken in proteins [15-17]. However, MT is a substrate for several aerobic and sulfate-reducing bacteria and methylotrophic methanogens as well (chapter 1). The inhibition effect of MT, ethanethiol and propanethiol on methanogenesis of anaerobic granular sludge from methanol, acetate and H_2/CO_2 has been studied in chapter 3. Table 8.1 shows the thiol concentrations at which methanol, acetate and H_2/CO_2 degradation is inhibited by 50% (IC_{50}).

Tabel 8.1. IC_{50} values (mM) of MT, ethanethiol and propanethiol for the methanogenesis from methanol, acetate and H_2/CO_2 (pH 7.2, 30°C) (after Table 3.3).

	Methanethiol	Ethanethiol	Propanethiol
Methanol	10	8	8
Acetate	8	6	7
H_2/CO_2	7	>14	14

Table 8.1 shows that the methanogenic activity is strongly inhibited at millimolar concentrations. This is important when anaerobic treatment of a wastewater containing thiols is considered.

Methanethiol is degraded during the toxicity experiments, but ethanethiol and propanethiol are not degraded. The specific methanogenic activity of the anaerobic granular sludge from methanol and acetate is, however, 20-40 times higher than from MT. MT is partially converted to DMS before it is degraded to H_2S, CH_4 and CO_2. This can be considered as a detoxification method by the microorganisms. Especially methanol is a good methyl donor. During degradation of 2-18 mM MT in the presence of 40 mM methanol, 10-99% of the MT is first converted to DMS. During the experiment with acetate and MT these percentages were lower (chapter 3). The presence of DMS is a stress or toxicity indicator of the biomass in anaerobic treatment [18]. Lomans *et al.* [11] and Stets *et al.* [19] mention methanol as methyl donor for DMS formation as well.

Also in the absence of an extra methyl donor DMS formation occurs during anaerobic MT degradation. This follows from several reactor experiments described in this thesis (chapter 2 and 4) and from literature data [8, 20, 21]. Possibly, this methylation is an 'accidental reaction' during methanogenesis [19].

8.4. Performance of continuously operated reactors for anaerobic methanethiol degradation

Reactor experiments have been carried out in laboratory-scale UASB reactors, inoculated with different inocula (Table 8.2). Because no anaerobic ethanethiol and propanthiol degradation occurs in batch experiments, reactor experiments were carried out with MT solely.

Table 8.2. Reactor performance (30°C) with different sludge types under different environmental conditions.

Inoculum	Reactor pH	Total Na^+ (M)* in reactor	HRT (hours)	Maximal degradation rate		Initial VSS (g)
				Volumetric (mmol MT·L^{-1}·d^{-1})	Sludge (mmol MT·$gVSS^{-1}$·d^{-1})	
Anaerobic granular sludge	7.2-7.5	< 0.03**	9	17	0.33	96
Wadden Sea sediment	7.2-7.5	0.5 NaCl	50-9	14	3.7	6
	8.2-8.4	0.5 $NaHCO_3$	9	37	11	5
	8.9-9.1	0.5 $NaHCO_3$ Na_2CO_3	9-6	22	7.0	5
	9.9-10.1	0.5 $NaHCO_3$ Na_2CO_3	9	0	0	5
Mixture salt lake sediments	9.9-10.1	0.8 $NaHCO_3$ Na_2CO_3 Na_2SO_4	12	13	0.77	27

* sodium salts responsible for this concentration
** total salt concentration < 0.03 M

Startup of the 3 UASB reactors

The UASB reactor, inoculated with "Eerbeek sludge" (granular sludge), was started up at an influent concentration of 6 mM MT. After 3 days of operation the influent concentration was decreased to 2 mM, but the MT removal efficiency remained around 50% during the experiment of 45 days. The relatively high MT concentration in the reactor during the first 4 days of the experiment can have had a toxic effect on the reactor sludge. The experiment was repeated with fresh anaerobic granular sludge at an influent concentration of 2 mM MT. Now, MT degradation started 8 days after the startup (chapter 2).

For process technological reasons, it is favorable to operate the LPG biodesulfurization process (Fig. 8.1) at elevated pH and salt concentrations. Therefore, subsequently this research focused on anaerobic MT degradation by saline and alkaline tolerant microorganisms, originating from the Wadden Sea and salt lakes. The UASB reactor, inoculated with Wadden Sea sediment (The Netherlands), has been started up in batch mode operation. During the first 20 days MT has been directly injected into the reactor to obtain a concentration of 2 mM MT in the reactor. After complete MT degradation, the system was

switched to a continuous mode at a hydraulic retention time (HRT) of 50 hours and a relatively low liquid upflow velocity of 1 m/h to prevent washout of sludge particles (chapter 4). Now, MT was degraded from day 1 onwards. Stepwise the HRT was decreased to 9 hours and in a subsequent experiment to 6 hours. The startup time in subsequent experiments with this reactor and sludge is always shorter than 4 days at an influent concentration of 2 mM MT. MT unfed periods in between reactor experiments of 4 to 8 weeks do not negatively affect the startup time.

MT degradation (influent 2 mM) in the UASB reactor, inoculated with salt lake sediments, started after 32 days of operation (chapter 6). This reactor was operated in the continuous mode from day 1 onwards. A MT-degrading reactor should thus be started up at a low influent concentration (≤ 2 mM) to prevent accumulation and toxicity of MT. The MT loading rate can be stepwise increased. Steps of about 2 mM are recommended.

MT degradation rate

The maximal anaerobic MT degradation rates in the different experiments are depicted in Table 8.2. The reactor experiment with Wadden Sea sediment at pH 8.2-8.4 clearly shows the highest MT degradation rates, both expressed per volume as per gram volatile suspended solid (VSS). Under these conditions a long term experiment has been performed (360 days) and in the last 20 days of the experiment the MT loading rate was successfully increased from 27 to maximal 37 mmol MT·g VSS^{-1}·d^{-1} (chapter 4). The isolated methanogenic bacterium from this reactor has a pH optimum of 8.4. The MT loading rate in reactor experiments with Wadden Sea sediment, carried out at pH 7.2-7.5 and 8.9-9.1 probably can be further increased if the experiment lasts longer. At pH 10, no MT degradation occurs in a reactor, inoculated with Wadden Sea sediment as solely inoculum (chapter 5).

The reactor inoculated with anaerobic granular sludge reaches a maximal volumetric degradation rate comparable to the reactor experiment with Wadden Sea sediment. However, the specific MT degradation rate is clearly lower because of the relatively high VSS amount in the reactor. Probably, the sludge loading in this reactor is not optimal, since in activity tests the maximal specific MT degradation rate is 1 to 5 mmol MT·gVSS^{-1}·day^{-1} (chapter 2 and 3). Sipma *et al.* [7, 21] report comparable volumetric MT degradation rates (10-50 mmol MT·L^{-1}·day^{-1}) in mesophilic (30°C) laboratory scale UASB reactors, inoculated with anaerobic granular sludge (pH 7.3-7.6; about 20 mM total salt).

Activity tests reveal that the specific MT degradation rate of sludge from the reactor, inoculated with Wadden Sea sediment, is 3-4 times higher than the specific degradation rate of anaerobic granular sludge at initial MT concentrations of 2-3 mM (chapter 2, 3 and 4).

In the reactor, inoculated with a Wadden Sea sediment and salt lake sediments, operated at pH 10, the MT degradation rate was slowly increased during a long term (365 days) experiment (chapter 6). This results in a maximal degradation rate of 13 mmol MT·L^{-1}·day^{-1} (Table 8.2) after more than 300 days. This relatively low degradation rate can be

explained by the fact that the responsible MT-degrading methanogens have a pH optimum below pH 10 (see section 8.5). The Wadden Sea sediment probably did not contribute to MT degradation in this reactor. Because two third of the initial sludge mass in this reactor is Wadden Sea sediment, the specific MT-degrading activity of the mixture of salt lake sediments can be estimated to be 3 times higher than presented in Table 8.2.

Use of co-substrates

The use of methanol as co-substrate for MT-degrading methanogens, to stimulate growth of methylotrophic (MT-degrading) methanogens, initially seems to have no negative effect on the MT degradation in the reactor inoculated with salt lake sediments (chapter 6). The volumetric sludge loading in the reactor is step by step increased to a maximum of 13 mmol $MT \cdot L^{-1} \cdot day^{-1}$. However, during the last 11 days of the experiment, methanol is converted to acetate, probably as a result of the inhibition of the methanogenesis by MT accumulation. Thus, during the last period of the experiment, the addition of methanol does not have the desired effect. Sipma *et al.* [7] add sucrose and acetate to an anaerobic MT-degrading reactor (pH 7.5, 30°C), inoculated with granular sludge, to stimulate granulation and/or to prevent disintegration of the granules. This does not result in a higher MT degradation rate, nor in the formation of stable granular sludge.

Sludge quality and biomass retention

The anaerobic sludge granules in the reactor, inoculated with "Eerbeek sludge" disintegrate slowly during the two reactor experiments, which lasted for 269 days (chapter 2). The strength of the granules decreases from $3.9 \cdot 10^5$ N/m^2 to $2.5 \cdot 10^5$ N/m^2. At the end of the experiment, 5% of the total sludge mass is present as suspended material, the remaining part are granules. The suspended fraction has a higher MT-degrading capacity (see section 8.5). The sludge washout varied from 27 to 165 mg/L total suspended solids (TSS).

In the reactor inoculated with Wadden Sea sediment, the sludge washout is less than 100 mg/L TSS and in the reactor inoculated with a mixture of salt lake sediments washout varied between 50 and 125 mg/L TSS. The particle size distribution of the Wadden Sea sludge varies between 1 and 1500 μm during all experiments (Table 8.3). The fraction between 50 and 500 μm increases and the fraction between 1000 en 1500 μm decreases during the consecutive reactor experiments (chapter 4). No clear floc formation or granulation occurs. A high salt concentration stimulates bacteria to grow individually or in small aggregates [22]. The calculated sludge growth in the "Wadden Sea reactor" is in general higher than the sludge washout (chapter 4).

The liquid upflow velocity in the reactors is 1-2 m/hour in all experiments. Higher velocities will lead to higher sludge washout. However, with a better sludge mixing it might be possible to further increase the sludge loading. To prevent sludge washout, other reactor types can be considered, like a membrane bioreactor or a sequencing batch reactor.

8.5. Microbial characterization of the sludge from the MT-degrading reactors

Table 8.3 gives an overview of the origin of the used inocula and the methanogenic archaea, which have been isolated from the reactor sludge.

Table 8.3. Origin of the reactor inocula and characteristics of the methanogenic archaea, which are the closest relatives of the MT-degrading species isolated from the reactors.

Reactor inoculum	Origin	Size granules/ particles	Organic matter content (%)	Methanogenic species	Characteristics		
					pH range	NaCl (M) range	Ref
Anaerobic granular sludge	UASB reactor for treatment of paper mill wastewater (30°C, pH 7) Eerbeek, The Netherlands	0.5-2 mm	60-70	*Methanomethylovorans hollandica* (in suspended material)	6.0-8.0	0-0.4	[8]
				Methanobacterium beijingense (in granules)	6.5-8.6	n.r.	[23]
Wadden Sea sediment	Wadden Sea, Den Oever, The Netherlands (pH 8, 0.5 M NaCl)	1-1500 µm	10	*Methanosarcina mazei*	5.5-8.5	<1.0	[24]
				Methanolobus taylorii	6.0-9.0	0.2-1.4	[25]
Soap Lake sediment (salt lake)	Soap Lake, Central Washington (USA) pH 10, sulfide ≤ 200 mM ~50 g/L total salt (NaCl and Na_2CO_3)	n.t.	14	*Methanolobus oregonensis*	7.6-9.4	0.1-1.5	[26]
Mixture Kalunda Steppe salt lakes	Tanatar Lake and Cock Soda Lake, Russia ~ 70 g/L total salt (NaCl and Na_2CO_3) pH 10	n.t.	6				

n.t.: not tested
n.r.: not reported

Anaerobic granular sludge

Initially, in the reactor inoculated with granular sludge, MT-degrading methanogenic archaea of the genus *Methanolobus* are present. Later, they are dominated by archaea related to *Methanomethylovorans hollandica,* the only known methanogenic MT- and DMS-degrading species isolated from freshwater environments [8]. During the reactor experiment part of the granular sludge disintegrated (paragraph 8.4). The suspended sludge forms aggregates of 10 to 100 µm. The suspended sludge has a 20 times higher specific MT-degrading capacity than the granules. *Methanobacterium beijingense* [23] remains the dominant methanogen in the granules during the entire reactor experiment of 269 days. In the genus *Methanobacterium* no MT-degrading species are known. Possibly, also other species within the family of *Methanosarcinacaea* contribute to MT degradation, but they were not enriched by serial dilutions.

Wadden sea sediment

During the first and second experiment in the reactor inoculated with Wadden Sea sediment (30°C, pH 7.2-8.3; chapter 4), methanogens related to *Methanolobus taylorii* and *Methanosarcina mazei* are the dominant MT-degrading species. *Methanolobus taylorii* is known to be able to grow on MT till pH 9.0 [25] and species within the genus *Methanosarcina* are known to degrade MT and DMDS till pH 8 [9]. *Methanosarcina mazei* is not known to degrade MT. During the third experiment in this reactor (chapter 5) the pH in the reactor is increased to 9.0. Hereafter, *Methanosarcina mazei* is not detected anymore in the reactor sludge, probably because *M. Mazei* grows at lower pH values (pH 5.5-8.5) [24, 27] and *Methanolobus taylorii* is the only dominant organism. During the last experiment at pH 10 in the reactor inoculated with Wadden Sea sediment (chapter 5), the fraction *Methanolobus taylorii* in the archaeal population decreases from about 80% to about 50%. MT is not degraded at pH 10 in this reactor. About 17% of the archaeal population are hydrogen-consuming methanogens. It can be concluded that Wadden Sea sediment can be used as an inoculum in anaerobic reactors for degradation of MT up to pH 9. DMS, DMDS, methanol, methylamine and trimethylamine can be degraded by the isolated methanogen from this reactor as well.

Salt lake sediments

The MT-degrading organism, which has been isolated from the reactor inoculated with sediments from the Soap Lake and the Kalunda Steppe, is closely related (97%) to *Methanolobus oregonensis*. *Methanolobus oregonensis* grows in media with 0.1-1.5 M Na^+ and at pH values between 7.6 and 9.4, between 30°C and 40°C [26]. At the operating conditions of the reactor (30°C, 0.8 M Na^+, pH 10.0) little growth will occur, because of the high pH, although almost complete MT degradation has been reached during the operation period of one year (chapter 6).

Conclusions for inoculum selection

Table 8.4 shows the conditions under which the used inocula can degrade MT or a mixture of MT, DMS and DMDS. Taking into account the origin of the "Eerbeek sludge" [28] it seems possible to treat a mixture of sulfur compounds, alcohols and volatile fatty acids. For the anaerobic treatment of MT-containing wastewaters up to pH 9, Wadden Sea sediment is a recommended inoculum, because of the high MT-degradation rates (Table 8.2) and, for the Dutch situation, easy availability. For MT degradation at pH 10, salt lake sediments are needed, in which halo-alkaliphilic methanogens are present, *e.g.* Soap Lake sediment, Kalunda Steppe sediment or other salt lake sediments [5, 14].

Table 8.4. Recommended conditions for anaerobic MT degradation with the tested inocula.

Inoculum	pH range	Na^+ (M)
Anaerobic granular sludge	6-8*	< 0.4*
Wadden Sea sediment	7-9	0.1-0.8
Soap Lake/mixture salt lake sediments	8-10**	0.1-1.5**

* This thesis and on basis of growth conditions of *Methanomethylovorans hollandica* [8]
** This thesis and on basis of growth conditions of *Methanolobus oregonensis* [26]

8.6. Chemical reactions between biologically formed sulfur and thiols

In the extractor column of liquid and gas desulfurization processes, thiols can contact biologically produced sulfur from a recycle stream and react. The effect on the process (Fig. 8.1) is described in chapter 7. The reaction between simple primary thiols and bio-sulfur has not been well studied yet. Primary thiols (MT, ethanethiol, propanethiol) are strong nucleophiles. The first step in a reaction series is the opening of the sulfur ring [29]:

$$RSH + S_8 \leftrightarrows RS_9^- + H^+ \quad (8.1)$$

In subsequent reactions inorganic and organic polysulfides and H_2S are formed:

$$2\ RSH + 1/8\ S_8 \rightarrow H_2S + RSSR \quad (8.2)$$
$$2\ RSH + n/8\ S_8 \rightarrow S_{n-x}^{2-} + 2\ H^+ + RS_xR \quad (8.3)$$

The main end-products in the reaction between MT and bio-sulfur (both 1-16 mM) are DMS, DMTS and H_2S. Inorganic polysulfides (S_3^{2-} - S_5^{2-}) are formed as well. An increase in the molar MT/S^0 ratio causes a decrease in the average sulfur chain length of the products.

The initial reaction rate increases with temperature (30-60°C), thiol and bio-sulfur concentration and with the pH of the reaction mixture. The activation energy for reaction 8.1 appears to be 70 kJ·mol^{-1} at pH 8.7 and 16 kJ·mol^{-1} at pH 10.3. At pH 10.3, 50% of the MT is present in the thiolate form (CH_3S^-). This is a stronger nucleophile than the MT molecule, which explains the lower activation energy at pH 10.3.

8.7. Application of the novel process for LPG biodesulfurization

The extractor

A computer simulation of the biodesulfurization process with a commercially available software package (OLI) shows that for MT removal from LPG to concentrations below 10 ppm the pH in the extractor and therefore through the complete process should be at least 9 (chapter 6). The amount of MT and H_2S that can be extracted from the LPG per volume unit of recirculation water determines the flow of the recirculation stream through the consecutive reactor units. An increased pH results in a better MT and H_2S removal from LPG, through

which a smaller recirculation stream is needed. In addition, smaller volume reactors are needed, resulting in lower investment and energy costs.

The bioreactors

The novel biodesulfurization process can treat MT- and H_2S-containing LPG. Anaerobic MT degradation is possible in a UASB reactor at pH 10 and 0.8 M Na^+. Anaerobic MT degradation at a higher Na^+ concentration might be possible, since the isolated methanogen is closely related to *Methanolobus oregonensis*, which grows till 1.5 M Na^+ [26]. At pH 10 a maximal volumetric loading rate of 13 mmol $MT \cdot L^{-1} \cdot day^{-1}$ is reached and 22 mmol $MT \cdot L^{-1} \cdot day^{-1}$ at pH 9 (Table 8.2). These are important parameters for reactor design.

MT inhibits methanogenesis for 50% at concentrations between 7 and 10 mM (Table 8.1). However, in reactor experiments, MT is directly mixed with a recycle flow, generally resulting in lower MT influent concentrations than the reported IC_{50} values. H_2S inhibits anaerobic MT degradation at concentrations below 10 mM (see section 8.3). In contrast, in the reactor inoculated with Wadden Sea sediment at pH 8.2-8.4, complete MT degradation occurs up to 37 mmol $MT \cdot L^{-1} \cdot day^{-1}$ (chapter 4) and up to 10 mM H_2S is formed, without noticeable inhibition.

To prevent H_2S toxicity in the anaerobic reactor, H_2S can be removed by a prewash, comparable to the Merox process (chapter 1). Subsequently, H_2S is led into the aerobic reactor. The biological sulfide oxidation is feasible at pH 10 and till a concentration of about 2 M Na^+ [5, 6]. The produced bio-sulfur is not a waste stream, but a valuable product that can be used for the production of sulfuric acid, H_2S and as fertilizer or fungicide in agriculture [30].

If thiols are incompletely degraded in the anaerobic reactor, they end up in the aerobic sulfur-producing reactor. Here, thiols are autooxidized to disulfides or they react with the produced bio-sulfur to (organic) polysulfides (see section 8.5). DMDS is probably not further oxidized to sulfate in the aerobic reactor, because of the oxygen limiting conditions [6] and the preferential biological oxidation of sulfide compared to DMDS [31]. The formed polysulfide can be chemically or biologically oxidized to elemental sulfur or thiosulfate [6]. Another drawback of the presence of MT in the anaerobic reactor is the inhibition of the biological sulfide oxidation at MT concentrations already below 1 mM (PLF van den Bosch, personal communication).

To prevent DMDS from entering the extractor with the recycle stream and to dissolve in the LPG, it is necessary to implement a DMDS removal step. Also sulfur particles that are not removed in the settler should be removed from the recycle stream. This is possible with *e.g.* an active coal filter.

However, possibly the recycling of sulfur particles has an enhanced effect on the absorption of thiols in desulfurization of gaseous streams (*e.g.* natural gas and biogas) as a result of the reactions with thiols, as described in section 8.5.

Alternative applications

The developed technology for anaerobic and aerobic degradation of methanethiol can be used for other purposes than LPG desulfurization. Other applications might be in natural gas and biogas desulfurization or sludge digestion, by which volatile organic sulfur compounds are released. Also condensates and thiol-containing wastewater streams from viscose, chemical and paper industry and breweries fall within the application field.

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8’

Samenvatting en algemene discussie

8.1. Inleiding

Het in dit proefschrift gepresenteerde onderzoek richt zich op de verwijdering en biologische afbraak van vluchtige organische zwavelverbindingen die in LPG (liquefied petroleum gas) voorkomen. LPG bestaat voornamelijk uit propaan en butaan met als belangrijkste zwavelverontreinigingen H_2S, methaanthiol (MT), ethaanthiol en propaanthiol [1, 2]. Het totale zwavelgehalte in LPG kan oplopen tot 5000 ppm, maar na ontzwaveling is dit normaliter minder dan 10 ppm.

LPG wordt momenteel ontzwaveld met fysisch-chemische methoden. Een alkalische extractie brengt de thiolen van het LPG naar een waterige fase. Vervolgens worden de thiolen chemisch geoxideerd tot in water onoplosbare disulfides, die van de waterfase worden afgeroomd en verder worden behandeld [3].

Dit proefschrift beschrijft onderzoek aan een nieuw, gepatenteerd [4] LPG ontzwavelingsproces, dat uitgaat van anaërobe en aërobe biologische afbraak van de zwavelverontreinigingen met als eindproduct elementair zwavel (Fig. 8.1). Het onderzoek richt zich met name op de anaërobe biologisch afbraak van methaanthiol naar H_2S, CH_4 en CO_2 (hoofdstuk 2-6). De extractiestap is niet met behulp van laboratoriumexperimenten onderzocht, maar door middel van flowsheet simulaties. De tweede biologische stap (sulfideoxidatie) is al beschreven door Sorokin en Kuenen [5] en de reactortoepassing door Van den Bosch *et al.* [6]. In dit hoofdstuk worden de volgende onderwerpen samengevat en bediscussieerd:

- Geschiktheid van verschillende entmaterialen voor anaërobe afbraak van vluchtige organische zwavelverbindingen
- Toxiciteit van vluchtige organische zwavelverbindingen voor methanogenese
- Prestaties van continu bedreven reactoren voor anaërobe methaanthiol afbraak
- Microbiële karakterisatie van het slib uit de MT afbrekende reactoren
- Chemische reacties tussen biologisch gevormd zwavel en thiolen
- Toepasbaarheid van het nieuwe biologische proces voor LPG ontzwaveling

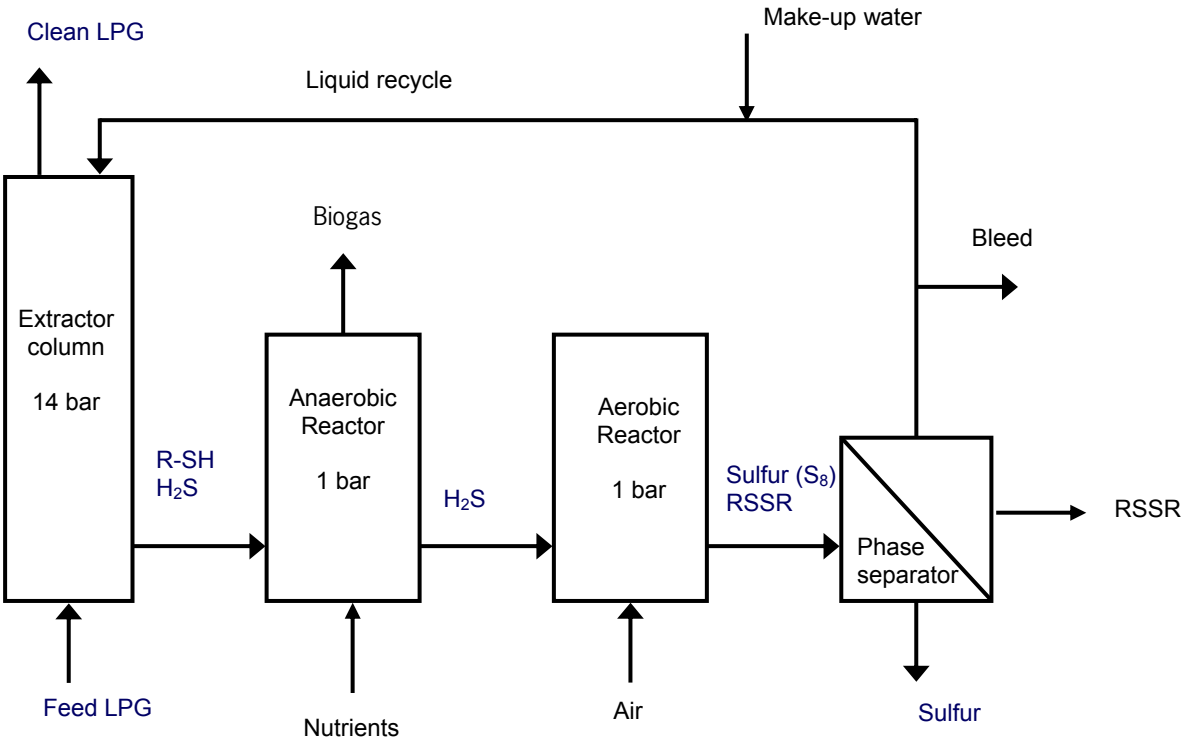

Fig. 8.1. Biologisch proces voor LPG ontzwaveling [4, 7].

8.2. Geschiktheid van verschillende entmaterialen voor anaërobe afbraak van vluchtige organische zwavelverbindingen

Diverse slibmonsters en sedimenten blijken MT afbrekende capaciteit te hebben. Dit omvat monsters van anaëroob (korrel)slib uit praktijkreactoren, (verontreinigd) havensediment, en zoetwater-, zoutmeer en estuarine sedimenten. De aan MT gerelateerde verbindingen dimethylsulfide (DMS) en dimethyldisulfide (DMDS) worden ook afgebroken door de monsters waarin MT afbraak plaatsvindt. In de meeste gevallen zijn methanogene archaea verantwoordelijk voor de afbraak, maar in enkele gevallen ook sulfaatreducerende bacteriën (hoofdstuk 3). Voor hogere thiolen (ethaanthiol en propaanthiol) kan met geen enkel slib- of sedimentmonster anaërobe afbraak worden vastgesteld.

Tot nu toe zijn MT en DMS afbrekende methanogenen vooral geïsoleerd uit mariene systemen en zoutmeren (hoofdstuk 1) en slechts één uit een zoetwatersediment [8, 9]. Uit dit proefschrift (hoofdstuk 2 en 3) en recent onderzoek van Sipma *et al.* [7] blijkt dat ook in anaëroob reactor(korrel)slib MT en DMS afbrekende capaciteit aanwezig is. Onder sulfaatreducerende omstandigheden verloopt de afbraak van MT langzamer dan onder methanogene condities. Volledige afbraak van 4 mM MT (pH 7,2) door anaëroob korrelslib (Eerbeek) duurt bijvoorbeeld vier keer langer onder sulfaatreducerende dan onder methanogene omstandigheden. Tot nu toe zijn slechts drie sulfaatreducerende bacteriën geïsoleerd die kunnen groeien op MT of DMS. Ze zijn allen geïsoleerd uit een thermofiele anaërobe vergister [10]. Lomans *et al.* [11] suggereren dat MT afbraak onder

sulfaatreducerende condities geschiedt door mengcultures waarbij waterstofoverdracht plaats vindt. Sulfaatreducerende bacteriën zouden dus steeds een syntrofe partner nodig hebben voor de afbraak van MT, hetgeen betekent dat geen reinculture kan worden verkregen.

8.3. Toxiciteit van vluchtige organische zwavelverbindingen voor methanogenese

H_2S toxiciteit voor methaanthiol omzetting

H_2S remt de activiteit van methanogene archaea [12]. Een initiële concentratie van 4,3 mM totaal H_2S (H_2S + HS^-) (pH 7,2) leidt tot 80% afname van de methanogene afbraaksnelheid van MT (2 mM) door anaëroob MT geadapteerd korrelslib (hoofdstuk 3). Ook met MT geadapteerd sedimentair slib (pH 8,3) als entmateriaal, dat afkomstig is uit de Waddenzee, vindt remming van methanogene MT afbraak plaats bij initiële H_2S concentraties rond 3 mM (hoofdstuk 4).

Vijftig procent remming van de methanogene afbraak van acetaat (pH 8,0-8,5) door H_2S vindt plaats bij 25 tot 30 mM totaal H_2S [13]. Hieruit kan geconcludeerd worden dat de remming voor de methanogenese van MT, ten gevolge van de aanwezigheid van sulfide, sterker is dan bij acetaat. Het toxische effect van H_2S is pH afhankelijk [12] aangezien het voor het grootste deel wordt veroorzaakt door het niet-gedissocieerde H_2S. Om H_2S toxiciteit te remmen is het daarom gunstig de reactor te bedrijven bij pH waardes hoger dan 8, waardoor meer dan 90% van het H_2S in de gedissocieerde vorm (HS^-) aanwezig is.

Toxiciteit van DMDS op methaanthiol afbraak

De eerste stap in de afbraak van DMDS is de reductie tot 2 moleculen MT. Deze reductie is waarschijnlijk de snelheidsbepalende stap in de volledige afbraak van DMDS tot H_2S, CH_4 en CO_2. De volledige afbraak van DMDS door anaëroob korrelslib (pH 7,2, 30°C) verloopt 10 keer langzamer dan de afbraak van MT en DMS bij concentraties tot 2 mM, en tot meer dan tien keer zo langzaam bij initiële concentraties tussen 2 en 8 mM (hoofdstuk 3). DMDS heeft een remmend effect op de anaërobe afbraak van MT. In een reactorexperiment met Waddenzee sediment als entmateriaal (pH 8.3, 30°C) veroorzaakt 1 mM DMDS reeds onvolledige MT afbraak. De efficiëntie daalt van 70% tot 40%. In een reactorexperiment met zoutmeer sedimenten als entmateriaal (pH 10,0, 30°C) veroorzaakt de aanwezigheid van 0,9-1,7 mM DMDS een daling van de verwijderingsefficiëntie van MT van meer dan 90% tot lager dan 50%. Na verwijdering van DMDS uit het influent is de activiteit binnen 3 dagen weer op het oude niveau terug (hoofdstuk 6). Ook Kiene *et al.* [14] vinden dat hoge DMDS concentraties de methanogenese remt: endogene methanogenese door sedimentmonsters uit (halo)alkalifiele milieus wordt gestimuleerd door 1-2 mM DMDS in vergelijking met controles zonder substraat toevoeging. Echter, bij 8 mM DMDS en hoger wordt de endogene methaanvorming met tenminste 80% geremd (22°C, pH 9,7) [14]. De aanwezigheid van

DMDS in het influent als gevolg van auto-oxidatie is dus ongewenst en moet worden voorkomen door een goede bedrijfsvoering en een goed ontwerp van de anaërobe reactor.

Toxiciteit van thiolen voor methanogenese

Thiolen werken boven een bepaalde concentratie remmend op de activiteit van methanogenen, mogelijk vanwege een inactivatie van bepaalde enzymen. Een thiol-disulfide uitwisselingsreactie, waarin een thiolaatgroep een zwavelatoom van een disulfidebinding aanvalt, is een bekende biochemische reactie waardoor disulfidebindingen in eiwitten kunnen worden gevormd en verbroken [15-17]. MT is echter ook een substraat voor verschillende aërobe bacteriën, sulfaat reducerende bacteriën en methylotrofe methanogenen (hoofdstuk 1). De remmende werking van MT, ethaanthiol en propaanthiol op de methanogenese van anaëroob korrelslib uit een MT afbrekende reactor (hoofdstuk 2) is in hoofdstuk 3 bepaald. Hiervoor zijn de substraten methanol, acetaat en H_2/CO_2 gekozen. De thiolconcentraties waarbij de afbraak van methanol, acetaat en H_2/CO_2 met 50% geremd wordt (IC_{50}), zijn weergegeven in Tabel 8.1.

Tabel 8.1. IC_{50} waardes (mM) van MT, ethaanthiol en propaanthiol voor de methanogene activiteit met methanol, acetaat en H_2/CO_2 (pH 7.2, 30°C) (zie Tabel 3.3).

	Methaanthiol	Ethaanthiol	Propaanthiol
Methanol	10	8	8
Acetaat	8	6	7
H_2/CO_2	7	>14	14

Uit Tabel 8.1 blijkt dat thiolen de methanogene activiteit al sterk remmen in millimolaire concentraties. Dit is een belangrijk gegeven wanneer anaërobe zuivering van een afvalwater met thiolen wordt overwogen.

Methaanthiol wordt afgebroken tijdens de toxiciteitsexperimenten, maar ethaanthiol en propaanthiol niet. De specifieke methanogene activiteit van het anaëroob korrelslib met methanol en acetaat is echter 20-40 keer hoger dan met MT. Methaanthiol wordt gedeeltelijk omgezet in DMS voordat het wordt afgebroken tot H_2S, CH_4 en CO_2. Dit kan worden beschouwd als een detoxificatiemechanisme van het slib. Vooral methanol blijkt een goede methyldonor te zijn. Bij de afbraak van 2-18 mM MT in de aanwezigheid van 40 mM methanol wordt 10-99% van het aanwezige MT eerst naar DMS omgezet. In een experiment met acetaat en MT zijn dit kleinere hoeveelheden (hoofdstuk 3). De aanwezigheid van DMS is een stress- of toxiciteitsindicator voor biomassa in anaërobe zuivering [18]. Ook Lomans *et al.* [11] en Stets *et al.* [19] noemen methanol als methyldonor voor DMS-vorming.

In afwezigheid van een extra methyldonor vindt eveneens DMS vorming plaats tijdens de MT afbraak. Dit blijkt uit verschillende reactorexperimenten die beschreven zijn in dit proefschrift (hoofdstuk 2 en 4) en uit de literatuur [8, 20, 21]. Mogelijk vindt de methylering van MT plaats als een bijreactie van methanogenese [19].

8.4. Prestaties van continu bedreven reactoren voor anaërobe methaanthiol afbraak

Reactorexperimenten zijn op laboratoriumschaal uitgevoerd met UASB reactoren, die zijn opgestart met verschillende entmaterialen (Tabel 8.2). Aangezien uit batch experimenten blijkt dat ethaanthiol en propaanthiol niet anaëroob worden afgebroken (hoofdstuk 2), zijn de reactorexperimenten alleen uitgevoerd met MT.

Tabel 8.2. Reactor prestaties (30°C) met verschillende slibtypes en onder verschillende milieuomstandigheden.

Entmateriaal	Reactor pH	Totaal Na^+ (M)* in reactor	HRT (uren)	Maximale afbraaksnelheid		VSS initieel (g)
				Volumetrisch (mmol MT·L^{-1}·d^{-1})	Slib (mmol MT·gVSS^{-1}·d^{-1})	
Anaëroob korrelslib	7,2-7,5	< 0,03**	9	17	0,33	96
Waddenzee sediment	7,2-7,5	0,5 NaCl	50-9	14	3,7	6
	8,2-8,4	0,5 $NaHCO_3$	9	37	11	5
	8,9-9,1	0,5 $NaHCO_3$ Na_2CO_3	9-6	22	7,0	5
	9,9-10,1	0,5 $NaHCO_3$ Na_2CO_3	9	0	0	5
Mengsel zoutmeer sedimenten	9,9-10,1	0,8 $NaHCO_3$ Na_2CO_3 Na_2SO_4	12	13	0,77	27

* natriumzouten, die verantwoordelijk waren voor deze concentratie
** totaal zoutgehalte < 0,03 M

Opstart van de drie UASB reactoren

De UASB reactor is opgestart met "Eerbeekslib" (anaëroob korrelslib) bij een influentconcentratie van 6 mM MT. Na 3 dagen werd de influentconcentratie teruggebracht naar 2 mM. De verwijderingefficiëntie van MT blijft gedurende de 45 dagen van het experiment rond de 50%. De relatief hoge MT concentratie in de reactor gedurende de eerste vier dagen kan een toxische werking hebben gehad op het reactorslib. Het experiment werd daarom herhaald met vers anaeroob korrelslib bij een influentconcentratie van 2 mM MT. Nu begint MT afbraak begint 8 dagen na de opstart (hoofdstuk 2).

Omdat het vanwege procestechnische redenen (Fig. 8.1) gunstig is om bij verhoogde pH en zoutconcentratie te werken, heeft het onderzoek zich hierna gericht op anaërobe MT afbraak met behulp van zoutminnende bacteriën afkomstig uit de Waddenzee en zoutmeren. De UASB reactor is batchgewijs opgestart met Waddenzee sediment als entmateriaal. Gedurende de eerste 20 dagen is pulsgewijs MT toegevoegd tot een concentratie van 2 mM in de reactor. Nadat MT een aantal keer volledig is afgebroken, is de reactor continu bedreven met een hydraulische verblijftijd van 50 uur en een vloeistof opstroomsnelheid van 1 m/h om uitspoeling van de sedimentdeeltjes te voorkomen (hoofdstuk 4). MT breekt nu vanaf dag 1

af. Stapsgewijs is de verblijftijd in deze reactor teruggebracht tot 9 uur en in een vervolgexperiment tot 6 uur. In vervolgexperimenten met deze reactor en dit entmateriaal is de opstarttijd steeds korter dan 4 dagen met 2 mM MT in het influent, ondanks dat de reactor tussen de experimenten door 4 tot 8 weken niet gevoed is.

MT afbraak (influent 2 mM) in de reactor met een mengsel van zoutmeer sedimenten start na 32 dagen (hoofdstuk 6). Deze reactor is vanaf dag 1 continu bedreven. Een MT afbrekende reactor moet dus met een lage influent concentratie opgestart worden (≤ 2 mM) om ophoping en toxiciteit van MT te voorkomen. Stapsgewijs kan de MT belasting omhoog gebracht worden (stapgroottes van ongeveer 2 mM zijn aan te bevelen).

MT omzettingscapaciteit

De maximale anaërobe MT afbraaksnelheden in de verschillende experimenten zijn weergegeven in Tabel 8.2. Het reactorexperiment met Waddenzee sediment bij pH 8,2-8,4 heeft duidelijk de hoogste MT afbraaksnelheden, zowel uitgedrukt per reactorvolume als per gram droge biomassa (VSS). Onder deze condities is ook het langst geëxperimenteerd en kon in de laatste 20 dagen van een langlopend experiment (360 dagen) de MT belasting met succes worden verhoogd van 27 tot maximaal 37 mmol MT·g VSS^{-1}·dag^{-1} (hoofdstuk 4). Het geïsoleerde methanogene archaeon uit deze reactor heeft een pH optimum bij 8,4. De reactorbelasting voor de experimenten met Waddenzee sediment bij pH 7,2-7,5 en 8,9-9,1 kunnen wellicht nog verder worden verhoogd, wanneer hiermee langer wordt doorgeëxperimenteerd. Bij pH 10 vindt met Waddenzee sediment als enig entmateriaal geen MT afbraak plaats (hoofdstuk 5).

Het experiment met anaëroob korrelslib (“Eerbeekslib”) geeft een maximale volumetrische afbraaksnelheid vergelijkbaar met reactorexperimenten met Waddenzee sediment. De specifieke MT afbraaksnelheid is echter beduidend lager door de relatief grote hoeveelheid VSS in de reactor. Waarschijnlijk is het slib in deze reactor niet optimaal belast, aangezien in activiteitstesten de maximale specifieke MT afbraaksnelheid 1 tot 5 mmol MT·$gVSS^{-1}$·dag^{-1} is (hoofdstuk 2 en 3). Sipma *et al.* [7, 21] rapporteren vergelijkbare volumetrische MT afbraaksnelheden (10-50 mmol MT·L^{-1}·dag^{-1}) in mesofiele (30°C) laboratoriumschaal UASB reactoren, aangeënt met anaëroob korrelslib (pH 7,3-7,6; ~ 20 mM totaal zout).

De specifieke afbraaksnelheid van MT in activiteitstesten met slib uit de reactor met Waddenzee sediment als entmateriaal is hoger dan met anaëroob korrelslib. Het verschil is een factor 3-4 bij initiële MT concentraties van 2-3 mM (hoofdstuk 2, 3 en 4).

In het reactorexperiment waarbij een mengsel van Waddenzee sediment en zoutmeer sedimenten is gebruikt als entmateriaal, is langdurig (365 dagen) getracht bij pH 10 de MT afbraaksnelheid te verhogen (hoofdstuk 6). Dit leidt tot een maximale afbraaksnelheid van 13 mmol MT·L^{-1}·dag^{-1} (Tabel 8.2) na meer dan 300 dagen. Deze relatief lage afbraaksnelheid is te verklaren door het feit dat de verantwoordelijke MT afbrekende methanogenen een pH

optimum onder de 10 hebben (zie paragraaf 8.5). Het Waddenzee sediment draagt waarschijnlijk niet bij aan MT afbraak in deze reactor. Aangezien tweederde van de initiële slibmassa in deze reactor Waddenzee sediment is, kan de specifieke activiteit van het mengsel van zoutmeer sedimenten een factor 3 hoger worden geschat.

Gebruik van co-substraten

De toevoeging van methanol als co-substraat voor MT afbrekende methanogenen, om groei van methylotrofe (MT afbrekende) methanogenen te stimuleren, lijkt in eerste instantie geen negatief effect te hebben op de MT afbraak in de reactor geënt met zoutmeer sedimenten (hoofdstuk 6). De volumetrische slibbelasting in de reactor kan langzaam worden opgevoerd tot maximaal 13 mmol $MT \cdot L^{-1} \cdot dag^{-1}$. Tijdens de laatste 11 dagen wordt methanol echter in acetaat omgezet, waarschijnlijk als gevolg van de remming van methanogenese door MT ophoping. Tijdens de laatste periode van het experiment heeft de toevoeging van methanol dus niet het gewenste effect. Sipma *et al.* [7] voegen in een anaërobe MT afbrekende reactor (pH 7,5, 30°C), geënt met korrelslib, sucrose en acetaat toe om korrelvorming te bevorderen en/of desintegratie van de korrels te voorkomen. Dit leidt echter niet tot een hogere MT afbraaksnelheid, noch tot de vorming van stabiele slibkorrels.

Slibkwaliteit en biomassaretentie

De anaërobe slibkorrels in de reactor geënt met "Eerbeekslib" desintegreren langzaam gedurende de twee reactorexperimenten die in totaal 269 dagen duurden (hoofdstuk 2). De sterkte van de korrels daalt van $3{,}9 \cdot 10^5$ N/m^2 tot $2{,}5 \cdot 10^5$ N/m^2. Aan het eind van het experiment is 5% van de slibmassa aanwezig als gesuspendeerd materiaal, de rest als korrels. Het gesuspendeerde materiaal heeft een hogere MT afbrekende capaciteit (zie paragraaf 8.5). De uitspoeling varieerde van 27 tot 165 mg/L totaal gesuspendeerde deeltjes (TSS).

Tijdens reactorexperimenten met Waddenzee sediment is de uitspoeling kleiner dan 100 mg/L TSS en tijdens het reactorexperiment met een mengsel van zoutmeren varieert de uitspoeling tussen 50 en 125 mg/L TSS. Het Waddenzee sediment bestaat uit deeltjes tussen de 1 en 1500 μm (Tabel 8.3 en hoofdstuk 4). Tijdens de achtereenvolgende reactorexperimenten met dit sediment neemt het aandeel deeltjes tussen 50 en 500 μm toe en daalt de hoeveelheid grote deeltjes (1000-1500 μm). Er vindt geen duidelijke vlok- of korrelvorming plaats. Een hoge zoutconcentratie stimuleert micro-organismen om in suspensie of in kleine aggregaten te groeien [22]. De berekende slibaangroei in de Waddenzee reactor is over het algemeen groter dan de slibuitspoeling (hoofdstuk 4).

De opstroomsnelheid in de reactoren is 1-2 m/uur in alle experimenten. Hogere opstoomsnelheden leidt tot slibuitspoeling. Echter, met een betere slibmenging is het misschien mogelijk het slib hoger te belasten. Om slibuitspoeling te voorkomen zijn dan andere reactortypes noodzakelijk, zoals een membraanbioreactor of een sequencing batch reactor.

8.5. Microbiële karakterisatie

Tabel 8.3 geeft een overzicht van de oorsprong van de gebruikte entmaterialen en van de methanogene archaea, die uit het reactorslib zijn geïsoleerd.

Tabel 8.3. Oorsprong van de entmaterialen en karakteristieken van de methanogenen, die het meest verwant zijn met de uit de reactoren geïsoleerde MT afbrekende soorten.

Entmateriaal reactor	Oorsprong	Korrel-grootte	Organisch stofgehal-te (%)	Methanogene archaea	Karakteristieken		
					pH range	NaCl (M) range	Ref
Anaëroob korrelslib	UASB reactor voor behandeling papierafvalwater (30°C, pH 7) Eerbeek, Nederland	0.5-2 mm	60-70	*Methanomethylovorans hollandica* (in gesuspendeerde materiaal)	6,0-8,0	0-0,4	[8]
				Methanobacterium beijingense (in korrels)	6,5-8,6	n.r.	[23]
Waddenzee sediment	Waddenzee, Den Oever, Nederland (pH 8, 0,5 M NaCl)	1-1500 µm	10	*Methanosarcina mazei*	5,5-8,5	<1,0	[24]
				Methanolobus taylorii	6,0-9,0	0,2-1,4	[25]
Soap Lake (zoutmeer)	Soap Lake, Central Washington (V.S.) pH 10, sulfide ≤ 200 mM ~50 g/L totaal zout ($NaCl$ en Na_2CO_3)	n.t.	14	*Methanolobus oregonensis*	7,6-9,4	0,1-1,5	[26]
Mengsel Kalunda Steppe zoutmeren	Tanatar Lake en Cock Soda Lake, Rusland ~ 70 g/L totaal zout ($NaCl$ en Na_2CO_3) pH 10	n.t.	6				

n.t.: niet getest
n.r.: niet gerapporteerd

Anaëroob korrelslib

In de Eerbeek-reactor zijn aanvankelijk MT afbrekende methanogene archaea aanwezig van het geslacht *Methanolobus*. Later worden deze overheerst door *Methanomethylovorans* soorten, de enige bekende methanogeen waarvan MT en DMS afbraak bekend is in zoetwater milieus [8]. Tijdens het reactorexperiment desintegreert een deel van het korrelslib (paragraaf 8.4). Het gesuspendeerde slib vormt aggregaten met een grootte tussen de 10 and 100 µm. De MT afbrekende capaciteit van dit slib blijkt vooral in het gesuspendeerde slib aanwezig, waarin de specifieke MT afbraaksnelheid 20 keer hoger is dan in de korrels. In de korrels blijft *Methanobacterium beijingense* [23] gedurende het gehele reactorexperiment van 269 dagen de belangrijkste methanogeen. In het geslacht *Methanobacterium* zijn geen MT afbrekende soorten bekend. Mogelijk dragen ook andere soorten binnen de familie van *Methanosarcinacaea* bij aan de MT afbraak, maar worden deze niet opgehoopt in verdunningreeksen.

Waddenzee sediment

Gedurende de eerste twee reactorexperimenten met Waddenzee sediment (30°C, pH 7,2- 8,3) (hooofdstuk 4) zijn methanogenen verwant aan *Methanolobus taylorii* en *Methanosarcina mazei* de dominante MT afbrekers in de reactor. Van *Methanolobus taylorii* is MT afbraak bekend en groei tot pH 9,0 [25] en het geslacht *Methanosarcina* bevat ook methanogenen die MT en DMS afbreken [9]. Van *Methanosarcina mazei* zelf is dit niet bekend. Tijdens het derde reactor experiment met Waddenzee sediment (hoofdstuk 5) is de pH in de reactor op 9,0 gebracht. Hierna is *Methanosarcina mazei* niet meer gedetecteerd in het reactorslib, waarschijnlijk, door het lagere pH bereik waarin *M. mazei* kan groeien (pH 5,5-8,5) [24, 27] en is *Methanolobus taylorii* het dominante organisme. Tijdens het laatste reactorexperiment met het Waddenzee sediment (pH 10) daalt het aandeel *Methanolobus taylorii* in de archaea populatie van 80% tot ongeveer 50%. MT wordt niet afgebroken bij pH 10. Tussen de 15 en 30% van de archaea wordt op dat moment ingenomen door waterstof consumerende methanogenen. Het Waddenzee sediment kan dus gebruikt worden voor anaërobe MT afbraak tot pH 9. Ook DMS, DMDS, methanol methylamine en trimethylamine kunnen door het uit de reactor geïsoleerde methanogeen worden afgebroken.

Zoutmeer sedimenten

In de sedimenten van het Soap Lake en de Kalunda Steppe is de voor MT afbraak verantwoordelijke methanogeen sterk gerelateerd (97%) aan *Methanolobus oregonensis*. Dit organisme kan groeien in media met 0,1-1,5 M Na^+ bij pH waardes tussen 7,6 en 9,4 en tussen 30°C en 40°C [26]. Bij de condities waaronder de reactor is bedreven (30°C, 0,8 M Na^+, pH 10,0) zal nauwelijks groei hebben plaatsgevonden gezien de hoge pH, hoewel vrijwel volledige MT afbraak is bereikt gedurende een jaar (hoofdstuk 6).

Conclusie voor entmateriaal selectie

In Tabel 8.4 zijn de condities aangegeven waaronder de gebruikte entmaterialen MT of een mengsel van MT, DMS en DMDS af kunnen breken. Gezien de oorsprong van het Eerbeekslib [28] lijkt het met dit slib mogelijk een mengsel van zwavelverbindingen, alcoholen en vluchtige vetzuren te kunnen behandelen. Voor de anaërobe behandeling van MT houdend afvalwater tot pH 9 is Waddenzee sediment een aan te bevelen entmateriaal gezien de hoge MT afbraaksnelheden (Tabel 8.2) en (voor de Nederlandse situatie) eenvoudige beschikbaarheid. Voor afbraak bij pH 10 zijn zoutmeer sedimenten nodig, waarin haloalkalifiele methanogenen aanwezig zijn, zoals het Soap Lake sediment, het Kalunda Steppe sediment of andere zoutmeer sedimenten [5, 14].

Tabel 8.4. Aanbevolen condities voor anaërobe afbraak van MT met de geteste entmaterialen.

Entmateriaal	pH range	Na^+ (M)
Anaëroob korrelslib	6-8*	< 0,4*
Waddenzee sediment	7-9	0,1-0,8
Soap Lake/mengsel zoutmeer sedimenten	8-10**	0,1-1,5**

* Dit proefschrift en op basis van de groeicondities van *Methanomethylovorans hollandica* [8]
**Dit proefschrift en op basis van groeicondities van *Methanolobus oregonensis* [26]

8.6. Chemische reacties tussen biozwavel en thiolen in anaëroob milieu

In de extractorkolom van vloeistof- en gasontzwavelingsprocessen kan biologisch gevormd zwavel in contact komen met thiolen en hiermee reageren. Het effect hiervan op het proces is beschreven in hoofdstuk 7. Over de reactie van eenvoudige primaire thiolen met biozwavel is nog weinig bekend. Primaire thiolen (MT, ethaanthiol, propaanthiol) zijn sterke nucleofielen. De eerste stap in de reactiereeks is de opening van de zwavelring [29]:

$$RSH + S_8 \leftrightarrows RS_9^- + H^+ \quad (8.1)$$

In vervolgreacties worden anorganische en organische polysulfides en H_2S gevormd:

$$2\ RSH + 1/8\ S_8 \rightarrow H_2S + RSSR \quad (8.2)$$
$$2\ RSH + n/8\ S_8 \rightarrow S_{n-x}^{2-} + 2\ H^+ + RS_xR \quad (8.3)$$

De belangrijkste eindproducten in de reactie tussen methaanthiol en biozwavel (beide 1-16 mM) zijn DMDS, DMTS en H_2S. Ook anorganische polysulfides (S_3^{2-} - S_5^{2-}) worden gevormd. Een toename van de molaire MT/S^0 ratio veroorzaakt een daling van de gemiddelde zwavelketenlengte van de producten.

De initiële reactiesnelheid neemt toe met temperatuur (30-60°C), thiol- en zwavelconcentraties en met de pH van het reactiemengsel. De activeringsenergie voor reactie 8.1 is vastgesteld op 70 kJ·mol^{-1} bij pH 8,7 en 16 kJ·mol^{-1} bij pH 10,3. Bij pH 10,3 is 50% van het MT in de thiolaatvorm (CH_3S^-) aanwezig. Dit is een sterker nucleofiel dan het moleculaire MT en verklaart de lagere activeringsenergie bij pH 10,3.

8.7. Toepasbaarheid van het nieuwe biologische proces voor LPG ontzwaveling

De extractor

Een computersimulatie van het ontzwavelingsproces met behulp van een commercieel verkrijgbaar softwarepakket (OLI) leert dat de pH in de extractor en daardoor in het gehele proces minimaal 9 moet zijn voor de verwijdering van thiolen uit LPG tot onder de 10 ppm (hoofdstuk 6). De hoeveelheid MT en H_2S die kan worden geëxtraheerd uit het LPG per volume-eenheid recirculatiewater bepaalt het debiet van de retourstroom door de

processtappen. Een verhoogde pH geeft een betere verwijdering van MT en H_2S uit LPG, waardoor een kleinere recirculatiestroom nodig is. Er zijn dan ook minder grote reactoren nodig, hetgeen leidt tot lagere investerings- en energiekosten.

De bioreactoren

Met het nieuwe ontzwavelingsproces (Fig. 8.1) is het mogelijk om methaanthiol en H_2S bevattend LPG te behandelen. De anaërobe afbraak van MT blijkt mogelijk in een UASB reactor bij pH 10 en 0,8 M Na^+. Verdere verhoging van het zoutgehalte is wellicht mogelijk, aangezien de geïsoleerde methanogeen sterk verwant is aan *Methanolobus oregonensis*, die activiteit vertoont tot 1,5 M Na^+ [26]. Bij pH 10 wordt een maximale volumetrische belasting van 13 mmol $MT \cdot L^{-1} \cdot dag^{-1}$ behaald en 22 mmol $MT \cdot L^{-1} \cdot dag^{-1}$ bij pH 9 (Tabel 8.2). Dit zijn belangrijke gegevens voor het uiteindelijke reactorontwerp.

MT remt de methanogenese al met 50% bij concentraties tussen 7 en 10 mM (Tabel 8.1). In de reactorexperimenten wordt MT echter direct gemengd met een retourstroom, waardoor de werkelijke ingaande concentratie lager is dan de gerapporteerde IC_{50} waardes. Ook H_2S remt anaërobe MT afbraak al bij concentraties onder de 10 mM (zie paragraaf 8.3). In een reactorexperiment met Waddenzee sediment (pH 8,2-8,4) vond echter volledige MT afbraak plaats tot 37 mmol $MT \cdot L^{-1} \cdot dag^{-1}$ (hoofdstuk 4), waarbij maximaal 10 mM H_2S in de reactor is gevormd.

Om H_2S toxiciteit in de anaërobe reactor te voorkomen kan H_2S verwijderd worden met een voorextractie, vergelijkbaar met het Merox proces (hoofdstuk 1). H_2S wordt daarna direct naar de aërobe reactor geleid. De biologische sulfideoxidatie tot elementair zwavel is mogelijk bij pH 10 en tot ongeveer 2 M Na^+ [5, 6]. Het gevormde biozwavel is geen afvalstroom maar een waardevol product, dat kan worden gebruikt voor de productie van zwavelzuur, H_2S en als meststof of fungicide in de landbouw [30].

Als thiolen onvolledig worden afgebroken in de anaërobe reactor komen ze terecht in de aërobe zwavelproducerende reactor. Hier vindt een auto-oxidatie plaats tot disulfides of een reactie met het geproduceerde biozwavel tot (organische) polysulfides (zie paragraaf 8.5). DMDS wordt waarschijnlijk niet verder geoxideerd tot sulfaat in de aërobe reactor, vanwege de zuurstof limiterende condities en de preferentiële biologische oxidatie van sulfide ten opzichte van DMDS [31]. De gevormde polysulfides kunnen chemisch of biologisch geoxideerd worden tot elementair zwavel of tot thiosulfaat [6]. Een ander nadelig effect van MT in de aërobe reactor is de remming van de biologische sulfideoxidatie bij MT concentraties onder de 1 mM (Van den Bosch, persoonlijke communicatie).

Om te voorkomen dat DMDS met de retourstroom in de extractor terechtkomt en oplost in het LPG is het nodig een DMDS verwijderingsstap te implementeren. Ook zwaveldeeltjes, die niet bezonken zijn moeten uit de retourstroom worden verwijderd. Dit kan onder andere middels een actief kool filter.

Mogelijk heeft het terugleiden van zwaveldeeltjes echter een versnellend effect op de absorptie van thiolen voor het ontzwavelen van gasvormige stromen (bijvoorbeeld aardgas en biogas) als gevolg van de reacties met biozwavel, zoals beschreven in paragraaf 8.5.

Alternatieve toepassingen

De ontwikkelde technologie voor anaërobe en aërobe afbraak van methaanthiol kan ook voor andere toepassingen dan LPG ontzwaveling gebruikt worden. Te denken valt aan aardgas ontzwaveling, biogasreiniging en slibvergisting, waarbij vluchtige organische zwavelverbindingen vrijkomen. Binnen het toepassingsgebied vallen ook condensaten en thiolhoudende afvalwaters uit de viscose en chemische industrie, papierindustrie en bierbrouwerijen.

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Robin

Netherlands Research School for the
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CERTIFICATE

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- **"Degradation of Methanethiol in an UASB-reactor" Second International Conference of Petroleum Biotechnology, 5-7 November 2003, Mexico City, Mexico**
- **"Anaerobic Degradation of Mercaptans" Symposium on Biotechnology From Bech to Market, 22 April 2005, Ede, The Netherlands**
- **"Degradation of Methanethiol in an UASB-reactor: reactor performance and salt tolerance" Congress on Biotechnology for Air Pollution Control, 9 – 13 July 2005, Coruna, Spain**
- **"Degradation of Methanethiol in anaerobic reactors for LPG desulphurization: reactor performance and salt tolerance" European Symposium on Environmental Biotechnology, 9 – 13 July 2006, Leipzig, Germany**

Deputy director SENSE
Dr. A. van Dommelen

Levensloop

Robert Cornelis (Robin) van Leerdam werd geboren op 22 juli 1977 te Delft. Na het VWO-examen aan het Christelijk Lyceum Delft in 1995 studeerde hij een jaar Scheikunde aan de Rijksuniversiteit Leiden. Na een jaar stapte hij over op de opleiding Milieukunde aan de Hogeschool Delft. Als afstudeeropdracht werkte hij bij IHE Delft (dr. Maria Kennedy en ir. Ingrida Bremere) aan de verwijdering van bariumsulfaat uit nanofiltratie concentraat ten behoeve van de drinkwaterbereiding. In 1999 begon hij aan de studie Milieuhygiëne aan de Wageningen Universiteit. Als afstudeervak bij de sectie Milieutechnologie werkte hij aan de chemische oxidatie van papierafvalwater met ozon (dr. Harry Bruning en dr. Hardy Temmink). In april 2002 begon hij met het promotieonderzoek "Biologische ontzwaveling van koolstofrijke gasstromen" bij de sectie Milieutechnologie van de Wageningen Universiteit, wat geleid heeft tot dit proefschrift. Momenteel werkt hij als onderzoeker bestrijdingsmiddelen bij Alterra in Wageningen.

Adres:
Van Uvenweg 28-1
6707BB Wageningen
robin.vanleerdam@wur.nl

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