THE INFLUENCE OF SULFATE AND NITRATE ON THE METHANE FORMATION BY METHANOGENIC ARCHAEA IN FRESHWATER SEDIMENTS

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Proefschrift

ter verkrijging van de graad van doctor

op gezag van de rector magnificus

van de Landbouwuniversiteit Wageningen,

dr. C.M. Karssen,

in het openbaar te verdedigen

op maandag 1 februari 1999

des namiddags te half twee in de Aula

Cover and Back: Edie Berendsen

ISBN 90 6464 057 2

This research was carried out at the Laboratory for Microbiology, Wageningen Agricultural University, The Netherlands, and was supported by the Netherlands Organization for Scientific Research (NWO).

"Ik eis van u geen oplossing. Maar ge moet leren althans de vragen te zien.„

Bill Clifford

Aan oom Iet

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

METHANE EMISSION

Methane (CH_4) is an atmospheric trace gas which plays an important role in the geochemistry of carbon (1). In addition, it exerts a strong influence on the earth's climate and the chemistry of the atmosphere. As one of the principal green house gases CH_4 became the object of study by scientists of many different disciplines. Several researchers reported that atmospheric methane is increasing at a rate of about 1-2% per year for at least the last decades (2,3). Gas bubbles in polar ice and other evidence show that the methane concentration has increased 2-3 times in the last 100 - 300 years (4,5). This increase is of great concern because of the potential role of methane in climate change and atmospheric chemistry. At this moment, it is not clear why the concentration of methane in the atmosphere is increasing. However, there is no doubt that the rise in methane is related to growing anthropogenic activities during the last 300 years. A methane budget in which all sources and sinks are included can lead to a better estimation of the increase of atmospheric methane (increase = sources - sinks). To determine such a budget detailed studies of anthropogenic and natural sources, sinks and factors controlling methane release to the atmosphere are needed.

About 60 % of the methane in the earth's atmosphere is of biological origin (6). The most important environments responsible for an increased methane production are natural wetlands, rice paddies, gastro-intestinal track of ruminants, insects and landfills (Table 1). Biological methane is produced by unique methanogenic archaea.

Table 1: Estimated natural and antrophogenic sources[a] of methane (7).

Sources	[a]Tg yr^{-1}
Natural	
Wetlands	55-150
Termites	10-50
Oceans	5-50
Other	10-40
Antrophogenic	
Fossil fuel related	70-120
Cattle	65-100
Rice paddies	20-100
Biomass burning	20-80
Landfills	20-70
Animal waste	20-30
Domestic sewage	15-80

[a]Tg = 10^{12} g

METHANOGENESIS

In methanogenic ecosystems, organic matter is degraded by complex consortia of anaerobic bacteria (8). Fermentative and acetogenic bacteria are absolutely necessary for the conversion of organic matter to methane because methanogenic archaea grow only on a small number of substrates like acetate, formate, methanol, methylamines and CO_2 and H_2 (9). Fermentative bacteria convert organic biopolymers to acetate, CO_2 and H_2, which can be directly used by methanogens, and to reduced organic compounds like propionate, butyrate, lactate and ethanol. Subsequently, these intermediates are converted by acetogenic bacteria into methanogenic substrates. Because of unfavorable thermodynamics these conversions are only possible if methanogens consume the formed products, H_2 and acetate, efficiently (10,11). This leads to obligate syntrophic conversions. As a consequence, at least three different trophic groups of microorganisms are involved in the complete anaerobic conversion of organic matter (Fig. 1). In the presence of inorganic electron acceptors, such as SO_4^{2-}, Fe^{3+} or NO_3^-, anaerobic respiration becomes important and that will influence the formation of methane. Methanogens may become outcompeted by nitrate- or sulfate reducers or by organisms that can utilize Fe^{3+} as an electron acceptor, since these organisms have better kinetic properties on substrates like H_2, formate and acetate (12-15).

In most methanogenic environments acetate is quantitatively the most important substrate for methanogens. In general, 60-70% of the biological methane is formed from acetate (16-18). Therefore, a better understanding of the acetate metabolism is crucial to predict the rate of methane production in anaerobic freshwater environments. All methanogens which are able to grow on acetate belong to the genera *Methanosarcina* and *Methanosaeta*. The two genera have considerable differences in their kinetic properties. *Methanosarcina* has a high growth rate but a low affinity for acetate, while *Methanosaeta* has a low growth rate but a high affinity for acetate (19). Consequently, *Methanosaeta* is dominant in methanogenic environments with low acetate concentrations. It has been shown that acetate is mainly consumed by sulfate reducers when sulfate becomes available in anaerobic environments. The outcome of competition between acetate-degrading methanogens and sulfate reducers can partially be explained by the better kinetic properties of sulfate-reducing bacteria (20-22). However, in some methanogenic environments like paddy soils and freshwater sediments it is less clear how acetate is degraded in the presence of inorganic electron acceptors like SO_4^{2-}, Fe^{3+} and NO_3^-. A complete conversion of acetate to methane has been reported, even in the presence of an excess of SO_4^{2-} and Fe^{3+} (23). Furthermore, it was shown that the inhibition of methanogenesis by NO_3^- is not the result of competition for substrate but due to the formation of toxic intermediates (24-26). Therefore, it is not yet clear how methane formation from acetate is influenced by the presence of inorganic electron acceptors.

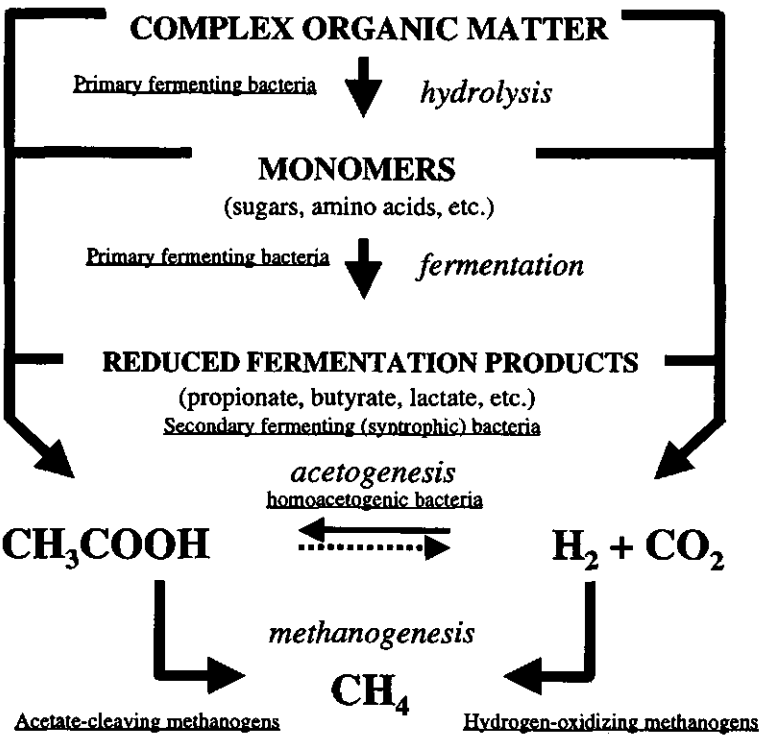

Figure 1. Model of oxidation of acetate in anaerobic freshwater sediment under methanogenic conditions. Adapted from Ward and Winfrey (27).

OUTLINE OF THE THESIS

The aim of the research presented in this thesis was to investigate how the syntrophic degradation of propionate and butyrate, and the methanogenic conversion of acetate in sediments is affected by the presence of sulfate and nitrate. The final goal was to determine the short- and long-term effects of changes in the environmental conditions (i.e. the presence of sulfate and nitrate) on methanogenic consortia and the emission of methane from sediments.

In Chapter 2 an overview is given of the physiological, ecological and biochemical aspects of acetate-utilizing anaerobes and their metabolic interactions. In Chapter 3 a brief description of the study area the polder Zegvelderbroek is given. The area is located between Leiden and Utrecht, and is representative for similar polders in The Netherlands. The polder contains peat grasslands with ditches that maintain stable

water levels. These ditches contain sediment which form a potential source of CH_4. The polder Zegvelderbroek is located in one of the most industrialized and agriculturally intensive areas of The Netherlands. SO_4^{2-} and NO_3^- can be present in significant concentrations e.g. due to water pollution as a result of anthropogenic activities or percolating water. Therefore, the presence of these compounds in groundwater may control the methane emission from sediments. In Chapter 4 the potential methanogenic and syntrophic activity in the sediment and the influence of SO_4^{2-} and NO_3^- on these potential activities is described. Intermediates that are of importance in the terminal steps in the degradation of organic matter in the sediment are described in Chapter 5. The anaerobic conversion of acetate in the presence and absence of sulfate or nitrate is reported in Chapter 6. The dominant acetate-utilizing microorganisms in the sediment were characterized with the Most Probable Number (MPN) technique (Chapter 6). In Chapter 7 the isolation and characterization of an acetate-utilizing methanogen, sulfate reducer and nitrate reducer obtained via the MPN dilutions are described. Finally, the conversion of acetate by methanogenic and sulfidogenic communities under acetate-limited conditions in a chemostat was studied in Chapter 8. The results presented in this thesis are summarized and discussed in Chapter 9.

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CHAPTER 2

ACETATE MINERALIZATION IN FRESHWATER SEDIMENTS

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Submitted for publication.

ABSTRACT

Acetate is quantitatively the most important substrate for methanogens in anaerobic freshwater environments. The presence of inorganic electron acceptors strongly affects acetate degradation by the methanogens because of the activity of anaerobic respiring microorganisms. Many anaerobic microorganisms are capable to grow on acetate. Some of these microorganisms are specialists, and utilize acetate as the sole substrate, but many others are generalists and grow on other substrates as well. In some cases the growth kinetic properties of acetate-utilizing organisms can be used to predict the outcome of the competition for acetate. Unfortunately, information about these parameters is still lacking for most of the anaerobically respiring microorganisms. The two important pathways, which can be distinguished in anaerobic acetate-degrading microorganisms, are the citric acid cycle and the acetyl-CoA cleavage pathway. It is not clear whether the type of pathway determines the activity of anaerobic acetate-degrading microorganisms. Several types of metabolic interactions might occur between anaerobic microorganisms and aceticlastic methanogens. These include competition for limiting amounts of acetate, antagonistic interactions, and inhibition caused by the formation of toxic inorganic compounds. In addition, the metabolic interactions between different respiring bacteria, competition for inorganic electron acceptors and other electron donors, are important as well. Carbon isotope fractionation, tracer and inhibition techniques are useful methods to get qualitative and quantitative information of the processes responsible for the consumption and production of acetate in the environment. Conventional identification and quantification techniques, immunodetection, membrane lipid analysis and molecular microbial detection techniques appear to be very useful to understand the fate of acetate and the interactions and dynamics of the different microorganisms in freshwater environments. In this review physiological, ecological and biochemical aspects of acetate-utilizing anaerobes and their metabolic interactions are presented to obtain a better understanding of the acetate metabolism in freshwater sediments.

INTRODUCTION

Biological methane formation is important in those anaerobic environments where inorganic electron acceptors are limiting in the conversion of organic matter. Wetlands, paddy fields, gastro-intestinal track of ruminants and other animals, and landfills are major natural and anthropogenic sources of biological origin which contribute to the increased level of methane in the atmosphere (1). This process is of great concern because of the potential role of methane in climate change and atmospheric chemistry. At this moment, it is not clear why the concentration of methane in the atmosphere is increasing. However, there is no doubt that the rise in methane is related to growing anthropogenic activities during the last 300 years (2). A methane budget in which all sources and sinks are included should give insight into the increase of atmospheric methane (emission = production – consumption) (3). As shown in Chapter 1 (Table 1), the

estimates of the individual sources are still very uncertain. For the determination of a conscientious budget more knowledge of anthropogenic and natural sources, sinks and factors controlling methane release to the atmosphere is needed. A better understanding of underlying microbial processes of methane formation and oxidation will contribute to an improved budget.

Methane is almost exclusively produced by a unique group of archaea known as methanogens (4). Methanogens use only a very limited number of substrates for growth. Other microorganisms provided these substrates during the degradation of complex organic matter. Thus, associations of methanogenic and non-methanogenic microorganisms are required for methanogenesis (5). Methane production is influenced by factors which can alter the interactions between methanogens and other microorganisms e.g. by the availability of electron acceptors, organic matter supply and temperature (6).

In most methanogenic environments acetate is quantitatively the most important substrate for methanogens (6-9). Therefore, a better understanding of the acetate metabolism is crucial to predict the rate of methane production in different environments. In this review physiological, ecological and biochemical aspects of acetate-utilizing anaerobes and their metabolic interactions are presented.

ACETATE, A KEY INTERMEDIATE IN THE ANAEROBIC DEGRADATION OF ORGANIC MATTER

Methanogens grow only on a limited number of substrates, H_2/ CO_2, formate, methanol, methylamines and acetate being the most important ones (4,5). Therefore, organic matter has to be metabolized to these compounds by other physiological types of microorganisms. In methanogenic environments, communities of fermenting, acetogenic and methanogenic microorganisms degrade organic compounds (Chapter 1, Fig. 1). Fermenting microorganisms excrete enzymes, which hydrolyze complex organic matter to compounds like sugars, amino acids, purines, pyrimidines and long chain fatty acids. These are subsequently fermented to acetate, H_2 and CO_2, and to reduced products like alcohols, lactate and volatile fatty acids (e.g. propionate, butyrate). Long chain fatty acids and the volatile fatty acids are converted by H_2-producing acetogenic bacteria to acetate, formate, H_2 and CO_2, which are then degraded by methanogens (10-12). It has been estimated that 66% or more of the methane formed in freshwater environments is derived from acetate (13). The conversion rate of acetate by methanogens is supposed to be the rate-limiting step in the degradation of soluble organic matter under methanogenic conditions (14).

Methanogenesis is strongly affected by the presence of inorganic electron acceptors. During the anaerobic degradation of organic matter, under non-methanogenic conditions, inorganic compounds such as nitrate, sulfate, sulfur or oxidized metal ions (Fe^{3+}, Mn^{4+}) can serve as electron acceptors (6). The bacteria involved in these processes are facultative (nitrate and iron reducers) or obligate anaerobic (most sulfate reducers) microorganisms. Some of the reactions, which can be carried out by anaerobically respiring microorganisms, are presented in Table 1. The oxidation of organic matter with NO_3^-, Fe^{3+} and SO_4^{2-} as the

possible electron acceptor is depicted in Figs. 1 and 2. So far, no obligate nitrate- or iron-reducing bacteria are known and some sulfate-reducing bacteria are able to reduce nitrate to ammonium in a true respiratory process coupled to electron transport phosphorylation (15-20). For thermodynamical reasons the energy yield of the oxidation of organic matter coupled to the reduction of various electron acceptors decreases in the order $O_2 > NO_3^- > Mn^{4+} > Fe^{3+} > SO_4^{2-} > CO_2$. In mixed microbial populations, the electron acceptor that gives the highest Gibbs free energy change and thus provides the highest growth yield is used (21). Therefore, the ability to use other electron acceptors might give facultative anaerobes a competitive advantage over obligate anaerobes. However, several factors play a role here, such as competition for substrates and biochemical limitations in the utilization of electron donors (see below).

Table 1. Relevant reactions involved in the degradation of acetate: methanogenesis, sulfate reduction, iron reduction and nitrate reduction.

Reaction	$\Delta G^{o'}$ [a] (kJ/reaction)
Methanogenic reactions	
1) $Acetate^- + H_2O \rightarrow CH_4 + HCO_3^-$	- 31.0
Sulfate reducing reactions	
2) $Acetate^- + SO_4^{2-} \rightarrow 2\ HCO_3^- + HS^-$	- 47.3
Iron reducing reactions	
3) $Acetate^- + 8\ Fe^{3+} + 4\ H_2O \rightarrow 2\ HCO_3^- + 8\ Fe^{2+} + 9\ H^+$	-290.1
Nitrate reducing reactions	
4) $5/4\ Acetate^- + 2\ NO_3^- + 3/4\ H^+ \rightarrow 21/2\ HCO_3^- + N_2 + + H_2O$	-990.1
5) $Acetate^- + NO_3^- + H^+ + H_2O \rightarrow 2\ HCO_3^- + NH_4^+$	-495.4

[a] $\Delta G^{o'}$-values are calculated from data in Thauer *et al.* (21).

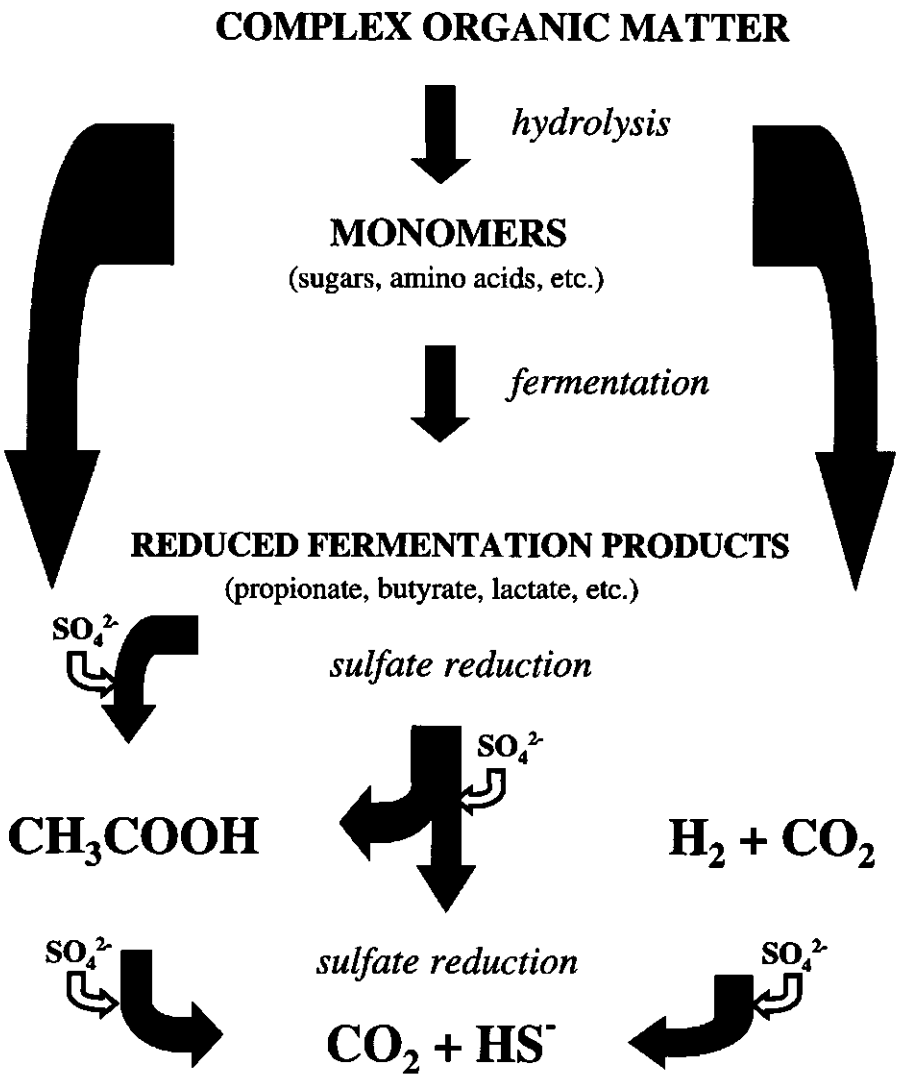

Figure 1. Model of oxidation of acetate in anaerobic freshwater sediment in the presence of SO_4^{2-}. Adapted from Ward and Winfrey (85).

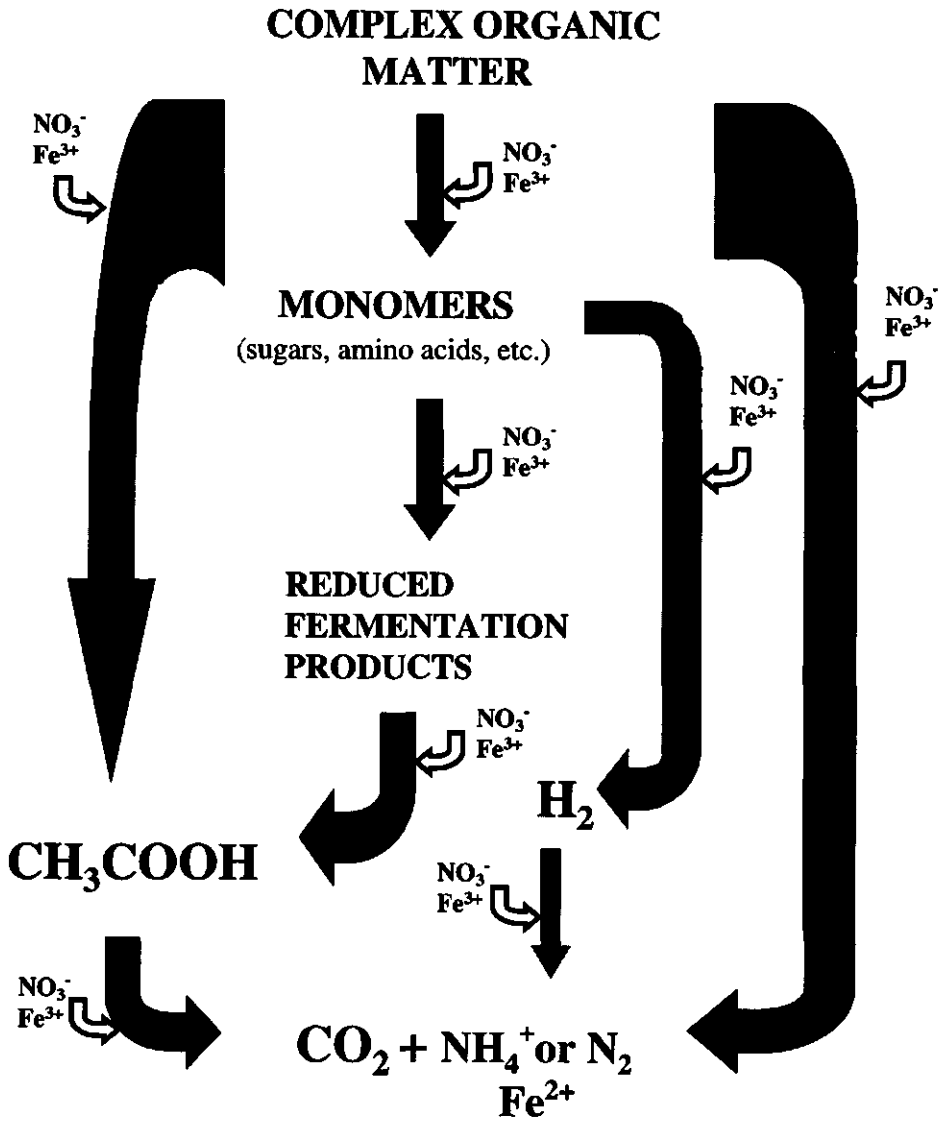

Figure 2. Model of oxidation of acetate in anaerobic freshwater sediment in the presence of Fe^{3+} and NO_3^-. Adapted from Lovley (24).

ANAEROBIC ACETATE-DEGRADING MICROORGANISMS

Many anaerobic microorganisms capable of growth on acetate as energy source have been described. Acetate may even be the sole substrate for some methanogenic and sulfate-reducing bacteria (4,22). Other anaerobic bacteria are generalists and can grow on other substrates as well (23,24). Some anaerobic microorganisms can oxidize acetate to 2 CO_2 and 4 H_2 but this reaction is only possible when the H_2 partial pressure is kept below 10^{-4} atm (25). Therefore, these microorganisms can only grow on acetate in syntrophic association with H_2-consuming microorganisms (26-28). Recently, two syntrophic acetate-oxidizing bacteria were obtained in pure culture and these bacteria appeared to be homoacetogens (26,29). The thermophilic acetate-oxidizing organism described by Lee and Zinder is also able to grow on H_2/CO_2, formate, CO, pyruvate and betaine (28). The ability to grow and obtain energy by reducing CO_2 to acetate with H_2 is a remarkable feature of this organism. *Clostridium ultunense* isolated by Schnürer et al. is able to grow on formate, pyruvate, glucose, ethylene glycol, cysteine and betaine, but H_2/CO_2 did not support growth (29).

Acetate is a common substrate for nitrate-reducing bacteria. The ability to denitrify is widely spread among bacteria and archaea, and shows representatives in almost 130 species within more than 50 genera (30). Most nitrate reducers are regarded as generalists and they are often able to grow with O_2 as an electron acceptor (23). So far, little is known about the role of nitrate reducers as anaerobic acetate-degraders in natural environments. This might be explained by the fact that in most cases the enrichment of denitrifying microorganisms was done with electron donors other than acetate or acetate was just one of them (31-33). Furthermore, denitrifiers were isolated with electron donors and electron acceptors other than acetate and nitrate (34-36). The isolated bacteria were just tested for their capacity to grow on acetate and nitrate.

Recently, *Geobacter metallireducens* and *Geobacter sulfurreducens* have been described which grow on acetate and other organic compounds with Fe^{3+} as the electron acceptor (37,38). Furthermore, *G. metallireducens* grows also on acetate with electron acceptors like Mn^{4+}, U^{4+} and NO_3^-. *G. sulfurreducens* is able to use Co^{3+}, S^0, fumarate or malate as alternative electron acceptors. Other known acetate-degrading Fe^{3+}-reducing microorganisms are *Desulfuromonas acetoxidans* and a bacterium (strain RA 6) belonging to the genus *Geobacter* obtained from rice paddy soil (39,40). Both microorganisms are also capable of oxidizing other organic compounds.

Acetate can be utilized by different genera of sulfate-reducing bacteria. Growth on acetate was demonstrated for *Desulfobacca acetoxidans*, *Desulforhabdus amnigenus*, and *Desulfobacterium*, *Desulfotomaculum* and *Desulfobacter* species (22,41-48). *Desulfobacca acetoxidans* and most *Desulfobacter* species are specialized in growth on acetate (42,47). *Desulfobacter* strains are mostly isolated from brackish and marine sediments, and may be enriched from freshwater environments using brackish water or marine media (46). However, these sulfate reducers probably are not important in the conversion of acetate in freshwater environments. In contrast, *Desulfobacca acetoxidans* shows best growth in freshwater media.

Desulforhabdus amnigenus, *Desulfobacterium* and *Desulfotomaculum* species are generalists, which use besides acetate a wide variety of substrates such as propionate, hydrogen and ethanol (41,43,44). Some sulfate reducers, e.g. *Desulfovibrio baarsii*, *Desulfosarcina variablis*, *Desulfococcus* and *Desulfobacterium* species, show very poor growth on acetate despite the fact that an acetate-degrading pathway is present (47). The reason for the marginal capacity or inability to use acetate as a growth substrate is not clearly understood. Furthermore, these sulfate reducers generally prefer substrates other than acetate. The utilization of mixed substrates was studied with the generalist *D. aminigenes* (49). Cells growing on acetate immediately stopped using acetate when ethanol, lactate or propionate was added. However, addition of hydrogen did not affect acetate oxidation. Hydrogen and acetate were used simultaneously, and this may increase the competitive advantage of *D. aminigenes* over other acetate-degrading microorganisms.

The methanogens that grow on acetate are *Methanosarcina* and *Methanosaeta* (formerly *Methanothrix*). The two methanogens developed different strategies for growth on acetate (50,51). *Methanosarcina* is a generalist and able to grow on several substrates including, H_2/ CO_2, methanol, methylamines and acetate (4,52). This methanogen contains two independent pathways for the degradation of acetate and the H_2-dependent reduction of CO_2. By means of methyl-transferases methanol and methylamines are channeled into the pathway of CO_2 reduction to CH_4. The ability of *Methanosarcina* to utilize C_1 and C_2 compounds is an interesting feature because it enables the methanogen to grow autotrophically, mixotrophically or heterotrophically (53). *Methanosaeta* species are specialists which use only acetate as energy source. The conversion of acetate in *Methanosaeta* occurs via a similar pathway, as in *Methanosarcina*, but the activation system is different (see below). The physiology of the aceticlastic methanogens has been reviewed previously (53,54).

Kinetic properties of microorganisms can be useful to explain which population is favored in an environment with certain substrate concentrations. Table 2 shows the physiological properties of some of these organisms (45,49,55-70). The affinity of *Methanosaeta* for acetate is higher than that of *Methanosarcina* (Table 2). Furthermore, the minimum threshold concentration of acetate utilization is lower for *Methanosaeta* (< 10 μM) than for *Methanosarcina* (0.2-1.2 mM). The influence of kinetic parameters (μ_m, K_s and threshold) on the growth rate of *Methanosaeta* and *Methanosarcina* at different substrate concentrations is represented in Fig. 3. From this figure it can been seen that *Methanosaeta* will be favored in environments with acetate concentrations below 1 mM (18). In general, sulfate reducers have a higher affinity for acetate and a lower threshold concentration of acetate compared with aceticlastic methanogens (Table 2).

Table 2. Comparison of growth kinetic data of acetate-degrading anaerobes.

Bacterial strain	physiology	K_s (mM)	μ_{max} (1/day)	Yield (g/mol acetate)	Threshold (mM)	K_m (mM)	V_{max} (μmol min^{-1} g^{-1} protein)	Reference
Methanogens								
Methanosarcina sp.	generalist	3-5	0.46-0.69	1.6-2.4	190-1180	3.0	-	55-61
Methanosaeta								
soehngenii	specialist	0.5-0.7	0.08-0.29	1.1-1.4	7-69	0.4-1.2	38-42	60-64
concilii	specialist	1.2	0.21-0.69	1.1-1.2	-	0.8	16	60,65,66
Sulfate reducers								
Desulfomaculum acetoxidans	generalist	-	0.65-1.39	5.6	-	-	-	67
Desulfobacter postgatei	specialist	-	0.72-1.11	4.3-4.8	-	0.07-0.23	53	45,68,69
Desulfobacca acetoxidans	specialist	-	0.31-0.41	-	<15	0.6	43	49
Desulforhabdus amnigenus	generalist	-	0.14-0.20	-	<15	0.6	28	49
Iron reducers								
Geobacter metallireducens GS-15	generalist	-	0.2-0.3[1]	5.6[1]				70

[1] estimated from published data (assumption 1 cell is 1 pg dw)

This lower threshold concentration for sulfate reducers can be explained thermodynamically. A threshold concentration exists below which the overall change in free energy of the degradation reaction is too low to couple it to the formation of metabolic energy (60,61,71). Because the energy yield of the oxidation of

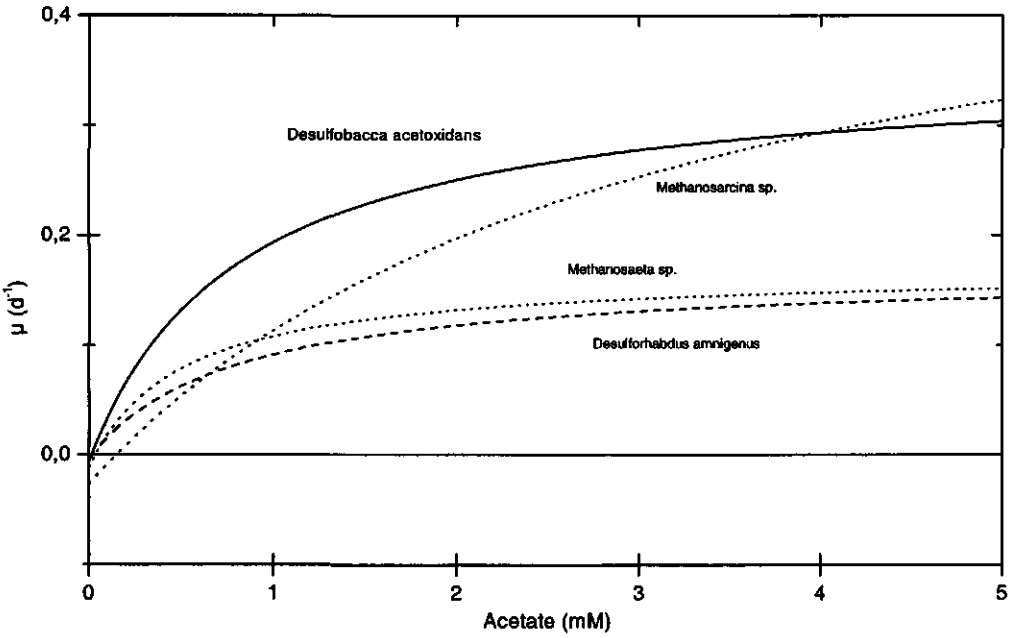

Figure 3. Relationship between specific growth rate (μ), threshold and acetate concentration for *Methanosaeta* sp., *Methanosarcina* sp., *Desulfobacca acetoxidans* and *Desulforhabdus amnigenus*. For the sulfate reducers it was assumed that K_s is equal to K_m (see Table 2 for kinetic parameters).

acetate is higher for sulfate reducers than for methanogens, sulfate reducers are able to carry out the degradation of acetate at lower concentrations. The kinetic parameters can also be used to predict the outcome of the competition for acetate between methanogens and sulfate reducers. From Fig 3. it is clear that the sulfate reducer *Desulfobacca acetoxidans* (specialist) is preferred in environments with acetate concentrations below 4 mM and where sulfate is not limiting. Furthermore, it shows that *Methanosarcina* is favored in the ecosystem when acetate concentrations are above 4 mM, even at an excess of sulfate. Remarkably, the model predicts that the generalist *Desulforhabdus amnigenus* will be outcompeted by *Methanosaeta* at each acetate concentration what so ever. However, it should be mentioned that predictions made on differences in the kinetic parameters could only partly explain the competition between sulfate-reducing bacteria and methanogens (see section 4). Many studies focused on the growth kinetic properties of aceticlastic methanogens and sulfate reducers. Unfortunately, information about these parameters is still lacking for Fe^{3+}/Mn^{4+} and NO_3^-- reducing microorganisms.

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The difference in affinity for acetate between the methanogens *Methanosaeta* and *Methanosarcina* coincides with differences in the acetate-activating enzyme systems (18). In *Methanosaeta* acetate is activated by acetyl-CoA synthetase (acetate thiokinase) (72). The enzyme activates acetate to acetyl-CoA by hydrolysis of one ATP to AMP and pyrophosphate. As pyrophosphate is cleaved into 2 phosphate while AMP and ATP is converted to 2 ADP in the adenylate kinase reaction, the activation of acetate requires net 2 ATP. *Methanosarcina* employs an acetate kinase and phosphotransacetylase to activate acetate at the expense of only one ATP (73). In this way, the abundance of *Methanosaeta* in environments with a low acetate concentration has a biochemical basis. Remarkably, up to now no respiring microorganisms have been isolated which activate acetate in a similar fashion as *Methanosaeta*; they all activate acetate by means of a kinase (15,74). Unfortunately, the K_m values of the enzyme in anaerobically respiring organisms have not been determined in detail. The affinity for acetate of the acetate kinase of *Desulfotomaculum acetoxidans* is higher than that of the *Methanosarcina* enzyme. The affinity of whole cells for acetate may also be determined by active transport systems for the uptake of acetate. Unfortunately, nothing is known about acetate uptake in strict anaerobes. *Desulfobacter sp.* and the sulfur-reducing bacterium *Desulfuromonas acetoxidans* use the citric acid cycle for the oxidation of acetate. These bacteria activate acetate by a succinyl-CoA:acetate HSCoA-transferase (75-77). Kinetic data for different activations systems are listed in Table 3. The K_m values of the different enzyme systems vary from 0.04 to 22 mM (18,75,77-79).

Two important pathways can be distinguished in anaerobic acetate-degrading microorganisms. These pathways have been discussed in detail elsewhere (14,15,19). General schemes of these pathways are depicted in Fig. 4a-c. One pathway is the citric acid cycle, which is operative in some anaerobic bacteria. In sulfate- and sulfur-reducing bacteria the TCA cycle is slightly different from the route in aerobic and nitrate-reducing bacteria; the citrate synthase reaction may be coupled to ATP synthesis, α-ketoglutarate dehydrogenase is ferredoxin dependent and the malate dehydrogenase may be membrane-bound. The second route is the so-called acetyl-CoA cleavage pathway. This pathway results in the disproportionation of acetate to CO_2 and CH_4 in methanogens (15). In sulfate reducers, which use this pathway, the C_1 units formed after cleavage of acetyl-CoA are further oxidized to CO_2 (14). The carbon monoxide dehydrogenase (CODH) is an important key enzyme in this pathway (80).

Table 3. Comparison of the pathways utilized by anaerobic acetate-degrading microorganisms.

Metabolic pathway	kinetic properties of enzymes involved in activation of acetate[1]								
microorganism	acetyl-CoA synthase		acetate kinase		phosphotrans-acetylase		succinyl-CoA: acetate CoA-transferase		Reference
	K_m^{Ac}	K_m^{CoA}	K_m^{Ac}	K_m^{ATP}	K_m^{CoA}	$K_m^{acetyl\text{-}PO3}$	K_m^{Ac}	$K_m^{succinyl\text{-}CoA}$	
acetate disproportionation									
Methanosaeta concilli	0.86	0.048	-	-	-	-	-	-	54
Methanosarcina barkeri	-	-	22	2.8	0.09	0.165	-	-	79
CO dehydrogenase pathway									
Desulfomaculum acetoxidans	-	-	2	2.4	0.10	0.40	-	-	78
TCA cyclus									
Desulfuromonas acetoxidans[2]	-	-	-	-	-	-	0.50	0.15	77
Desulfobacter postgatei	-	-	-	-	-	-	0.04	ND	75

[1] K_m values in mM
[2] Sulfur-reducing bacterium

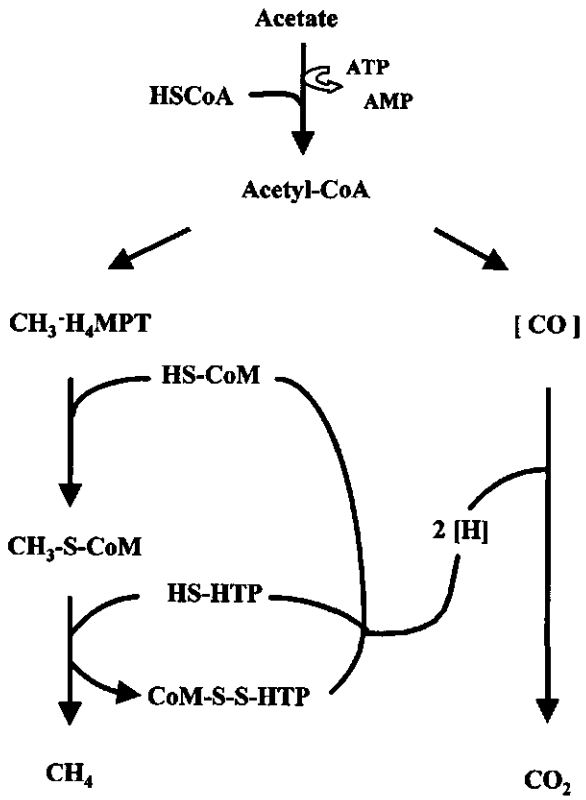

Figure 4a. Pathway of acetate fermentation in the methanogen *Methanosaeta soehngenii*. Abbreviations: HSCoA, coenzyme M; CH_3-H_4MPT, methyltetrahydromethanopterin; CH_3-S-CoM, methyl-coenzyme M; HS-HTP, 7-mercaptoheptanoylthreoninephosphate; CoM-S-S-HTP, heterodisulfide of coenzyme M and HS-HTP. Adapted from Blaut (53).

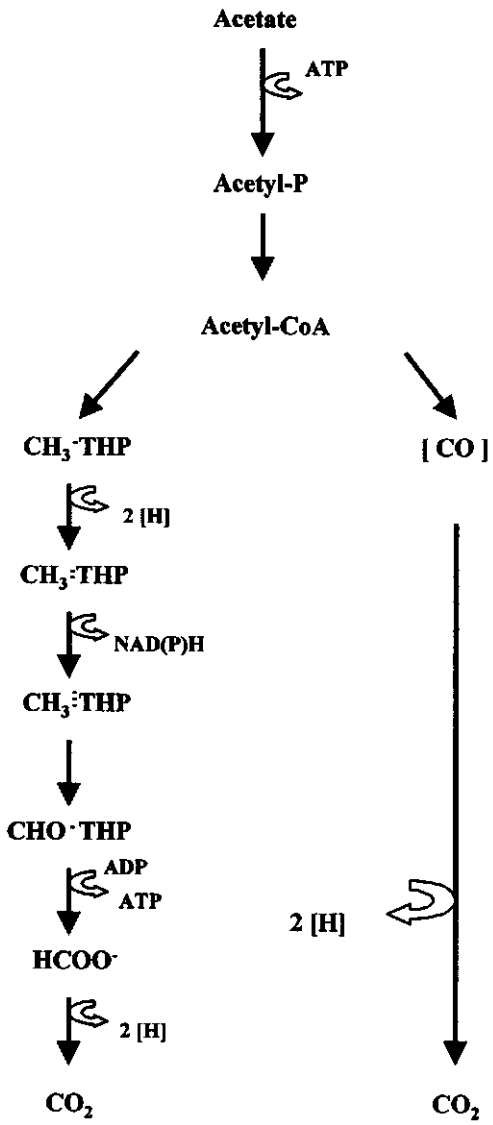

Figure 4b. The carbon monoxide dehydrogenase pathway operative in the acetate-degrading sulfate reducer *Desulfotomaculum acetoxidans*. Abbreviations: Acetyl-P, acetyl phosphate; [H], unknown physiological electron or hydrogen carrier; THP, tetrahydropterin. Adapted from Widdel and Hansen (22).

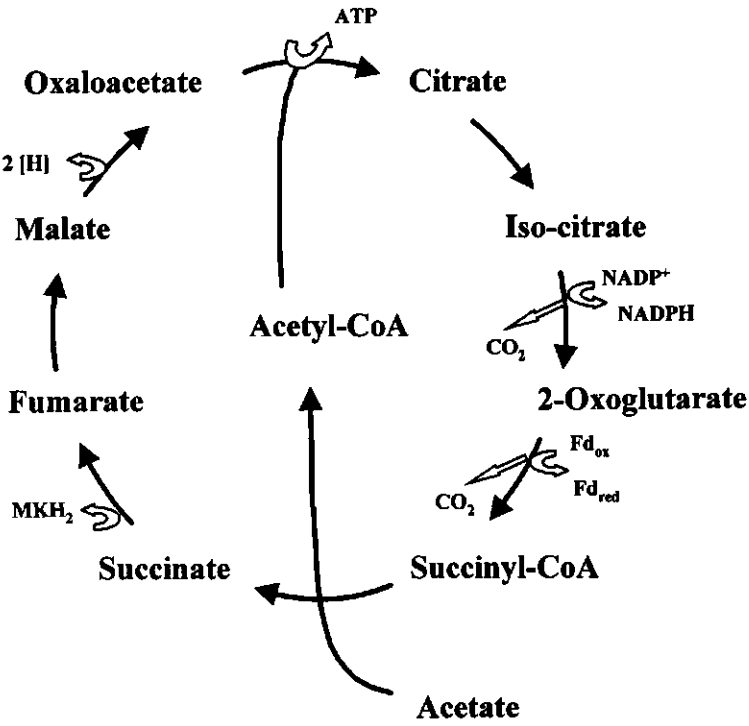

Figure 4c. The citric acid cycle operative in the acetate-degrading sulfate reducer *Desulfobacter postgatei*. Abbreviations: [H], unknown physiological electron or hydrogen carrier; $Fd_{red/ox}$, reduced/oxidized ferredoxin; MKH_2, menaquinone. Adapted from Widdel and Hansen (22).

INTERACTIONS BETWEEN ANAEROBIC MICROORGANISMS INVOLVED IN ACETATE METABOLISM

Changes in the environmental conditions will influence the microbial interactions between the community members present. For example, several studies have shown that addition of electron acceptors like NO_3^-, Fe^{3+} or SO_4^{2-} to methanogenic environments resulted in the inhibition of methanogenesis (81-85). However, it was also reported that acetate was almost completely converted to methane at an excess of sulfate and iron (86). This indicates that it is not always clear how aceticlastic methanogenesis is influenced in the presence of inorganic electron acceptors. In the following paragraphs several interactions between the anaerobic microorganisms involved in the acetate metabolism and their effect on the formation of CH_4 will be discussed.

Competition for acetate

Methanosaeta and *Methanosarcina* are the two known acetate-degrading methanogenic genera. The competition for acetate between these methanogens has been reviewed before (18). Although *Methanosaeta* and *Methanosarcina* sp. compete for acetate, this competitive interaction does not affect the formation of CH_4 as such.

Previous studies have shown that sulfate reducers can outcompete methanogens for acetate when sufficient sulfate is present (82-85). Sulfate reducers have better enzyme kinetic and growth kinetic properties than methanogens (Table 2 and 3), and they conserve more energy per mole of acetate than methanogens. The kinetic properties can be used to predict the outcome of the competition (see section 3). A simulation of the competition between methanogens and sulfate reducers revealed that the outcome of the competition could be predicted with Monod kinetics as well (87). In the applied model, the affinities for acetate and sulfate, decay and growth rates and growth yields were considered. It was assumed that acetate affinities, growth rates and decay rates for acetate-utilizing methanogens and sulfate reducers were in the same range. However, the biomass yield on acetate for sulfate reducers was two times higher than that for methanogens (Table 2). It was calculated that, due to the small difference in growth rates and affinities, the relative number of methanogens and sulfate reducers determines how long it will take before methanogens are outcompeted. These simulations were done assuming that sulfate is present in excess. However, many environments exhibit low or alternating sulfate concentrations, and this is of major importance for the outcome of the competition. Oude Elferink et al. mentioned that mixed substrate utilization by generalists might play a role as well in the competition for acetate (49). The kinetic properties of *Methanosaeta* sp. (specialists) are slightly better than those of the generalist *Desulforhabdus amnigenus*. On basis of these parameters one would expect that *Methanosaeta* sp. should outcompete the sulfate reducer (see Table 2 and

Fig. 3). However, *D. amnigenus* outcompeted acetate-degrading methanogens in a bioreactor treating complex wastewater. This indicates that the ability to use other substrates besides acetate may give *D. amnigenus* a competitive advantage over *Methanosaeta* sp. Several other factors which can affect the competition between methanogens and sulfate reducers include temperature, pH and the toxicity of sulfide (see below) (88).

The addition of NO_3^- and Fe^{3+} to sediments inhibited methanogenesis and sulfate reduction (81,82,84,89). It was suggested that Fe^{3+}-reducing microorganisms can inhibit methane production and sulfate reduction by outcompeting methanogens and sulfate reducers for acetate (89). However, a different result was reported for observations in a paddy field (86). Here aceticlastic methanogens competed successfully with acetate-utilizing Fe^{3+}-reducing bacteria for the available acetate. Achtnich et al. suggested that the iron reducers in the paddy soil had a higher threshold for acetate than the methanogens. This might explain the outcome of the competition for acetate between both populations but information about the threshold is still lacking for Fe^{3+}-reducing bacteria (see section 3). For denitrifying bacteria it was suggested that the inhibition of methanogenesis is not only the result of competition for substrate but is also due to the complete oxidation of precursors of acetate or formation of toxic intermediates (see below). Experiments with labeled acetate in a freshwater sediment showed that acetate stimulated the production of labeled methane but methanogenesis was inhibited when SO_4^{2-} was added as well (82). Obviously, the mechanism behind this inhibition was the competition for acetate. This hypothesis was supported by results obtained in inhibition studies (6). Addition of acetate in combination with NO_3^- resulted in the complete inhibition of methanogenesis. The inhibition of methanogenesis could not be explained by competition alone, because in that case some formation of methane should have been observed in the acetate and NO_3^- incubations.

Competition for electron acceptors and other electron donors

Anaerobically respiring microorganisms can compete with methanogens for the available acetate. Growth of aceticlastic methanogens depends only on the acetate concentration, whereas that of anaerobically respiring microorganisms depend on the acetate and electron acceptor concentration. At low electron acceptor concentrations the growth of these bacteria will be limited and therefore they become less effective competitors. This could enable methanogens to outcompete sulfate reducers for acetate (90). Laanbroek et al. studied the competition for ethanol and sulfate among three sulfate reducers in a sulfate-limited chemostat (91). Sulfate limitation resulted in the incomplete oxidation of ethanol to acetate, which was not degraded further under sulfate limitation. It was also shown that sulfate reducers stopped consuming acetate when other electron donors (lactate, propionate, ethanol) became available (49). So, under limiting conditions, the complete oxidation of substrates will result in an advantageous situation for sulfate-reducing bacteria. However, when sulfate becomes limiting for these microorganisms the formation of methane is favored.

Perhaps other anaerobic microorganisms respond in a similar way as sulfate reducers on electron acceptor limitation or the availability of other electron donors (mixotrophic growth). Unfortunately, such information is still lacking for Fe^{3+}/Mn^{4+} and NO_3^-- reducing microorganisms. Studies in energy-limited chemostats with defined mixed cultures of anaerobic acetate-utilizing microorganisms can give insight in on how these factors affect the competition for acetate between methanogens and anaerobic respiring microorganisms.

Syntrophic degradation of acetate

In methanogenic habitats, interspecies hydrogen transfer plays an important role in the anaerobic degradation of organic matter (9,10,92). Zinder and Koch described a thermophilic acetate-degrading coculture consisting of an acetate-degrading homoacetogen and a H_2-consuming methanogen (93). Also mesophilic acetate-oxidizing syntrophic methanogenic (27-29) and sulfate-reducing cocultures (94) have been described. The free energy change of acetate oxidation to H_2 and CO_2 is temperature dependent. It has been suggested that the syntrophic degradation of acetate under methanogenic conditions is only favorable at temperatures above 35 °C (92). However, the sulfate-dependent syntrophic oxidation is energetically more favorable than the methanogenic syntrophic acetate oxidation (Fig. 5). Therefore, the temperature dependence of the syntrophic degradation of acetate under sulfate-reducing conditions is less than under methanogenic conditions. Recently, syntrophic acetate degradation has been reported to occur at 30 °C (95). It was shown that *Geobacter sulfurreducens* could oxidize acetate in syntrophic cooperation with partners such as *Wolinella succinogenes* or *Desulfovibrio desulfuricans* with nitrate as the electron acceptor. The relative importance of syntrophic acetate degradation in nature is not exactly known. However, an interesting feature is that the CO dehydrogenase pathway in the acetate-cleaving bacteria is similar to the pathway of some acetate-degrading sulfate-reducing bacteria. Therefore, it has been speculated that acetate-degrading sulfate reducers oxidize acetate in syntrophy with methanogens when sulfate becomes depleted (96).

Antagonistic interactions involved in the degradation of acetate

A remarkable example of sulfate-dependent interspecies H_2 transfer has been described between an acetate-utilizing *Methanosarcina* and H_2-utilizing *Desulfovibrio* species (97). *Methanosarcina* species are known to produce H_2 during growth on acetate (98,99). In coculture, *Desulfovibrio vulgaris* kept the H_2 partial pressure low enough to shift the catabolism of the methanogens more to CO_2 instead of CH_4 formation. This phenomenon appeared to be disadvantageous for *Methanosarcina barkeri* because of the potential loss of energy conservation that is coupled to methanogenesis. The interaction appears to be an antagonistic one (97). It was proposed that the sulfate-dependent inhibition of aceticlastic methanogenesis in freshwater sediments and paddy fields is in part due to this type of interspecies H_2 transfer rather than to direct competition between methanogens and sulfate reducers for acetate (86,100,101). Conrad discussed whether a similar explanation

might hold for the inhibition of aceticlastic methanogenesis by Fe^{3+} and NO_3^- and mentioned the possibility that H_2-utilizing sulfate reducers like *Desulfovibrio desulfuricans* can function as ferric iron or nitrate reducers (102). This metabolic potential could be of importance in situations where the interactions between respiring bacteria and methanogens can not be explained by the competition models (11,87).

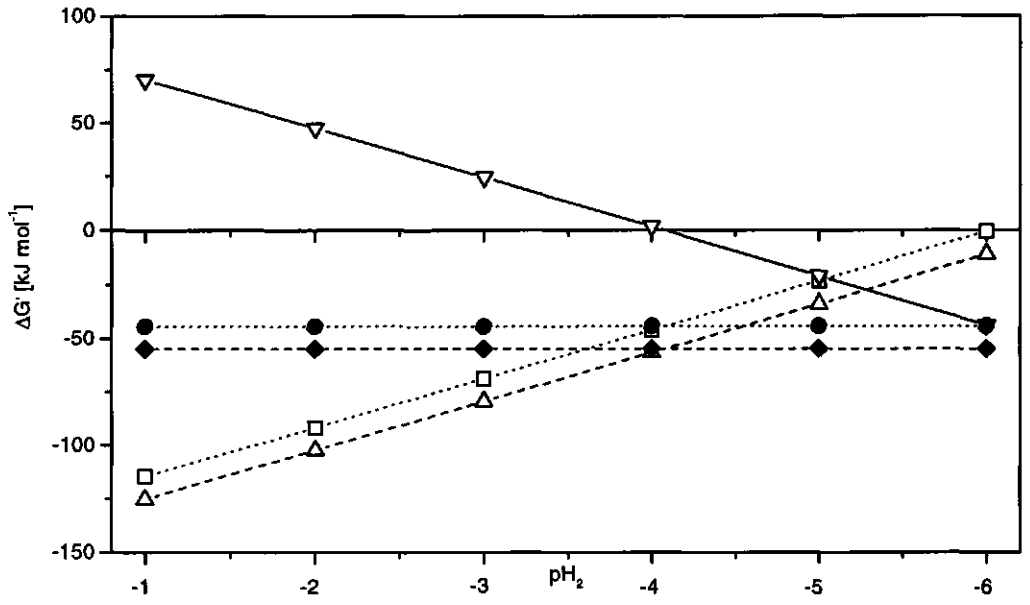

Figure 5. Gibbs free energy changes of acetate and hydrogen metabolism at different hydrogen partial pressures. Conditions: temperature: 298.0 K; pH=7; CH_3COO^-: 40 mM; SO_4^{2-}: 20 mM; CH_4: 100 Pa; HCO_3^-: 20 mM and HS^-: 5 mM. ∇: acetate conversion to hydrogen and bicarbonate; Δ: H_2 consumption by sulfate reducer; □: H_2 consumption by methanogen; ◆ : syntrophic acetate degradation under methanogenic conditions; ●: syntrophic acetate degradation under sulfidogenic conditions.

Inhibition caused by inorganic compounds

Sulfide is the endproduct of sulfate reduction. Cappenberg suggested that the distribution of sulfate reducers and methanogens in sediment in a freshwater lake might be due to sensitivity of methanogens to hydrogen sulfide (H_2S) (103). However, it was shown that sulfide inhibits both sulfate reducers and methanogens (104-109). Also the reduction of nitrate in denitrifying *Pseudomonas fluorescens* and *Desulfovibrio desulfuricans* was shown to be inhibited by sulfide (16,110). Values for the free H_2S concentration at which

methanogenesis was inhibited for 50% vary between 1.5 and 8 mM H_2S (104-109). For sulfate reduction a value of 2.5 mM H_2S was reported (104). Reis et al. showed that sulfate-reducing bacteria are inhibited directly by H_2S (111,112). Free H_2S binds to many biomolecules (e.g. cytochromes) which are than inactivated due to the binding of sulfide. This inactivation might explain the toxic effect of H_2S on sulfate reducers and other microorganisms (113). An example of sulfide inhibition which could be of importance in natural situations is discussed below.

It has been suggested that inhibition of methanogenesis by NO_3^- is due to the formation of toxic intermediates of the denitrification process (83,84). It was shown that methanogens are not only inhibited by competition for acetate between denitrifying bacteria and methanogens, but also by reduced nitrogen forms during denitrification (114,115). This inhibition effect can not be caused by an increased redox potential (116). Instead, the available information on this type of inhibition suggest that nitrogen oxides (NO_3^-, NO_2^-, NO and N_2O) cause an inhibition of the enzyme activity in methanogens. This was confirmed by studies where the inhibitory effects of NO_3^-, NO_2, NO and N_2O on pure cultures of methanogens were investigated (114,117,118). Gaseous nitrogen oxides caused a greater decrease in methanogenesis than nitrite and nitrate.

Two pathways of nitrate reduction can be distinguished: denitrification by which nitrogen oxides (NO_3^- and NO_2^-) are reduced to dinitrogen gases (N_2O and N_2), and dissimilatory nitrate reduction to ammonium. Evidence from studies with freshwater sediment showed that the free sulfide concentration determines the type of nitrate reduction (119). At low sulfide concentrations nitrate was reduced via denitrification whereas at high sulfide concentrations, dissimilatory nitrate reduction to ammonium and incomplete denitrification to gaseous nitrogen oxides took place. It was proposed that sulfide inhibited the activity of NO- and N_2O reductases (119). The accumulation of gaseous nitrogen oxides due to the presence of sulfide might cause a prolonged inhibitory effect of nitrate on methanogenesis. However, in most sediments sulfide concentrations are low as long as sulfate reduction is inhibited (e.g. as they are outcompeted for acetate). Moreover, the effects of sulfide inhibition will be small as sulfide concentrations in freshwater sediments always remain below the inhibiting concentrations for nitrate reduction and methanogenesis (120).

Besides, inorganic compounds like H_2S might influence methanogenesis in a different way as it can also be used as electron donors by facultative anaerobic chemolithoautotrophs that respire NO_3^- (121). Sulfate reducers produce H_2S and thus provide substrate to nitrate reducers. Such chemolithoautotrophic denitrifiers may compete with heterotrophic microorganisms for the available NO_3^-. A similar interaction can occur under influence of a coupled iron oxidation with nitrate reduction (122). Methanogenesis might be increased under influence of these conversions, as the competition for acetate is less severe for sulfate or ferric iron than for nitrate (as explained above). Incubations of freshwater sediments with high acetate, nitrate and sulfate concentrations could only be described if a significant part of the nitrate reduction was attributed

to the oxidation of sulfide rather than to the oxidation of acetate (120). This indicates that such conversions might play an important role in freshwater sediments.

QUANTIFICATION AND IDENTIFICATION OF THE ACETATE DEGRADING MICROORGANISMS

To get a more detailed insight in the role of the different groups of acetate-degrading microorganisms in freshwater sediments it is essential to combine acetate turnover studies with the characterization of the acetate-degrading microbial populations. Some conventional and molecular tools, which can be used in sediment studies, are discussed below.

Conventional methods

Conventional identification and quantification techniques are often based on selective growth media. The Most Probable Number (MPN) technique for example is a technique in which serial sample dilutions are inoculated in selective liquid media. By assuming that single cells will grow at the highest dilutions, the number of a certain group of microorganisms in the ecosystem can be estimated (123). This method can give very useful information of acetate-degrading microorganisms that are present in high numbers and that are able to grow in artificial media. However, many microorganisms cannot be cultivated in artificial media yet. Furthermore, this method will underestimate the number of microorganisms if these are attached to solid substrates or are associated to each other, like the threaded *Methanosaeta* (2). In some cases it is not even possible to quantify the numbers of a certain group of bacteria; e.g. in a methanogenic sludge from a bioreactor the relative low numbers of acetate-degrading sulfate reducers could not be quantified because they were overgrown by the methanogens which were present in much higher numbers (124).

Substrate conversion rates are often used for microbial ecosystem characterization (see below). These calculated rates give information on maximum possible metabolic activity of the different microbial groups. Unfortunately, they cannot be used for the identification or quantification of the microorganisms in complex environments (125,126). However, the calculated number of microorganisms based on turnover rates can be compared with numbers obtained with MPN counts. Because of the independent approach the "turnover" method can be used to validate the MPN technique (82).

Direct microscopic analyses have always played an important role in the characterization of microbial populations. The major drawback of most microscope techniques is the fact that the identification of microbes is usually based on cell morphology only, which for most bacteria is not very distinctive. An exception form methanogens, which can be identified by epifluorescence microscopy by detecting the coenzyme F420-dependent autofluorescence (127). However, *Methanosaeta* does not exhibit

autofluorescence but these methanogens have such a typical morphology that they can be distinguished easily (128).

Despite the limitations of morphology and conventional microbial identification techniques based on isolation and cultivation, these methods are useful for a rough characterization of the microbial populations. However, for detailed characterization studies direct identification methods for microorganisms are essential. Such methods are available and allow a direct identification of acetate-degrading microorganisms.

Immunodetection

Immunodetection is a very powerful tool for the identification of microorganisms in complex environments, because it is easy to use, inexpensive, and mostly very specific. This technique has, for example, been applied successfully for the detection of aceticlastic methanogens in anaerobic bioreactors (129-133) or for the identification of sulfate reducers in sediments (134-136). A good result with quantitative analysis of methanogens in sludge has been achieved with enzyme-linked immunoasorbent assays (137). Nevertheless, it should always be taken into account that antibodies can cross-react with other non-related strains (133,134).

Membrane lipid analysis

Another approach for studying the microbial composition of complex environments is the identification of microorganisms by analyzing bacterial components that are specific for individual species. Membrane lipids and their associated fatty acids have been used extensively in this respect (138). Microorganisms can be characterized by the patterns of their methylated phospholipid ester-linked fatty acids, known as (PL)FAME-patterns or phospholipid fatty acid (PLFA) profiles. Aceticlastic methanogens are characterized by their phospholipid-derived ether lipids (PLEL). FAME-patterns can be very useful for the characterization of unknown bacterial isolates (139). Unfortunately FAME-patterns are not always suitable for the characterization and quantification of microorganisms, due to the lack of (specific) biomarker lipids for many groups of microorganisms (140,141). In addition, some lipid biomarkers may be less specific than previously thought. For example the i17:1ω7 lipid, which was considered as specific biomarker for *Desulfovibrio* sp. is also present in high amounts in some *Syntrophobacter* sp. (141). Nevertheless, lipid analysis has been successfully applied for the identification of *Methanosaeta*, *Methanosarcina*, *Desulfobacter*, *Desulfotomaculum* and *Geobacter* in complex environments such as bioreactors, sediments, peatlands and rice fields (37,141-146). Combining lipid analyses with labelled precursor molecules (e.g. ^{13}C-labelled acetate) seems promising for sediment characterization studies. These labelling studies make it possible to link specific microbial processes with the organisms involved (147).

Molecular microbial detection techniques

Detection and identification based on molecular biological methods have become extremely important for microbial ecology studies (148-150). Nowadays, ribosomal RNA(rRNA)-based detection methods, are applied more and more in microbial ecological studies. These methods are based on the genetic variability of rRNA and/or DNA sequences. One of the rRNA-based methods for analysis of microbial environments is the hybridization with 16S or 23S rRNA oligonucleotide probes (151,152). The probes can be applied after extraction of the rRNA from the ecosystem (dot-blot hybridization), or can be used *in situ* in combination with fluorescent microscopy or confocal laser scanning microscopy. The major advantage of oligonucleotide hybridization methods over other hybridization methods (e.g. immunolabelling) is that the probe specificity can be controlled (150). rRNA based hybridization probes have been successfully applied for the detection (and quantification) of aceticlastic methanogens (153), aceticlastic sulfate- and sulfur-reducing bacteria (153-156) and denitrifying populations (157) in different environments.

An important tool for molecular microbial detection methods is the polymerase chain reaction (PCR) amplification technique. With this technique target genes may be amplified to make them detectable and quantifiable (158). The selection of the PCR primers determines which gene or part of a gene will be amplified. With the PCR amplification technique not only the detection of microorganisms, but also the detection of genes encoding for specific enzyme functions, or the detection of mRNA is possible (159). However, in mixed microbial environments PCR amplification is often applied for the amplification of 16S rRNA genes. When selective 16S rRNA primers are used, it is possible to amplify 16S rRNA-genes from specific groups of microorganisms present in the environmental sample. Kudo et al. used for example PCR amplification with methanogen-specific primers in combination with cloning to identify the methanogenic population in paddy soil (160). A similar approach, in combination with restriction fragment length polymorphism analysis was used by Hiraishi et al. to identify the methanogenic population in anaerobic sludge (161).

The past years several PCR-based quantification techniques have been developed for complex microbial ecosystems (162-164). However, unfortunately these techniques have some severe limitations (149). PCR-based quantification techniques are dependent on a high DNA extraction efficiency and PCR efficiency. A well-documented problem is preferential PCR amplification, i.e. the selective amplification of a template in a sample with mixed templates, which can cause a large difference between the estimated and the actual number of microorganisms in the sediment (165).

To circumvent the cloning technique it is also possible to separate the PCR products with denaturing gradient gel electrophoresis (DGGE) or temperature gradient gel electrophoresis (TGGE). With these electrophoresis techniques DNA fragments of the same length, but with different nucleotide sequences can

be separated. By using these electrophoresis techniques in combination with selective PCR amplification before electrophoresis, or specific DNA probes after electrophoresis, complex microbial populations can be studied (166). For example, the combination of DGGE and specific DNA probes has been used to reveal the presence of sulfate reducers in anaerobic sludge (166).

STUDY OF ACETATE CONSUMPTION AND PRODUCTION IN THE ENVIRONMENT

Accurate determination of the formation and turnover of acetate will give a direct estimate of the anaerobic organic matter degradation. However, the role of acetate in the carbon flow within the sediment is complex. On the one hand acetate, is formed during the decomposition of organic matter while on the other hand, acetate is consumed. Conventional tracer and inhibition techniques have been, and still are, used to quantify the relative importance of acetate in the anaerobic degradation of organic matter. These methods and some new developments in these techniques will be discussed below.

Inhibition techniques

Specific inhibitors have been used extensively to study competition between sulfate reducing bacteria and methanogens in different environments (85,167). Molybdate, an analogue of sulfate, is used as an inhibitor of sulfate-reducing bacteria and bromo-ethane sulfonic acid (BES), an analogue of methyl-coenzyme M, as an inhibitor of methanogens (168). Inhibition methods can also be used to identify intermediates, which are of importance in the terminal steps in mineralization of organic matter. Since methanogens and sulfate reducers consume the products of organic matter breakdown, these substances accumulate when microorganisms are inhibited. Therefore, the application of BES and molybdate can be used to quantify the role of acetate and other intermediates in anaerobic environments. Nevertheless, the use of inhibitors has its pitfalls and in some cases the results must be interpreted with caution. For example, molybdate inhibits the reduction of sulfate in sulfate-reducing bacteria. However, studies done with sulfate reducers isolated from marine environments showed that the sulfate-independent processes of these bacteria were not affected (169). As a result of this, sulfate reducers may oxidize lactate in syntrophic cooperation with H_2-utilizing methanogens (170). In this case, acetate accumulates instead of lactate and hereby the role of lactate as an intermediate is underestimated. In case the biochemical basis of the inhibition mechanism is not precisely known results obtained with inhibitor should be interpreted with caution. An example of such an inhibitor is chloroform ($CHCl_3$) (concentrations of a maximum of 100 μM are used), which is occasionally used to inhibit methanogenesis or to estimate the production of acetate (86,171-173). It was shown that $CHCl_3$ was an inhibitor of growth and product formation by methanogenic archaea, homoacetogenic bacteria and a sulfate-reducing bacterium (*Desulfotomaculum acetoxidans*) operating the acetylCoA-cleavage pathway

(174). A possible explanation for this can be given. In this pathway, a carbon monoxide dehydrogenase (CODH) is involved in the cleavage of acetylCoA and the further oxidation of the formed CO to CO_2 (18,19). This cleavage reaction was shown to be inhibited by CCl_4 and the mechanism behind the inhibition of this enzyme was postulated (175). One can speculate that a similar mechanism occurs when $CHCl_3$ is used as an inhibitor of methanogenesis (86,171-173). So one should be aware of the inhibitory effects of $CHCl_3$ on other microorganisms beside methanogens. However, the acetate-utilizing sulfate reducer *Desulfobacter acetoxidans*, which uses the citric acid cycle for the oxidation of acetate, was not inhibited by $CHCl_3$. This indicates that $CHCl_3$ might be used in another way. In principle it allows a better elucidation of the role of different metabolic types of sulfate reducers to sulfate reduction in natural environments.

Another approach for studying the contribution of acetate is the use of fluoroacetate, an inhibitor of acetate metabolism. It was shown that both aceticlastic methanogenesis and aceticlastic sulfate reduction were inhibited (172,176,177). Cappenberg suggested that the methyl-transfer reactions involved in methane formation be inhibited by fluoroacetate (176). This might also explain the inhibition of some species of acetate-utilizing sulfate-reducing bacteria, which oxidize acetate via the CO dehydrogenase pathway (see above).

Tracer techniques

Another approach to quantify the relative importance of acetate is the use of ^{14}C or ^{13}C labelled substrates. This method is based on the measurement of the fate of the labeled C-atoms. In lake sediments and paddy soil, acetate was identified as the most important metabolite formed from radioactive glucose (178-180). Also ^{14}C-labeled phytoplankton or cellulose have been used in lake sediment and rice paddies (181,182). The formation of acetate by CO_2 reduction was quantified with radioactive HCO_3^- (183,184).

The fate of acetate can be determined by measuring the appearance of labeled CO_2 or CH_4 from ^{13}C or ^{14}C-labeled acetate, unless there is a high CO_2 fixation rate (9). This method was successfully used to identify the main acetate-consuming processes in anoxic environments (7,82,85,172). The respiratory index (RI) is used to indicate the relative importance of acetate respiration and methanogenesis from acetate. The RI is defined as the ratio of labeled CO_2 divided by the sum of labeled CO_2 + CH_4 produced from [2-$^{13/14}C$] acetate. RI values of below 0.5 indicate that methanogenesis controls the consumption of acetate while when RI values approach 1 anaerobic respiration processes dominate (7,85). However, tracer techniques showed overestimations of the acetate turnover compared to independent measurements of anaerobic mineralization (e.g., sulfate reduction and ammonium release) (185,186). Those overestimations were mainly ascribed to overestimations of the free porewater acetate concentration (187). However, carboxyl exchange of [U-$^{13/14}C$] acetate and deviation from pseudo-first-order kinetics may also be sources of errors in the determination of acetate turnover (172,188). Therefore, acetate labeled in the methyl group should be used to obtain the most reliable estimate of the acetate turnover. Furthermore, these acetate turnover rate estimates should be

compared with independent estimates of acetate turnover (acetate accumulation after inhibition and methane formation rates) to determine if these estimates are correct (172,184).

Carbon isotope biochemistry

The carbon isotope biochemistry of acetate is also used to study the formation and consumption processes of acetate in anoxic environments. Although little is known about the isotope effects associated with the anaerobic metabolism of acetate, it is clear that metabolic pathways create a unique isotopic signature in the acetate that is produced (189). Blair and Carter summarized the isotopic composition of acetate from various biological sources (190). They mentioned that the potential for isotopic effects during the consumption of acetate is also of importance. Anaerobic acetate-consuming microorganisms convert acetate via two important pathways (see section 3). In general, acetate is oxidized to CO_2 or disproportionated to CH_4 and CO_2. This will probably result in different carbon isotopic compositions of CO_2 and/or CH_4. Only a few papers are available in which the carbon isotope effects associated with aceticlastic methanogens were studied (191,192). However, the method of carbon isotope fractionation can give qualitative information of the processes responsible for the formation and consumption of acetate. For a quantitative estimate of the flow of carbon through acetate, information concerning the fractionations occurring during the metabolism of acetate is required (190). This technique mainly is valuable to study the production and consumption of acetate in anoxic habitats that are exposed to diurnally and seasonally changing environmental conditions (e.g. temperature, water level, and carbon sources) (193).

CONCLUSIONS

Acetate is quantitatively the most important substrate for aceticlastic methanogens in many anaerobic freshwater environments. A better understanding of acetate metabolism is therefore crucial to predict the rate of methane production in different environments. However, acetate degradation by methanogens is strongly affected by the presence of inorganic electron acceptors. This makes it difficult to understand the fate of acetate in anaerobic environments because of the different interactions between the different microbial populations present.

Competition for acetate between methanogens and sulfate-reducing bacteria is in many situations the type of interaction responsible for the inhibition of methane formation. The physiological parameters of these microorganisms are useful to explain which population is favored in an environment with a certain acetate concentration. Unfortunately, information concerning these properties is still lacking for Fe^{3+}/Mn^{4+} and NO_3^-- reducing microorganisms. Therefore, more research is necessary to determine the physiological properties of these anaerobic acetate-degraders. In addition, the mechanism of acetate

uptake deserves more attention as it may determine the affinity of different types of microorganisms for acetate. Furthermore, aceticlastic methanogens are not only inhibited by the competition for acetate between some anaerobic respiring bacteria and methanogens, but also by reduced inorganic compounds formed by anaerobic respiring bacteria.

Modern molecular detection methods have become available for the detection of physiological groups of microorganisms. The use of these techniques offers the possibility to study the population dynamics of different acetate-degrading microorganisms in anaerobic environments upon changes in the environmental conditions. In addition, insight in the population dynamics of different microorganisms upon changes in the environmental conditions may give clues how to isolate the organisms.

ACKNOWLEDGEMENTS

This research was made possible by a grant from the Netherlands Organization for Scientific Research (NWO) and the Innovative Oriented Program (IOP) Environmental Biotechnology.

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CHAPTER 3

DESCRIPTION OF THE POLDER ZEGVELDERBROEK

THE POLDER ZEGVELDERBROEK

The polder Zegvelderbroek (52°07'N, 4°52'E) is located between Leiden and Utrecht, close to most densely populated parts of the Netherlands (Fig. 1). The polder is representative for other polders in the Netherlands. In previous centuries the peatlands in this area were largely drained and reclaimed, and divided into polders. Peat extraction for fuel was practiced at a large scale (1,2). In this century, water management of the polders was profoundly intensified in favor of agriculture. Today, it is a peat grassland area in which ditches are lying at roughly every 40 to 100 meter. Between the ditches lie small long strips of grassland and smaller cross-ditches. The water level varies from 2.4 to 2.1 m below NAP (Normal Amsterdam Level – the Dutch reference level) (3). A cross-section of the polder is given in Figure 2. Sediment in the ditches has a high CH_4 emitting potential because it is anoxic at shallow depth and has high organic matter contents (see below). Indeed, it was shown that these sediments emit high quantities of CH_4 (4). SO_4^{2-} and NO_3^- can be present in significant concentrations e.g. due to water pollution as a result of anthropogenic activities or percolating water. The presence of these compounds in groundwater may control the CH_4 emission. Thus far, it is not known to which extent SO_4^{2-} and NO_3^- control CH_4 emission from the sediment.

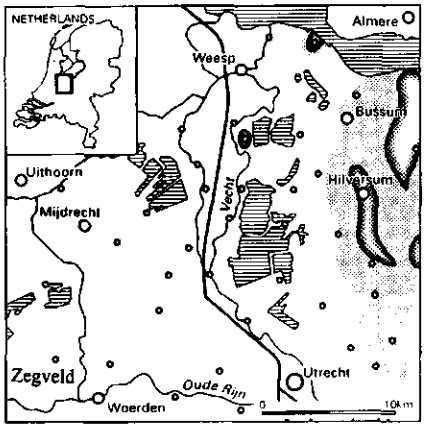

Figure 1. Overview of the study area in The Netherlands.

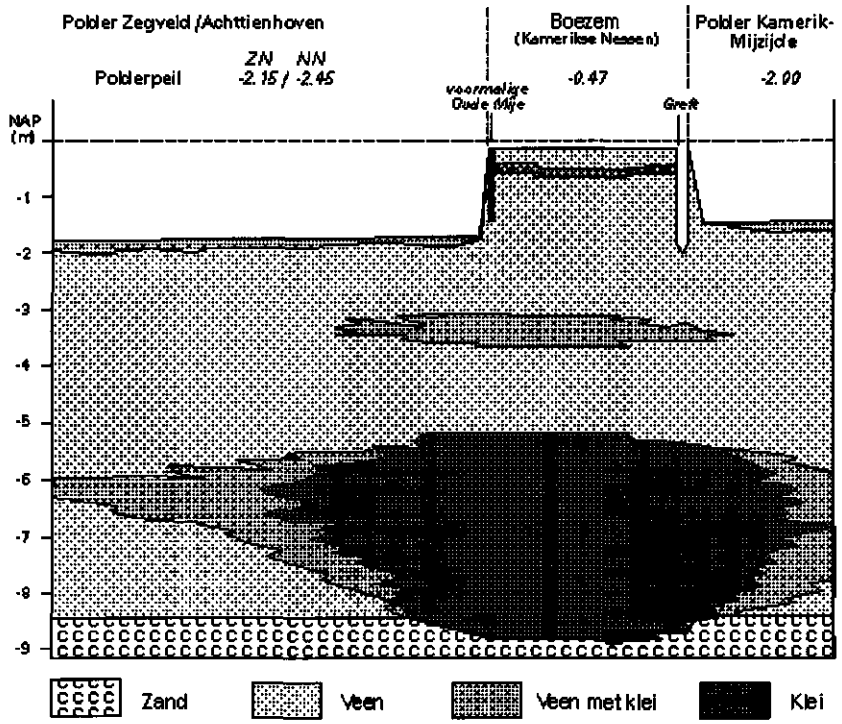

Figure 2. Schematic cross-section of the polder Zegvelderbroek.

SEDIMENT AND POREWATER

The organic matter content of the sediment was in general between 90-95%. The porewater pH varied from 6.5-6.9. The pH of the water above the sediment showed a higher value varying from 7.0-7-5. Anions like Cl^-, NO_3^-, NO_2^-, SO_4^{2-} and $S_2O_3^{2-}$ were analyzed in the porewater. The chloride concentrations in the porewater seemed to be reasonably constant during the year varying from 1-2 mM. Sulfate concentration in the porewater fluctuates strongly during the season. The concentrations in winter and early spring were much higher (2-5 mM) compared to summer and early autumn (0.05-1 mM). Other anions (see above) were only detected occasionally. The sediment temperature varied from 4-6 °C in winter and early spring to 10-17 °C in summer and autumn.

ACKNOWLEDGEMENT

We thank Hans de Mars for providing the Figures 1 and 2 (5).

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CHAPTER 4

THE EFFECT OF SULFATE AND NITRATE ON METHANE FORMATION IN A FRESHWATER SEDIMENT

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Published as:

Scholten, J.C.M. and A.J.M. Stams. 1995. The effect of sulfate and nitrate on methane formation in a freshwater sediment. Antonie van Leeuwenhoek **68**:309-315.

ABSTRACT

A freshwater sediment from a ditch of a peat grassland near Zegveld (Province of Utrecht, The Netherlands) was investigated for its potential methanogenic and syntrophic activity and the influence of sulfate and nitrate on these potential activities. Methanogenesis started after a 10 days lagphase. After 35-40 days aceticlastic methanogens were sufficiently enriched to cause a net decrease of acetate. In the presence of sulfate methane formation was only slightly affected. The addition of nitrate led to an outcompetion of aceticlastic methanogens by nitrate reducers. When inorganic electron acceptors were absent, substrates like propionate and butyrate were converted by syntrophic methanogenic consortia. Addition of inorganic electron acceptors resulted in an outcompetition of the syntrophic propionate and butyrate degrading consortia by the sulfate and nitrate reducers.

INTRODUCTION

Methane (CH_4) is an important atmospheric trace gas which plays an important role in the geochemistry of carbon. From research on gas bubbles in polar ice it is known that the last 100 - 200 years the amount of methane in the atmosphere has increased 2 - 3 times. This increase is probably caused by anthropogenic activities. Each year the emission of methane rises with 1 to 2 % (1,2). About 60 % of the methane in the atmosphere is of biological origin. The most important biogenic sources are (in Tg/ year): oceans and lakes (1-7), termites (2-5), ruminants (72-99), man (4-7) and wetlands, such as paddy fields (30-59). These values were estimated values for 1975 (3). Because of the potential role of methane in climate change and atmospheric chemistry more detailed studies of the anthropogenic and natural sources and factors controlling methane release to the atmosphere is needed.

Biological methane formation is important in those anaerobic environments where only bicarbonate and protons are available as electron acceptors in the conversion of organic matter. Different physiological types of bacteria (fermentative and acetogenic) perform a series of reactions, starting from complex polymers (polysaccharides, proteins, lipids) and leading to acetate, formate, and CO_2 and H_2, which are the main substrates for methanogenic bacteria (4,5). Therefore, methanogens are dependent on fermentative and acetogenic bacteria for their substrate supply. On the other hand, methanogens enable acetogens to degrade fatty acids like propionate or butyrate, by efficiently removing the hydrogen and formate formed by those organisms. In the presence of sulfate, nitrate or other inorganic electron acceptors like Mn^{4+} or Fe^{3+}, anaerobic respiration becomes important. This influences methane formation. Methanogens may become outcompeted by nitrate- or sulfate reducers or by organisms that can utilize Mn^{4+} or Fe^{3+} as electron acceptors, since these organisms have better growth kinetic properties on substrates like H_2, formate and acetate (4-10). Sulfate is present in excess in marine environments and salt marches. High nitrate concentrations can be present in the

groundwater as a result of intensive agricultural activities. Therefore, in the Netherlands, sulfate and nitrate concentrations in the groundwater may control the methane emission from wetlands and sediments.

The aim of this study was to investigate: i) the potential methanogenic and syntrophic activity in a freshwater sediment and ii) the influence of sulfate and nitrate on these potential activities.

MATERIALS AND METHODS

Site description and sampling collection. Freshwater sediment was collected on the 27 th of february 1993 from a ditch of a peat grassland near Zegveld (Province of Utrecht, The Netherlands). The sediment surface of the sampling site was overlaid with 40 cm of water. The temperature of the sediment was 4 °C and that of the water 6 °C. Sampling of the fresh water sediment was done with a sediment corer. The sediment samples were collected in 1-l serum bottles with 500 ml freshwater to get a 50 %(v/v) sediment slurry. Bottles were closed with butylrubber stops and aluminium screw caps. During and after transport the bottles were stored at 4 °C. After 3 days the sediment slurry was processed further.

Media. The incubations were done in a basal bicarbonate buffered medium with a composition as described by Huser et al. (11). To one litre of medium 0.5 g of yeast extract, 1 ml of a trace elements solution (12) and 1 ml of a vitamin solution (13) were added. The vitamin solution was sterilized separately. The pH of the medium was 6.8 - 6.9.

Batch Experiments. The experiments were performed in duplicate in anaerobic serum bottles of 300 ml. After the addition of the vitamin solution to the basal medium, 20 ml of the sediment slurry was transferred anaerobically into the bottle. The gas phase was changed to 80%N_2 :20%CO_2 by flushing. In case, sulfate or nitrate reducing conditions were required sodium sulfate or sodium nitrate were added from 1-M stock solutions. Sodium acetate, sodium propionate or sodium butyrate were added as substrates. The flasks were incubated in the dark (20 °C). Samples were taken periodically to determine substrate or/and electron acceptor utilization and product formation.

Analytical methods. Methane and hydrogen were measured on a 406 Packard gas chromatograph equipped with a thermal conductivity detector (TCD), 100 mA. The gases were separated with argon as the carrier gas on a molecular sieve column (13X, 180 cm by ¼ inch, 60-80 mesh) at 100 °C. N_2O was determined on a CP9001 Packard gas chromatograph equipped with a thermal conductivity detector (TCD), 100 mA. The gases were separated with argon as the carrier gas on a poraplot Q column (250 cm by 0.53 mm) at 40 °C. The utilization of fatty acids was analyzed by high-pressure liquid chromatography on a Merck-column (Polyspher OA HY). The mobile phase was 0.01 N H_2SO_4 at a flow of 0.6 ml/min at 60 °C. Sulfate and nitrate were analyzed by high-pressure liquid chromatography. Ions were separated on a Dionex column (Ionpac AS9-SC) with an eluent consisting of 1.8 mM Na_2CO_3 and 1.7 mM $NaHCO_3$ at a flow of 1 ml/min at room temperature. The anions were detected with suppressed conductivity.

RESULTS AND DISCUSSION

Incubations without electron donor. In the batch experiments the potential activity of different functional groups of bacteria was determined. The incubations of the sediment with or without added electron acceptors are given in figure 1. In the incubation without an electron acceptor the formation of methane started after 10 days. At the end of the incubation 4 mmol CH_4 had been formed per liter medium. Accumulation of acetate started within 10 days and accumulated to a concentration of 4 mM. The acetate concentration remained constant over 20 days but declined after 30 days. After 50 days the acetate concentration was below the detection limit. Incubation of the sediment with sulfate showed a similar pattern for the acetate accumulation and consumption. Methane formation on the other hand was much lower compared with the incubation without sulfate. After 60 days only 2 mmol CH_4 was formed per liter medium. Only about 3 mM sulfate was consumed, which suggests that acetate was degraded via methanogenesis and sulfate reduction. In the incubations with nitrate about 8 mM nitrate was consumed within 20 days. After 20 days the nitrate concentration did not decline anymore which indicates that the electron donors had become limiting. Part of the nitrate was converted to N_2O indicating that not all nitrate was reduced to N_2. After 15 days 2.5 mmol N_2O had been formed per liter medium. No accumulation of acetate and methane formation was observed in these incubations.

Incubations with acetate, propionate and butyrate under methanogenic conditions. The incubations with acetate showed a lagphase of about 20 days (Fig. 2). Acetate was converted to methane presumably by aceticlastic methanogens. This was supported by the fact that we could enrich *Methanosarcina* and *Methanothrix* species from the sediment with acetate. Rajagopal, Belay & Daniels reported the isolation of a *Methanosarcina* sp. from anoxic soil as the dominant acetate consuming bacterium (14). Also profile studies done with littoral sediment from Lake Constance suggested that acetate turnover was mainly due to the activity of methanogens. In situ acetate concentrations were in a range of 25-50 μM, which is below the threshold concentration for *Methanosarcina* spp. (>0.2 mM) but above the threshold concentration for *Methanothrix* spp. (>7 μM). The authors therefore suggested that only *Methanothrix* or similar species were able to grow at these low substrate concentrations (15).

When inorganic electron acceptors were absent, the substrates propionate and butyrate were converted syntrophically (Fig. 3 and 4). The consortia oxidized both substrates to acetate and presumably hydrogen or formate. However, the reduced equivalents produced by the propionate or butyrate oxidizing bacteria were directly consumed by methanogens. The observed CH_4/electron donor ratio for the incubations with

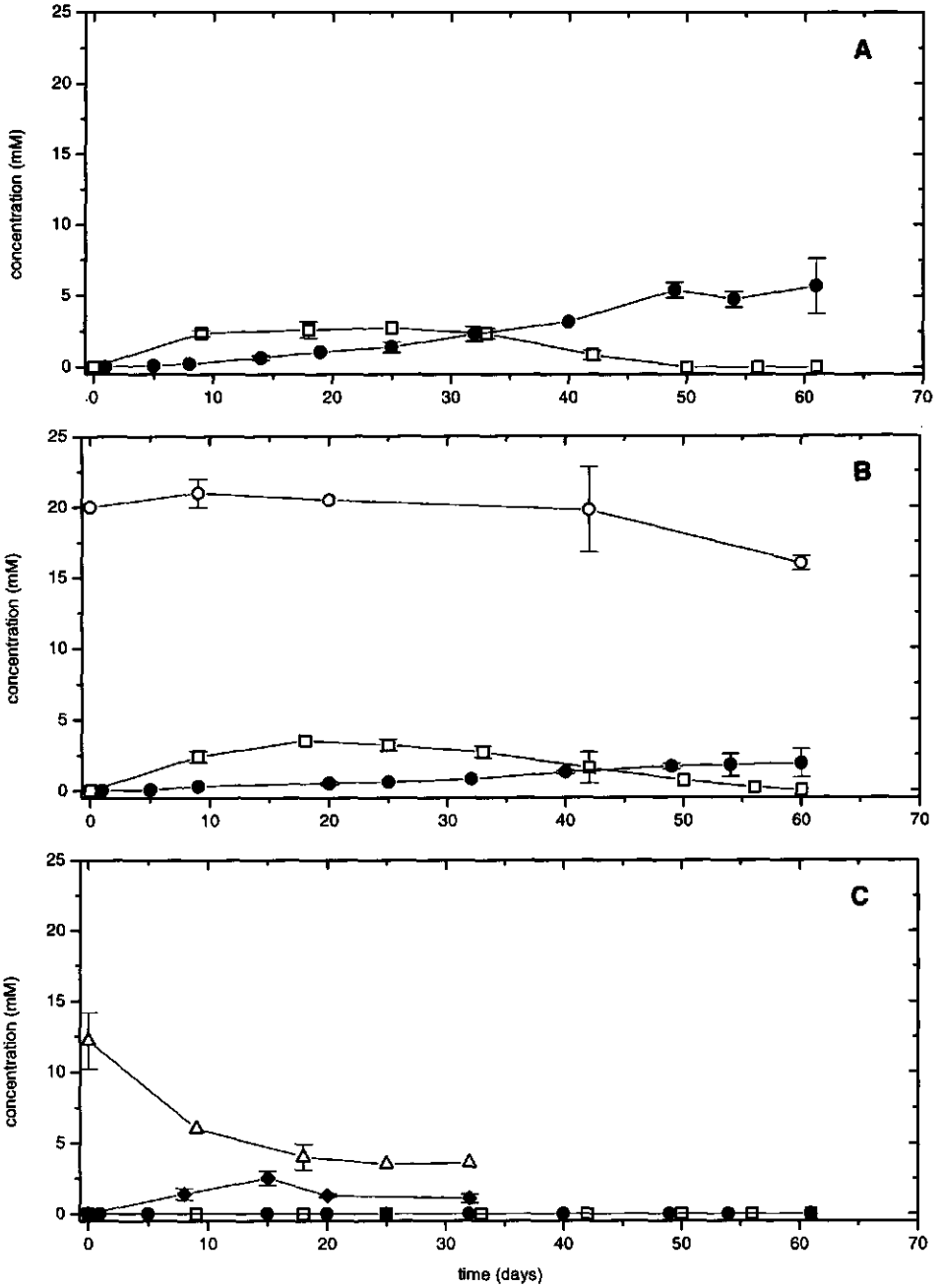

Figure 1. The product formation in freshwater sediment slurries (Zegveld) incubated at 20 °C (A) with the addition of 20 mM sulfate (B) and 20 mM nitrate (C). Symbols: □: acetate; ●: methane; ○: sulfate; Δ: nitrate and ◆: N_2O. Error bars represent standard error of mean (n=2).

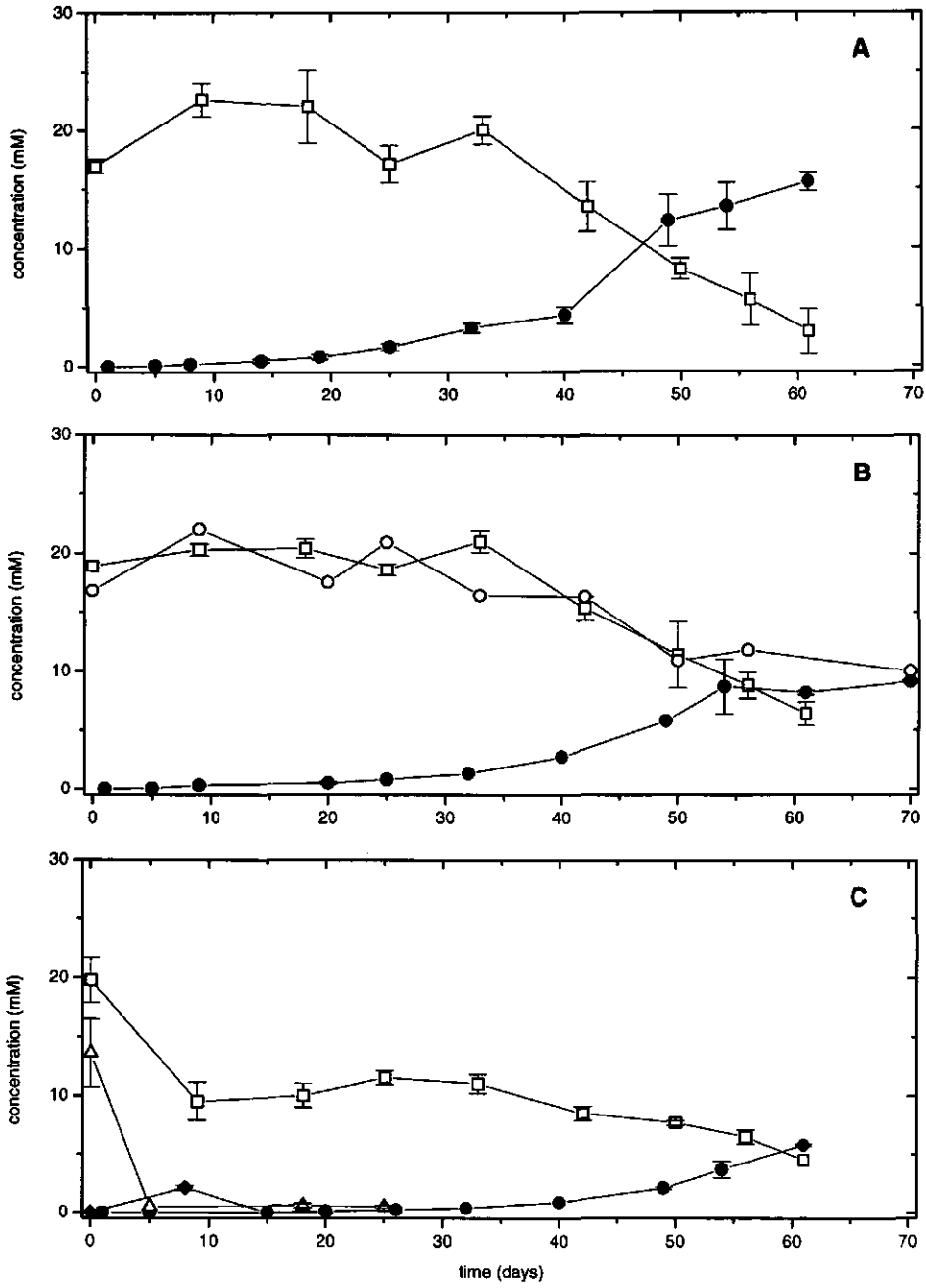

Figure 2. The acetate utilization and product formation in freshwater sediment slurries (Zegveld) incubated at 20 °C with the addition of 20 mM acetate (A), 20 mM acetate + sulfate (B) and 20 mM acetate + nitrate (C). Symbols: □: acetate; ●: methane; ○: sulfate; Δ: nitrate and ◆: N_2O. Error bars represent standard error of mean (n=2).

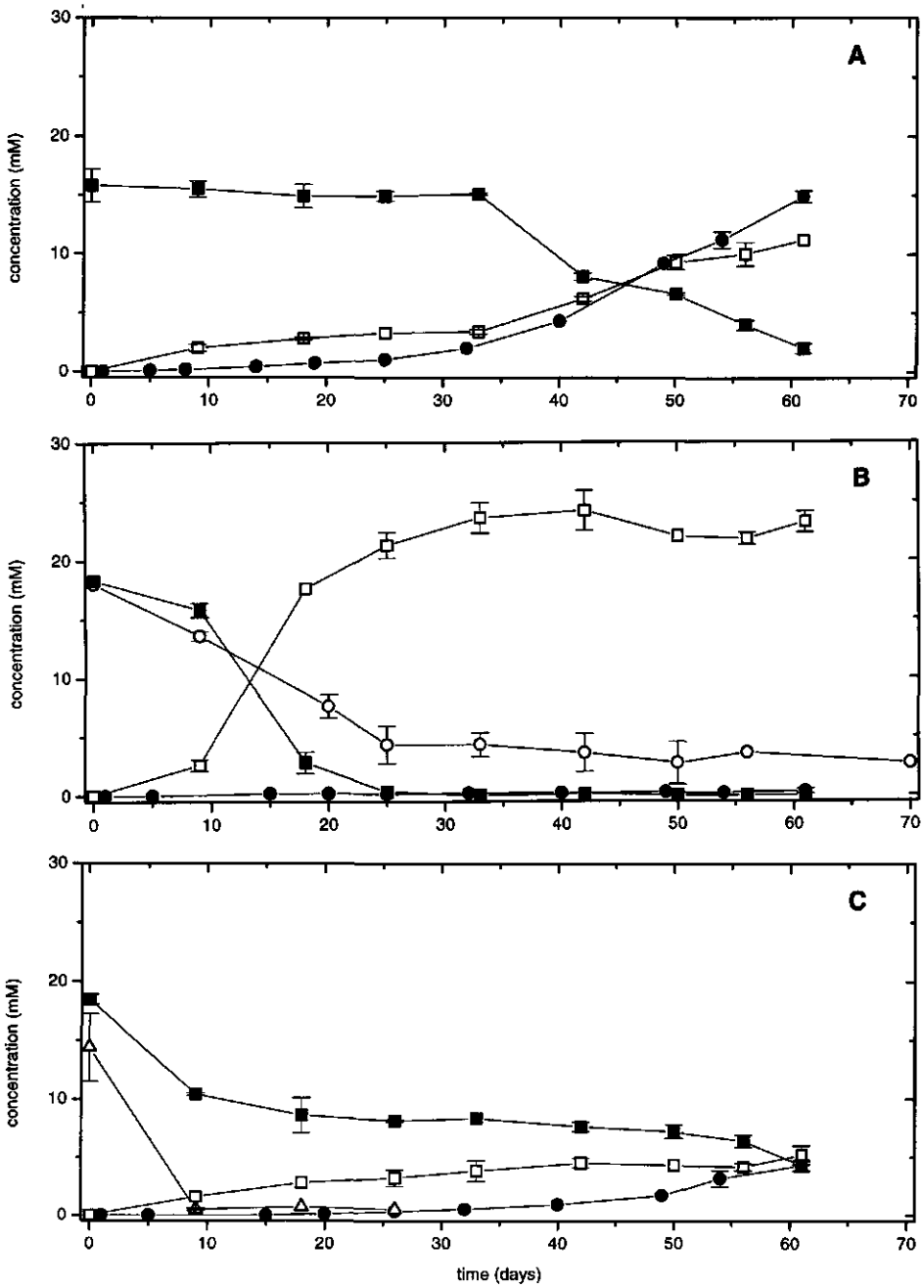

Figure 3. The propionate utilization and product formation in freshwater sediment slurries (Zegveld) incubated at 20 °C with the addition of 20 mM propionate (A), 20 mM propionate + sulfate (B) and 20 mM propionate + nitrate (C). Symbols: ■: propionate; □: acetate; ●: methane; ○: sulfate and Δ: nitrate. N_2O data not shown. Error bars represent standard error of mean (n=2).

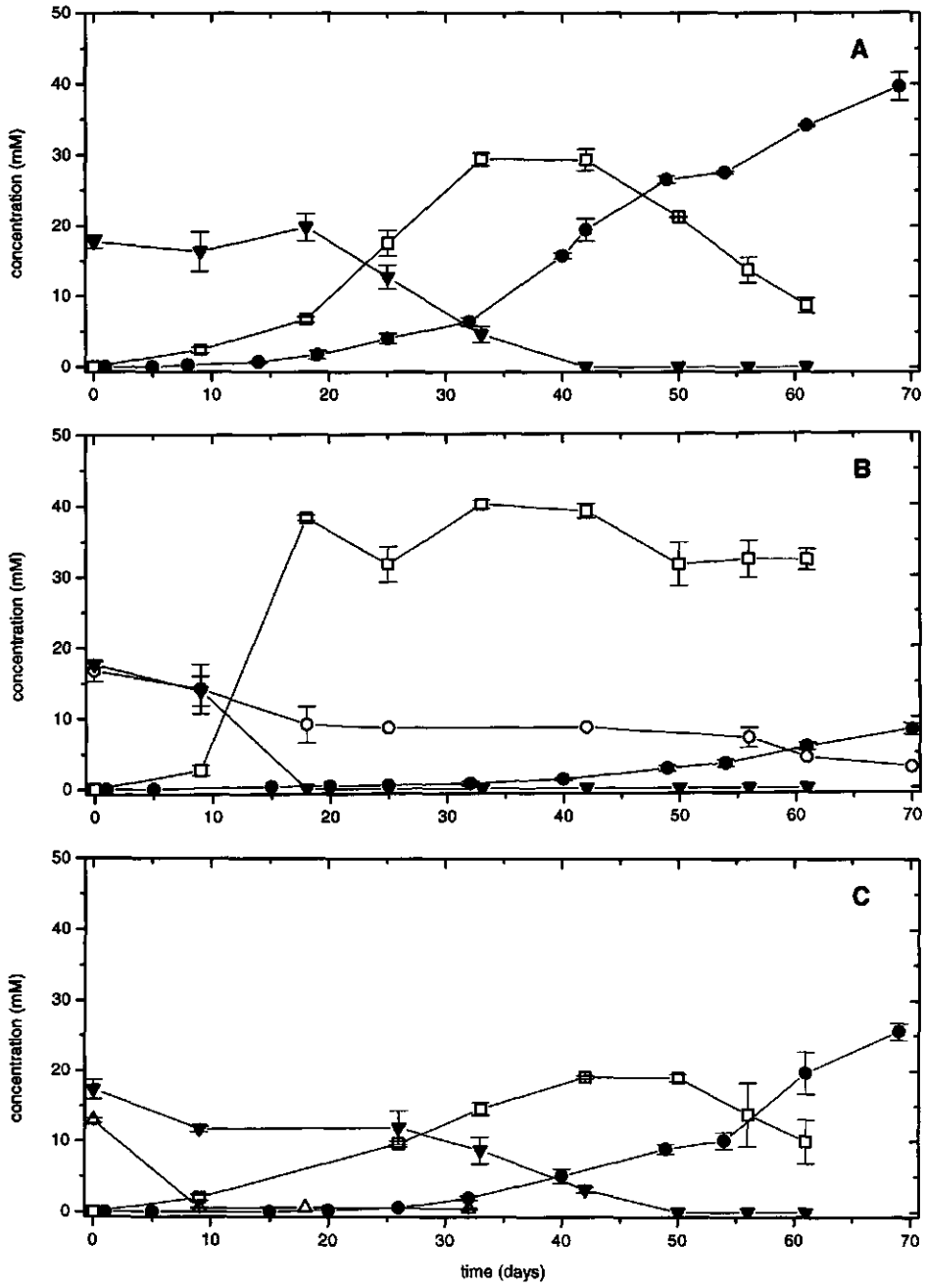

Figure 4. The butyrate utilization and product formation in freshwater sediment slurries (Zegveld) incubated at 20 °C with the addition of 20 mM butyrate (A), 20 mM butyrate + sulfate (B) and 20 mM butyrate + nitrate (C). Symbols: ▼: butyrate; □: acetate; ●: methane; ○: sulfate and Δ: nitrate. N_2O data not shown. Error bars represent standard error of mean (n=2).

propionate and butyrate was 0.7 and 0.3 (table 1) respectively, which corresponded with the expected ratio. The observed acetate/ electron donor ratios were according to the expected ratios. Aceticlastic methanogens converted acetate to methane after 40 days as described above. If acetate was consumed immediately by methanogens the expected ratio CH_4/electron donor would have been higher in both cases. Syntrophic degradation of propionate and butyrate has been described before and is found in different methanogenic habitats (4,5,16). In the sediment from Zegveld a potential syntrophic activity was present. However, the quantitative importance of this activity is unclear.

Incubations with acetate, propionate and butyrate under sulfate reducing conditions. Sulfate reduction was only responsible for part of the acetate consumption (Fig. 2). With some assumptions it can be calculated that the ratio of SO_4^{2-}/acetate is 0.2, which is much lower than expected. This indicates that aceticlastic methanogens and acetate-oxidizing sulfate reducers are both responsible for the consumption of acetate in the sediment. Studies by others have shown that sulfate reducers can out-compete methanogens even at freshwater sulfate concentrations of 60-100 μM (6,7,17). In this study the consumption of acetate was dominated by aceticlastic methanogens. However, acetate concentrations used in our experiments were in the millimolar range while in situ concentrations in freshwater sediments are in general much lower (15,18). At these high substrate concentrations methanogens were able to compete successfully with the sulfate reducers for the available acetate.

The oxidation of propionate and butyrate in the presence of sulfate took place at a higher rate than in the absence of this electron acceptor (Fig. 3 and 4). In addition, the conversion of these substrates started earlier. In the presence of sulfate, propionate and butyrate were oxidized incompletely to acetate by sulfate reducing bacteria within 20 days of incubation. The observed ratios electron acceptor/electron donor and product/electron donor for both incubations were according to the expected ratios (Table 1). The incomplete oxidation of fatty acids has been described for many sulfate reducing bacteria isolated from different marine and freshwater ecosytems (19). The addition of sulfate influenced the hydrogenotrophic methanogens to a great extent in the first 20 days because no methane was formed in the presence of propionate and butyrate. Probably the syntrophic consortia were outcompeted by the sulfate reducers. However, Oude Elferink *et al.* (20) discussed the possibility that fatty acids like propionate and butyrate can be oxidized to acetate and hydrogen by acetogenic bacteria while the hydrogen is oxidized by sulfate reducers. This means that the role of sulfate reducers may be that of hydrogen consumers rather than that of propionate or butyrate oxidizers. Their argument was based on the fact that hydrogenotrophic sulfate reducers have a higher affinity for sulfate than propionate oxidizing sulfate reducers. Profile studies revealed high sulfate concentrations (2-5 mM) in the porewater of the sediment during autumn and winter, and low sulfate concentrations (< 0.1 mM) during summer (unpublished data). Probably this could explain the high potential for sulfate reduction. It remained unclear if acetate-oxidizing sulfate reducers showed some activity in these incubations during the 70 days of incubation.

Table 1. Expected and observed[a] ratios between electron acceptor/ electron donor, methane/ electron donor and acetate/ electron donor for the conversion of acetate, propionate and butyrate under different conditions.

Incubation	Expected			Observed		
	e-acceptor/ e-donor	CH_4/ e-donor	acetate/ e-donor	e-acceptor/ e-donor	CH_4/ e-donor	acetate/ e-donor
Acetate	-	1	-	-	0.7	-
Propionate[b]	-	0.75	1	-	0.7	0.9
Butyrate[b]	-	0.5	2	-	0.3	2.1
Acetate + SO_4^{2-}	1	-	0	0.2	1.0	0
Propionate + SO_4^{2-}	0.75[c]	-	1	0.8	0	1.0
Butyrate + SO_4^{2-}	0.5[c]	-	2	0.4	0	2.0
Acetate + NO_3^-	1.6[d]	-	-	0.6[e]	0	0
Propionate + NO_3^-	2.8[d]	-	-	1.0[e]	0	0
Butyrate + NO_3^-	4[d]	-	-	1.2[e]	0	0

[a] Corrections made for background levels.

[b] Assuming that only hydrogenotrophic methanogenesis appears.

[c] Assuming that only incomplete oxidation appears.

[d] Assuming that complete oxidation of substrate and reduction of nitrate ($NO_3^- \rightarrow N_2$) appears.

[e] Values calculated in the first 10 days

Incubations with acetate, propionate and butyrate under nitrate reducing conditions. When nitrate was present all the three substrates seemed to be completely oxidized by nitrate reducing bacteria. In all the incubations lagphases for denitrification were not observed and the formation of methane or accumulation of acetate did not occur during the first 10 days when nitrate respiration was the dominating process (Fig. 2, 3 and 4). The observed ratios of electron donor degraded versus nitrate reduced were lower than expected (Table 1) due to the presence of other electron donors than the added substrates. Part of the nitrate was converted to N_2O. After 9 days in all the three incubations 2.0 mmol N_2O was formed per liter medium. The latter indicates that not all nitrate was reduced to N_2 at an excess of nitrate. However, when nitrate became limiting N_2O disappeared from the headspace. When nitrate and N_2O were depleted propionate and butyrate were further converted, most likely by syntrophic methanogenic consortia. The addition of nitrate stimulated

the anaerobic respiration process to a great extent. The bacteria involved in the respiration process with protons or bicarbonate as electron acceptors were completely outcompeted by the nitrate reducers. Probably the same is true for sulfate reducing bacteria although no incubations were done with the addition of sulfate and nitrate at the same time.

A lot of work has been done on denitrification in marine sediments (21-24). In these studies it has been demonstrated that nitrate in anaerobic sediments could be reduced completely to ammonium as well as being denitrified to gaseous products. King and Nedwell mentioned the ecological significance of these processes (21). They stated that at low nitrate concentrations nitrate reduction to ammonium conserves nitrogen within the aquatic environment, whereas under high nitrate concentrations the loss of fixed nitrogen from the ecosystem through denitrification to gaseous products is stimulated. In this study high concentrations of nitrate were added to the sediment. Indeed, high nitrate concentrations lead to a loss of fixed nitrogen from the sediment through denitrification to gaseous products. It should be mentioned that no ammonium measurements were done.

From the batch experiments it can be concluded that a potential methanogenic activity was present in the sediment which was slightly affected by the presence of sulfate but completely inhibited by the presence of nitrate. It is unknown whether the sediment emits high quantities of methane. Intriguingly, measurements in the field at the same location have shown that a net methane consumption in the area is possible (25). They found that when ever an oxic top layer in the grassland is present, the grassland acts as a sink for atmospheric methane. These results indicate that methane produced in the ditches and originating from other sources may be oxidized again by the grassland soils.

ACKNOWLEDGEMENTS

This study was subsidized by a grant from the Netherlands Organization for Scientific Research (NWO). The authors would like to thank Wim Roelofsen for technical assistance.

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ABSTRACT

The relative importance of methanogenesis and sulfate reduction with acetate in a freshwater sediment was investigated. Addition of acetate stimulated both methane formation and sulfate reduction, indicating that an active aceticlastic population of methanogens and sulfate reducers was present in the sediment. Sulfate reducers were most important in the consumption of acetate. However, when sulfate reducers were inhibited, acetate was metabolized at a similar rate by methanogens. Acetate, propionate and valerate accumulated only when both processes were inhibited by the combined addition of 2-bromo-ethane sulfonate and molybdate. The relative amounts of acetate, propionate and valerate were 93, 6 and 1 mole%, respectively. These results demonstrate the role of acetate as a key intermediate in the terminal step of organic matter mineralization in the sediment. Addition of chloroform inhibited both methanogenesis and sulfate reduction. We studied the inhibitory effect of $CHCl_3$ on homoacetogenic bacteria, sulfate-reducing bacteria and methanogens. The results showed that the inhibition of $CHCl_3$ correlates with microorganisms which operate the acetylCoA-cleavage pathway. We propose that the use of chloroform can be used to elucidate the role of different metabolic types of sulfate reducers to sulfate reduction in natural environments.

INTRODUCTION

Methanogenesis and sulfate reduction are important processes in the anaerobic degradation of organic matter in freshwater and marine sediments. Volatile fatty acids are important key intermediates in the transformation of organic matter in anaerobic environments. Quantitatively, acetate is the main substrate for methanogenesis in freshwater sediments, and for sulfate reduction in marine sediments (1-6). Sulfate reducers can potentially compete with methanogens for acetate in freshwater sediments and they have been shown to oxidize a part of the available acetate even at very low sulfate concentrations (7-9). Thus, aceticlastic methanogens and acetotrophic sulfate reducers might be of importance in the consumption of the available acetate in freshwater sediments.

The relative importance of acetate as a precursor of methanogenesis and effects of electron acceptors on methanogenesis from acetate have been determined by studying the fate of ^{14}C or ^{13}C labelled acetate (2,10,11). Specific inhibitors have been also used to quantify the relative contribution of sulfate reducers and methanogens in sediments (1,7,12,13). Sulfate reduction is specifically inhibited by the addition of molybdate and methanogenesis by the addition of 2-bromo-ethane sulfonate (BrES). Occasionally, also chloroform has been used to inhibit methanogenesis (4,11,14).

Most of these studies indicate that in freshwater sediments the contribution of sulfate reducing bacteria in the carbon turnover is relatively unimportant. However, the contribution may be important in

sediments covered with microbial mats, where an active sulfur cycling takes place by photosynthetic O_2 input in the presence of light (15). In a previous study, we measured the potential sulfate-reducing and methanogenic activities with acetate in sediment slurries from ditches in a peatland area (16). Acetate was converted by methanogens, but the addition of sulfate only slightly affected the formation of methane. However, we have found that porewater of the sediment contained high sulfate concentrations (2-5 mM) during autumn and winter, and low sulfate concentrations (<0.1 mM) during summer (16). Therefore, in the sediment, acetate-utilizing sulfate-reducing bacteria may be present and compete with aceticlastic methanogens for the available acetate. The aim of this research was to determine the relative importance of sulfate reduction and methanogenesis from acetate in these sediments by using inhibitors. In addition, we studied the inhibition by $CHCl_3$ of microorganisms which contain the acetylCoA-cleavage pathway.

MATERIALS AND METHODS

Site description and sample collection. Sediment samples were collected April 15, 1995 with a sediment corer (acrylic glass tubes, 50 cm in length and an inner diameter of 6.4 cm) from ditches next to peat grassland. At the sampling date the sediment sulfate concentration was 2-2.5 mM. The grassland area is located near Zegveld (Province of Utrecht: 52°07'N, 4°52'E, The Netherlands). The sediment surface of the sampling site was overlaid with 30-40 cm of water at the day of sampling. The temperature of the sediment was 8 °C and that of the water 7 °C. After transport the cores were stored at 10 °C. The sediment was further processed within 4 days.

Set up of incubation experiments. All handlings were done under anaerobic conditions in a glove box. Homogenized sediment from the 0-10 cm layer was distributed in 10-ml portions into 26-ml tubes and closed with butyl rubber stoppers. Viton stoppers were used in the experiments with chloroform ($CHCl_3$). The tubes were repeatedly evacuated and gassed with N_2 (172 kPa), and stored at 10 °C. After 10 hours, acetate and inhibitors were added. Controls without acetate were prepared as well. Acetate was added to a final concentration of 1 mM. To inhibit methanogenesis, 20 mM of 2-bromo-ethane sulfonate (BrES) or 10 μM of $CHCl_3$ were added. Sulfate reduction was inhibited by the addition of sodium molybdate (5 mM). Killed controls were prepared by addition of formaldehyde to a concentration of 3.75% (v/v). The incubation experiments were done with a set of 12 tubes. For the controls a set of 10 tubes was used. The tubes were incubated in the dark at 15 °C. Gas samples were taken from the headspace and analyzed by gas chromatography for the accumulation of H_2, CH_4 and CO_2 while keeping the tubes on ice. After analyses of the head space the tubes were analyzed for dissolved intermediates. The contents of the tubes were taken under anoxic conditions and centrifuged at 13,000 rpm. The supernatant was centrifuged again and stored at -20 °C for analysis by gas chromatography and high pressure liquid chromatography (HPLC). Statistical

comparisons of the concentrations of acetate and sulfate consumed, and methane produced in the different incubations were evaluated with the Student's *t*-test ($P < 0.05$).

Medium preparation. A bicarbonate-buffered, sulfide-reduced mineral medium was prepared as described previously by Hüser *et al.* (17). To one litre of medium 1 ml of a vitamin solution (18), and 1 ml each of an acid and an alkaline trace elements solution was added (19) . The vitamin solution was filter-sterilized separately. The gas phase above the medium was 172 kPa N_2/CO_2 (80%/20%) and the pH of the medium was 6.8-6.9. Substrate and other supplement solutions (0.5 or 1 M) were sterilized by autoclaving or membrane filtration. Growth substrates were added just prior to inoculation. Hydrogen was added to the headspace at 60-kPa overpressure. Sodium acetate was used at 20 mM for aceticlastic methanogens and sulfate-reducing bacteria. Sodium lactate was used at 20 mM for sulfate-reducing bacteria. Methanol was used at 20 mM for methanogens and acetogens. Fructose was used at 20 mM for acetogens. In some cases, 2 mM acetate was added as a supplementary carbon source.

Pure culture incubations. The effect of $CHCl_3$ on different anaerobic microorganisms was followed in 120-ml serum bottles containing 50 ml medium. The growth conditions and media listed in Table 1 were used in these experiments. Experiments were started with an inoculum of 10 or 20 % of a freshly grown, stationary-phase culture. $CHCl_3$ was added to the required concentrations (20 and 50 µM) from stock solutions ($CHCl_3$ dissolved in methanol, ethanol or 1-propanol). Control incubations without $CHCl_3$ but with methanol, ethanol or 1-propanol were done as well. All experiments were performed in duplicate and incubated at 30 °C. Samples were taken periodically to determine substrate or/and electron acceptor utilization and product formation.

Archaeal and bacterial strains. *Methanosaeta concilii* $GP6^T$ (DSM 3671), *Methanosarcina barkeri* MS^T (DSM 800), *Methanospirillum hungatei* $JF1^T$ (DSM 864), *Methanobacterium bryantii* (DSM 862), *Acetobacterium woodii* (DSM 1030), *Sporomusa acidovorans* (DSM 3132), *Desulfovibrio vulgaris* strain Marburg (DSM 2119), *Desulfotomaculum acetoxidans* (DSM 771), *Desulfobacter postgatei* (DSM 2034) and *Syntrophobacter fumaroxidans* (DSM 10017) were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany).

Degradation of $CHCl_3$. Degradation of $CHCl_3$ in growing cultures of *A. woodii* (energy source: fructose), *D. vulgaris* (energy source: lactate) and *D. postgatei* (energy source: acetate) was followed in 120-ml serum bottles containing 50 ml of medium. Experiments were started as described above. $CHCl_3$ was added from a stock solution dissolved in ethanol to give a concentration of 20 or 50 µM. Control incubations without $CHCl_3$ but with ethanol were done as well. All experiments were performed as described above. During growth, 2-ml samples were taken from the gas phase and transferred to sterile anaerobic 15-ml serum bottles. The gas samples were stored at room temperature until further analysis. The degradation of $CHCl_3$ and formation of dichloromethane (CH_2Cl_2) was followed by gas chromatography.

Analytical techniques. Methane was measured on a 417 Packard chromatograph equipped with a flame ionization detector (FID) and a molecular sieve 5A column (Chrompack). The column temperature was 70 °C and the carrier gas was nitrogen at a flow rate of 20 ml/min. The accumulation of alcohols and fatty acids was determinated on a CP9001 gas chromatograph (Chrompack) equipped with a FID. Alcohols were separated on a fused silica WCOT CP-Sil 5 CB column (25 m long by 0.32 mm [i.d.]) with nitrogen, 35 kPa inlet pressure, as carrier gas. The samples (1 µl) were introduced via a splitter injection port (250 °C) with a split ratio of 25. The temperature of the column and the detector was 35 °C and 300 °C, respectively. Fatty acids were was quantified by gas chromatography (19). The samples (5 µl) were introduced via a packed column injection port (250 °C). The gas chromatograph was operated at an initial oven temperature at 160 °C. Then, the temperature was raised 2.5 °C/min to 200 °C, which was held for 1 min. The detector temperature was 300 °C. Organic acids were analyzed by HPLC on an ICE-AS6 column (DIONEX, Breda, the Netherlands). The mobile phase was 0.4 mM heptafluorbutyrate at a flowrate of 1.0 ml/min at room temperature. The organic acids were detected by chemical suppressed conductivity using an Anion-ICE micromembrane suppressor (DIONEX). Sulfate was analyzed by HPLC on an AS9-SC column (DIONEX). The mobile phase was 1.8 mM Na_2CO_3 and 1.7 mM $NaHCO_3$ at a flow rate of 1.0 ml/ min at room temperature. Sulfate was detected with suppressed conductivity using an anion self-regenerating suppressor (DIONEX). For the pure culture experiments H_2 and CH_4 were quantified by gas chromatography (20,21). Acetate, lactate and fructose consumption, and acetate production was measured by high-pressure liquid chromatography as described by Krümböck and Conrad (22). Sulfate consumption was analyzed by ion chromatography (23). $CHCl_3$ and CH_2Cl_2 were measured by gas chromatography as described by van Eekert et al (24).

RESULTS

Addition of acetate to sediment samples stimulated the consumption of sulfate but also enhanced methane formation (Table 2 and Fig. 1). About 69% of the added acetate served as an electron donor for sulfate reduction and about 20% was used for methanogenesis. This suggests that sulfate-reducing bacteria and methanogens were competing for the available acetate. Measuring the concentration of several electron acceptors showed a high natural pool of sulfate (2-3 mM). The concentrations of electron acceptors like thiosulfate, nitrate and nitrite were below the detection limit (1 µM).

In the acetate incubations with $CHCl_3$, the sulfate reducing activity (SRA) and methane producing activity (MPA) were completely inhibited (Table 2 and Fig. 2a). No depletion of sulfate occurred and the amount of CH_4 in the gas phase was the same as in the killed controls. Acetate and valerate accumulated immediately after the addition of $CHCl_3$. After 89 hours of incubation 584 µM of acetate and 12 µM of valerate were formed (Fig 2b). In the controls to which no acetate was added only acetate accumulated (Table 2). Other

Table 1. Growth inhibition of various microorganisms by 20 and 50 μM $CHCl_3$[a].

Microorganism	Acetyl-CoA pathway[b]	Energy source	Product formation	Inhibition[d]
Methanosaeta concilii GP6	present	acetate	CH_4	+
Methanosarcina barkeri MS	present	acetate	CH_4	+
		hydrogen	CH_4	+
		methanol	CH_4	+
Methanospirillum hungatei JF1	present[c]	hydrogen[f]	CH_4	+
Methanobacterium bryantii	present[c]	hydrogen[f]	CH_4	+
Acetobacterium woodii	present[c]	fructose	acetate	-
		hydrogen	acetate	+
		methanol	acetate	+
Sporomusa ovata	present[c]	fructose	acetate	+
		hydrogen	acetate	+
		methanol	acetate	+
Desulfovibrio vulgaris	not present	lactate + sulfate	acetate + H_2S	-
		hydrogen + sulfate	H_2S	-
Desulfotomaculum acetoxidans	present	acetate + sulfate	H_2S	+
		ethanol + sulfate	acetate + H_2S	+[e]
Desulfobacter postgatei	not present	acetate + sulfate[g]	H_2S	-
Syntrophobacter fumaroxidans	present	fumarate	succinate	+

[a]All cultures were grown in mineral medium at 30 °C without shaking, unless otherwise noted.

[b]When acetyl-CoA pathway is present, acetyl-CoA is degraded by CODH complex. [c]Acetyl-CoA is formed by acetyl-CoA synthase.

[d] +, inhibition of product formation (rate of product formation in the presence of $CHCl_3$ <1% of that in cultures without $CHCl_3$); -, no inhibition of product formation (rate of product formation in the presence of $CHCl_3$ >75% of that in cultures without $CHCl_3$).

[e] +, inhibition of product formation (rate of product formation in the presence of 20 μM $CHCl_3$ <60% and 50 μM $CHCl_3$ <10% of that in cultures without $CHCl_3$)

[f]Medium 119 of the DMSZ was used.

[g]Medium 193 of the DSMZ was used.

Table 2. The amounts[a] of acetate[b], sulfate and methane which were consumed or formed in the absence or presence of inhibitors during 89 hours of incubation at 15 °C using sediment samples supplemented or unsupplemented with acetate.

Incubation	consumption (μM)		formation (μmol/l)
	acetate	sulfate	methane
acetate	1293±235	1600±200	302±140
-	-	700±200	48±5
acetate+$CHCl_3$	-584±195	200±200	67±22
$CHCl_3$	-420± 50	50±223	32±13
acetate+MoO_4^{2-}	1275±273	200±420	1391±580
MoO_4^{2-}	-	300±100	634±140
acetate+BrES	1673±175	1400±316	208±98
BrES	-	1000±300	62±35
acetate+MoO_4^{2-}+$CHCl_3$	- 20± 314	10±224	66±35
MoO_4^{2-}+$CHCl_3$	-593±125	200±223	62±5
acetate+MoO_4^{2-}+BrES	- 10±262	100±280	226±146
MoO_4^{2-}+BrES	-454±190	180±310	82±24

[a]Means±standard deviation (n=3) are shown.
[b]Acetate formation is indicated by negative values.

products were not detected. Accumulation of H_2 was not observed in the different incubations (detection limit 10 Pa).

When molybdate was added to the tubes with acetate, the SRA was completely inhibited (Fig. 3a), resulting in a significantly higher methane formation (Table 2) compared to the controls without acetate and the incubations without molybdate. The stimulation of the CH_4 production by the addition of molybdate again indicates that sulfate reducers and methanogens were strongly competing for acetate. In the acetate and molybdate incubations 11 μM of propionate had accumulated after 17 hours of incubation, but propionate disappeared later on (Fig. 3b). No other intermediates were detected during the experiment. Our results suggest that the production of CH_4 mainly proceeds via acetate. In the sediment incubations with $CHCl_3$ about 420 μM of acetate accumulated after 89 hours of incubation. This amount of acetate could account for about 70% of the CH_4 (634 μM after 89 hours) produced in the presence of molybdate (Table 2).

Acetate incubations with BrES showed results comparable to the incubations without BrES (Fig. 4). However, the initial acetate consumption rate of 67 μM h^{-1} was 60% of the rate (110 μM h^{-1}) in the absence of the inhibitor. This suggests that the aceticlastic methanogens were inhibited by the addition of BrES but that the inhibition was not complete (Table 2 and Fig. 3). No other intermediates accumulated and the sulfate reduction rate was not significantly different from that in the incubations with acetate.

By the combined addition of molybdate and $CHCl_3$ (data not shown) or molybdate and BrES (Fig. 5a), a complete inhibition of the SRA and MPA with acetate was observed. Sulfate was not consumed and CH_4 was not produced. Propionate, butyrate and valerate accumulated immediately after addition of the inhibitors (Fig. 5b). However, the amount of acetate which accumulated was low (Table 2). In the controls without acetate similar results were obtained but here more acetate accumulated. However, the control incubations with molybdate and BrES did not show accumulation of butyrate. The combined use of inhibitors showed a similar effect on the SRA and MPA as the use of $CHCl_3$ alone. In all cases the inhibition resulted in the accumulation of fatty acids. However, the types of fatty acids which accumulated were different (Fig. 2b and 5b). These results suggest that the presence of molybdate influenced the degradation pathway(s) of fatty acids as well.

The effect of the addition of 20 and 50 μM $CHCl_3$ on the metabolism of pure cultures of various anaerobic bacteria was evaluated by measuring the consumption of substrates and/ or production of metabolic end products (Table 1). In all cases, the inhibitory effect of $CHCl_3$ could be clearly

evaluated by the kinetics of end product formation compared to that of control experiments without $CHCl_3$. All methanogens growing on acetate or H_2 were inhibited by 20 and 50 μM $CHCl_3$. Both species of

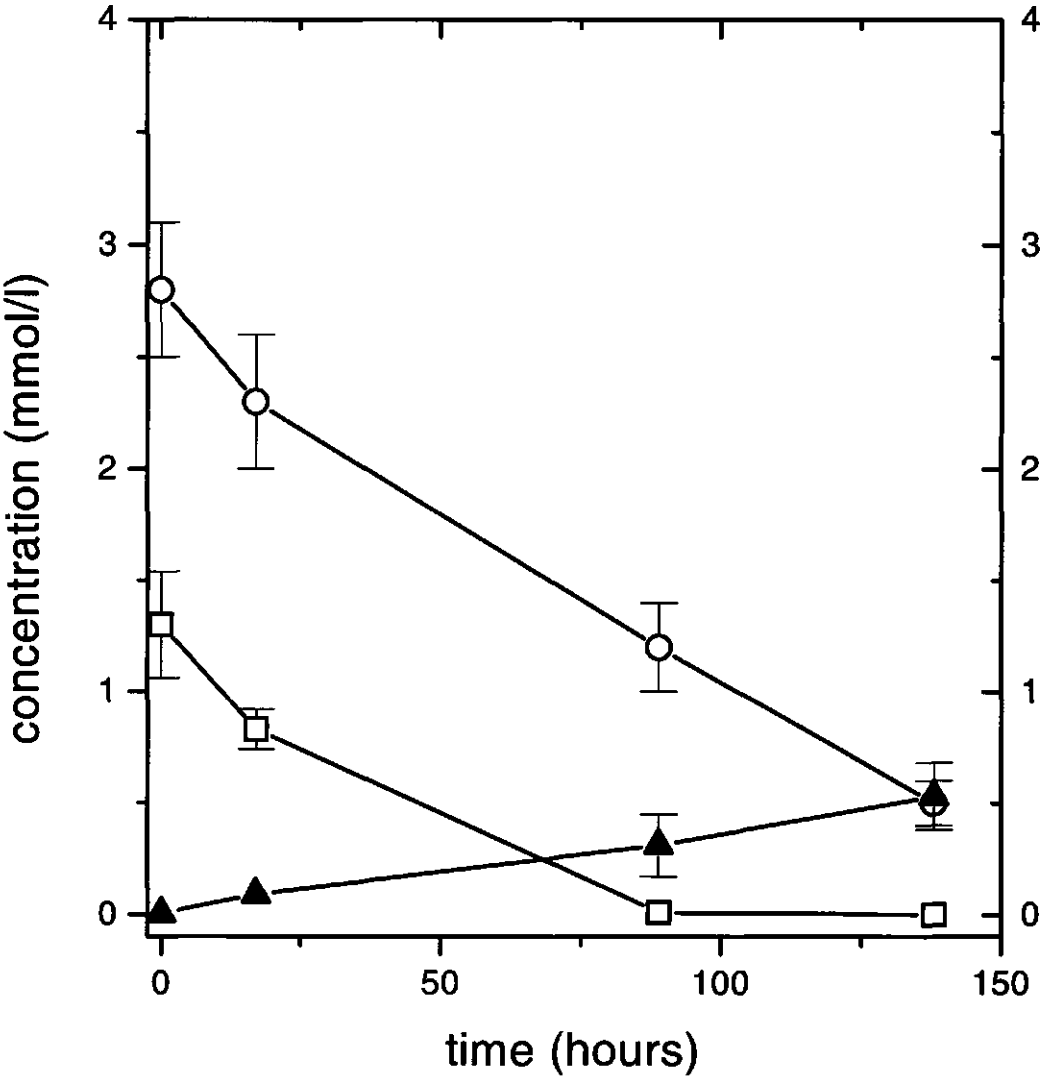

Figure 1. The consumption of acetate and sulfate, and formation of methane in freshwater sediment (Zegveld, 15 April 1995) incubated at 15°C with the addition of 1 mM acetate. Symbols: □: acetate; Δ: methane and O: sulfate. Error bars represent standard error of mean (n=3).

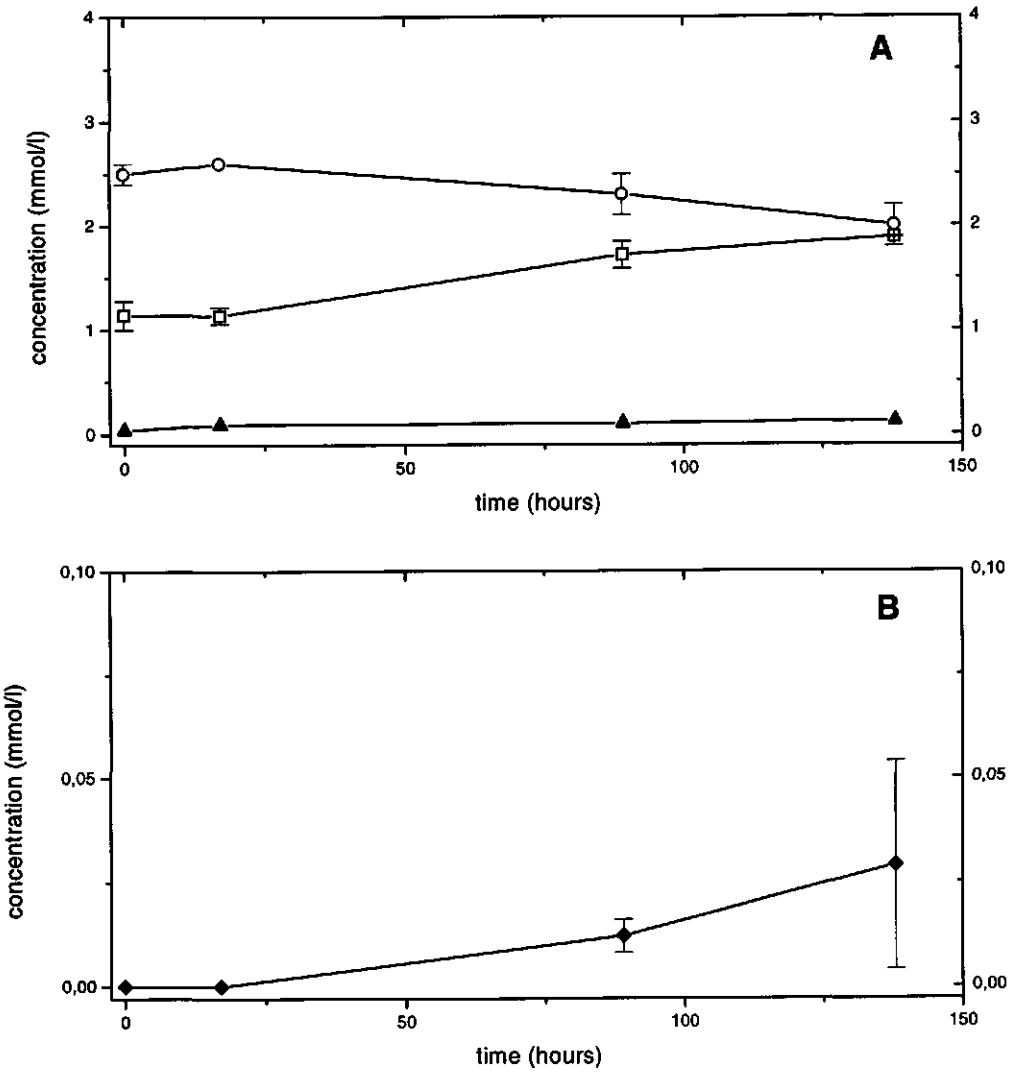

Figure 2. The consumption of acetate and sulfate, and formation of methane in freshwater sediment (Zegveld, 15 April 1995) incubated at 15°C with the addition of 1 mM acetate + 10 µM $CHCl_3$ (a), and the formation of valerate in same incubation (b). Symbols: □: acetate; Δ: methane; ○: sulfate and ◆: valerate. Error bars represent standard error of mean (n=3).

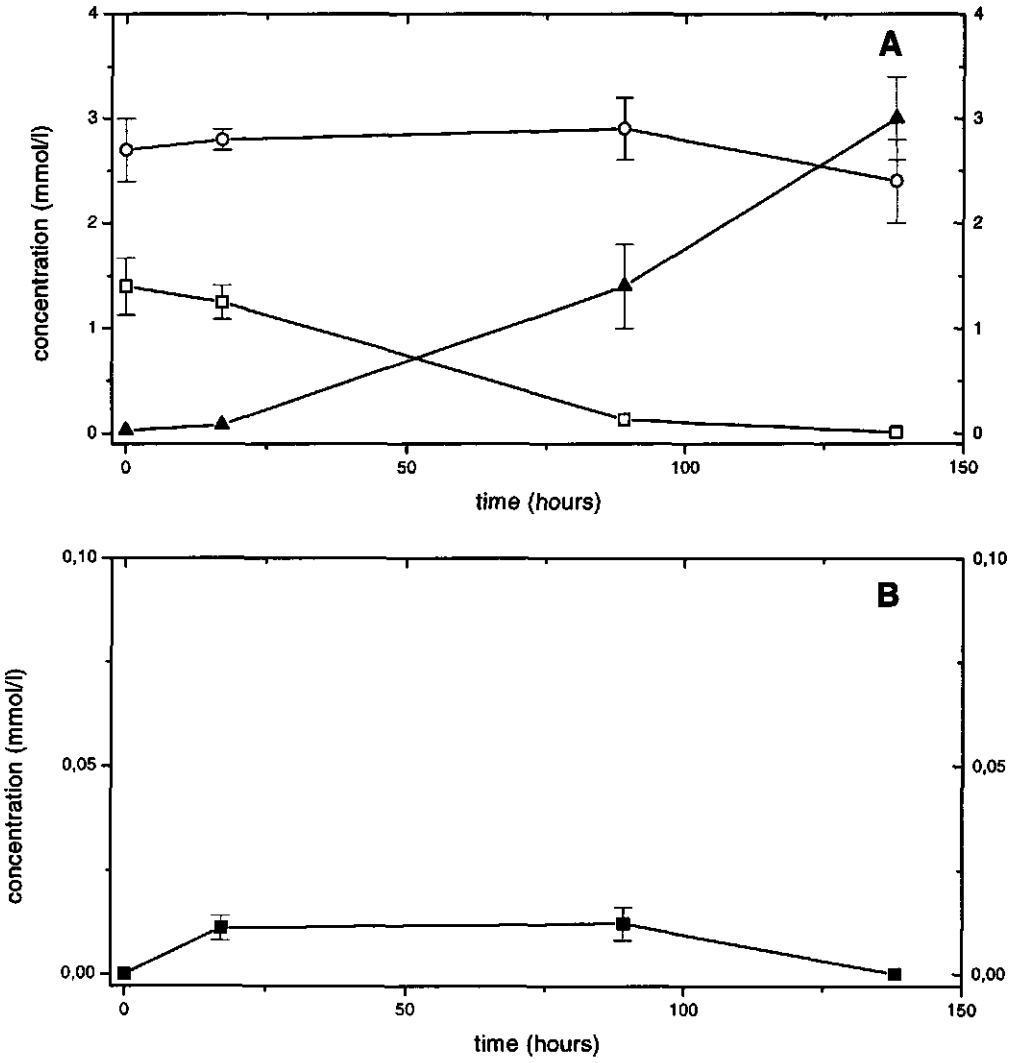

Figure 3. The consumption of acetate and sulfate, and formation of methane in freshwater sediment (Zegveld, 15 April 1995) incubated at 15°C with the addition of 1 mM acetate + 5 mM molybdate (a), and the formation of propionate in same incubation (b). Symbols: □: acetate; Δ: methane; ○: sulfate and ■: propionate. Error bars represent standard error of mean (n=3).

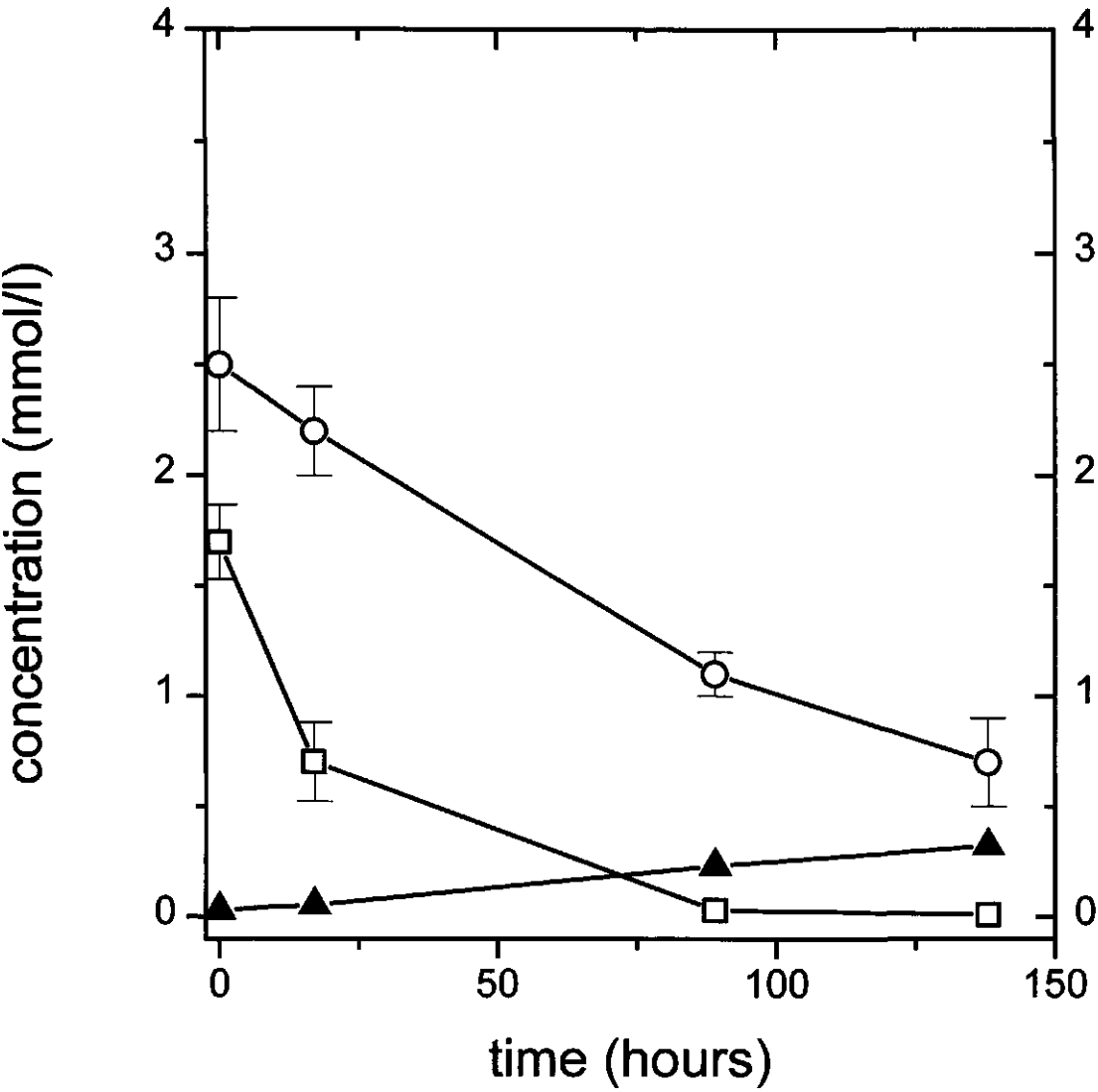

Figure 4. The consumption of acetate and sulfate, and formation of methane in freshwater sediment (Zegveld, 15 April 1995) incubated at 15°C with the addition of 1 mM acetate + 20 mM BrES. Symbols: □: acetate; ▲: methane and ○: sulfate. Error bars represent standard error of mean (n=3).

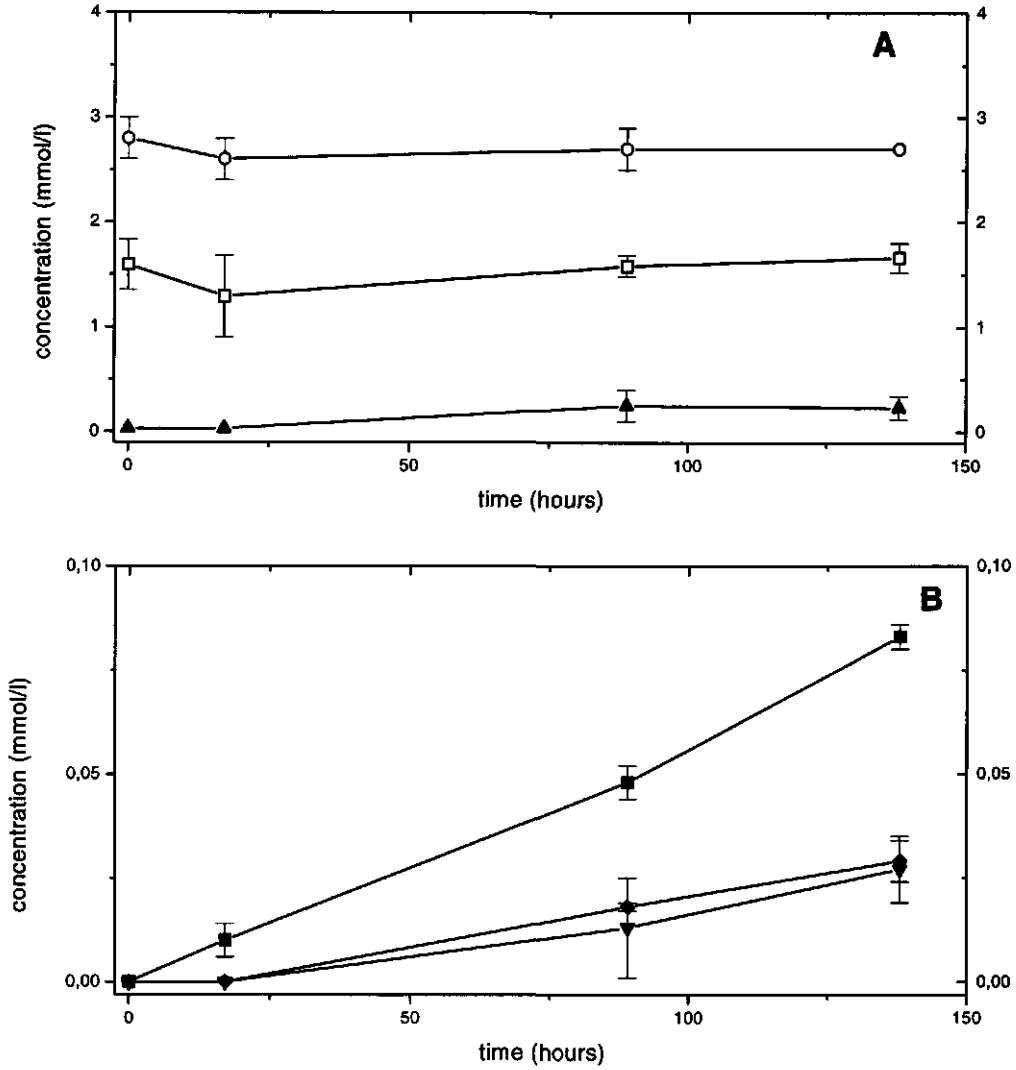

Figure 5. The consumption of acetate and sulfate, and formation of methane in freshwater sediment (Zegveld, 15 April 1995) incubated at 15°C with the addition of 1 mM acetate + 5 mM molybdate + 20 mM BrES (a), and the formation of propionate, butyrate and valerate in same incubation (b). Symbols: □: acetate; Δ:methane; ○: sulfate; ■: propionate; ▼: butyrate and ◆: valerate. Error bars represent standard error of mean (n=3).

homoacetogenic bacteria were inhibited at these $CHCl_3$ concentrations when growing on H_2. *A. woodii* growing on fructose was not inhibited but *S. ovata* was inhibited by 20 and 50 μM $CHCl_3$. The sulfate reducer *D. acetoxidans* growing on acetate or ethanol was inhibited at both $CHCl_3$ concentrations. *D. vulgaris* and *D. postgatei* were not inhibited by $CHCl_3$. The syntrophic bacterium *S. fumaroxidans* growing on fumarate was inhibited at both $CHCl_3$ concentrations. Degradation of $CHCl_3$ was only observed in growing cultures of *A. woodii*. In these cultures CH_2Cl_2 could be detected as an intermediate but $CHCl_3$ was not completely degraded.

DISCUSSION

Acetate stimulated sulfate reduction and the production of methane indicating that an active acetotrophic population of sulfate reducers and aceticlastic methanogens was present in the sediment of a ditch of a grassland area. Stimulation of sulfate reduction and CH_4 production by the addition of acetate indicates that this substrate is limiting for both methanogens and sulfate reducers. Thus, these two organisms compete directly for the available acetate. The amount of methane measured in the sediment incubations was 48 μmol/l (Table 2) and equal to the amount measured in killed controls (data not shown). This suggests that at *in situ* concentrations the sulfate reducers outcompeted the methanogens for the available acetate. This outcompetition has been explained before by the more efficient uptake systems of sulfate reducers for acetate and their ability to maintain the concentration low enough to exclude methanogens (25-28). The initial consumption of added acetate was lower in the BrES-inhibited incubations than in the uninhibited control, indicating that at relatively high acetate concentration methanogens successfully competed with the sulfate reducers for the available substrate. However, sulfate reducers were the dominant acetate-utilizers as they consumed 69% and methanogens only 20% of the added acetate. In the sediment sulfate was sufficiently high to allow sulfate reduction to be the dominant process. A previous study showed that the sulfate concentration in the sediment changed during the season (16). This will affect the outcome of the competition between sulfate reducers and methanogens. When the sulfate reducers were inhibited, acetate was metabolized at similar rates by methanogens. From our inhibition experiments it can be concluded that methanogenesis may become the dominant pathway in the consumption of acetate when sulfate becomes depleted from the sediment.

The accumulation of organic compounds gives an indication of their role in anaerobic mineralization. Many sulfate reducers utilize substrates like hydrogen, acetate, propionate, butyrate and valerate. Some of these bacteria oxidize fatty acids completely to CO_2, whereas other sulfate reducers oxidize these compounds only to acetate (28). In the case of methanogenesis, acetate and H_2 are the substrates for methanogens and these accumulate when CH_4 production is inhibited. Compounds like propionate, butyrate and valerate are degraded by proton-reducing bacteria to acetate and H_2. For thermodynamical reasons these reactions must be coupled to methanogenesis (29,30). When methanogens are inhibited, the accumulation of these

compounds becomes obvious. The relative amount of intermediates that accumulated in sediment incubations when both the sulfate reduction and methanogenesis were inhibited at the same time was 93 acetate, 6 propionate and 1 mole% valerate. These results confirm the role of acetate as a key intermediate in the terminal step of organic matter mineralization in the sediment. Several studies have reported similar results about the importance of acetate and other intermediates in freshwater sediments. When more than 90% of the organic carbon is converted to acetate, homoacetogens must play an important role (1,3,5,12,31,32).

Addition of molybdate in combination with acetate resulted in the accumulation of propionate which did not occur in the control incubations. The ability of sulfate reducers like *Desulfobulbus propionicus* to reduce acetate and bicarbonate with hydrogen to propionate could be an explanation for this observation (33). This suggests that sulfate reduction by sulfate reducers is inhibited by the addition of molybdate but that sulfate-independent processes of these bacteria are not affected. This was also found in studies done with sulfate reducers isolated from marine environments (34). However, in some studies it was found that molybdate inhibits the syntrophic utilization of propionate (i.e. sulfate-dependent processes are inhibited) as well (35,36). These authors assumed that sulfate reducers acted as the propionate-degrading syntrophic bacteria. Furthermore, molybdate has been shown to inhibit *Desulfovibrio desulfuricans* growing syntrophically (37). This does not agree with the finding that propionate accumulated in our incubations and was degraded afterwards.

Chloroform is occasionally used to inhibit methanogenesis (4,11,14). Our results showed that beside methanogens, acetate-utilizing sulfate reducers were also inhibited by the addition of chloroform. This inhibition might be explained by the type of pathway acetate-utilizing sulfate reducers use to degrade acetate. Some sulfate reducers degrade acetate via the acetylCoA-cleavage pathway while others use the citric acid cycle (38). In the acetylCoA-cleavage pathway, a nickel iron-sulfur corrinoid enzyme (carbon monoxide dehydrogenase (CODH) complex) is involved in the cleavage of acetylCoA and the further oxidation of the formed CO to CO_2 (38, 39). This cleavage reaction was shown to be inhibited by CCl_4. The formation of highly reactive carbenes during reductive dehalogenation of CCl_4 by corrinoids was postulated as the mechanism behind the inhibition of the CODH complex (40). Inhibition studies with different anaerobic microorganisms showed that the growth and product formation by homoacetogenic bacteria, *D. acetoxidans* and *S. fumaroxidans* was inhibited by $CHCl_3$. Only the homoacetogen *A. woodii* grown on fructose and the sulfate reducers *D. postgatei* and *D. vulgaris* were not inhibited by $CHCl_3$ (Table 1). The homoacetogenic bacteria, *D. acetoxidans* and *S. fumaroxidans* operate the acetylCoA-cleavage pathway during growth. Only during fructose-dependent growth homoacetogens do not need the pathway for energy and biosynthesis. Under these conditions the main function of the acetylCoA-cleavage pathway is to recycle the reduced electron carriers generated during the oxidation of fructose (41). But during growth on fructose, intermediates like hydrogen and formate accumulated in the incubations with $CHCl_3$ (data not shown). These

results suggest that $CHCl_3$ inhibits the CODH complex in a similar way as postulated for CCl_4 and that other anaerobic microorganism which uses the acetylCoA-pathway will be inhibited as well.

Although methanogens also operate the acetylCoA-pathway the observed inhibition by $CHCl_3$ is not necessarily due to the inhibition of the CODH complex (40), but may as well be caused by the inhibition of the methyl-coenzyme M reductase present in methanogens (42). This enzyme does not contain a corrinoid as a prosthetic group but coenzyme F_{430}, a nickel tetrapyrrole (40). Furthermore, it should be mentioned that methanogens and acetogens contain other corrinoid enzymes which might also be inhibited by $CHCl_3$ (43). Nevertheless, the finding that $CHCl_3$ inhibits microorganisms which operate the acetylCoA-cleavage pathway offers interesting possibilities in ecological studies. Chloroform may be used to elucidate the role of different metabolic types of sulfate reducers to sulfate reduction in natural environments.

ACKNOWLEDGEMENTS

This study was subsidized by a grant from the Netherlands Organization for Scientific Research (NWO) and the Max-Planck-Society. We thank M. van Eekert and R. Thauer for useful discussions.

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CHAPTER 6

ANAEROBIC ACETATE CONVERSION BY A FRESHWATER SEDIMENT UNDER DIFFERENT REDOX CONDITIONS

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ABSTRACT

The anaerobic conversion of acetate in a freshwater sediment under different redox conditions was investigated. Addition of [2-^{13}C] acetate stimulated the production of labeled methane. The dominant aceticlastic methanogen in the sediment was a *Methanosaeta* species. The presence of sulfate inhibited aceticlastic methanogenesis almost completely, most likely caused by the competition of sulfate reducers and methanogens for acetate. Acetate-utilizing sulfate reducers were limited by the availability of electron acceptor. At sulfate concentrations around 70 μM they were unable to compete with the methanogens. Unexpectedly, nitrate-reducing bacteria hardly competed with methanogens and sulfate reducers for the available acetate when nitrate was added. The electron-acceptor/acetate ratio indicated that denitrification was coupled to the oxidation of reduced sulfur compounds or other electron donors rather than to the oxidation of acetate. Nevertheless, addition of nitrate led to an inhibition of methanogenesis and sulfate reduction. Nitrate reduction seems to have a direct inhibitory effect on methanogenesis, and an indirect effect as a consequence of the oxidation of reduced sulfur-compounds to sulfate.

INTRODUCTION

Methanogens play an important role in the terminal processes of anaerobic organic matter degradation in electron acceptor-limited environments (5,21). In such methanogenic environments, organic matter is degraded by consortia of different physiological types of microorganisms. In sequences of reactions fermenting and acetogenic bacteria degrade biopolymers (carbohydrates, proteins and lipids) to acetate, formate, and CO_2 and H_2, which are the substrates for methanogens. It has been estimated that about 70% of the methane formed in mesophilic methanogenic environments is derived from acetate (15). Also in other habitats, acetate has been identified as the most important precursor of methane (8,32,33,43).

Methane is a green-house gas which contributes to the climate change on earth. One of the regulating factors of methane formation in nature is the availability of inorganic electron acceptors like sulfate and nitrate. Insight in the effect of sulfate on methanogenesis has been obtained in sediment studies and in studies with pure cultures (22,24,27). Much research was focused on the competition of methanogens and sulfate reducers for H_2. It was shown in batch cultures that sulfate reducers outcompete methanogens for H_2 because of their higher affinity and higher growth yield (22,31). However, it was also proposed that the threshold concentration for H_2 rather than the kinetic parameters determine the relative contribution of the two processes (24,25). Sulfate reducers are able to maintain lower threshold concentrations than methanogens, and these differences in H_2-thresholds are related to the Gibbs free energy change of the reactions (9,37).

Less is known of the competition of methanogens and sulfate reducers for acetate. Schönheit *et al* (35)

showed that *Desulfobacter postgatei* has a higher affinity for acetate than *Methanosarcina barkeri*. This could explain why *Desulfobacter* species are the main acetate-degrading microorganisms in marine sediments. However, *Methanosaeta* rather than *Methanosarcina* species are the dominant acetate-degrading methanogens in various methanogenic environments (19,23,32). These methanogens display comparable growth kinetic properties as acetate-utilizing sulfate reducers isolated from freshwater environments (28,29). Thus, the effect of the presence of sulfate on the fate of acetate is not so clear cut as observed for H_2 (27).

We have done experiments with freshwater sediments in which over the year relatively high sulfate concentrations are present (34). By the use of specific inhibitors we could show that both sulfate reducers and methanogens are involved in acetate degradation. The aim of this study was to elucidate the role of sulfate reducers and methanogens by the use of ^{13}C-labeled acetate. For comparison, the effect of nitrate was determined as well. To get insight into the importance of inorganic electron acceptors on methanogenesis *in situ*, the different groups of microorganisms involved in the acetate metabolism in the freshwater sediment were quantified.

MATERIALS AND METHODS

Site description and sample collection. Sediment samples were collected September 4, 1995 and April 4, 1996 with a sediment corer as previously described (34). The temperature of the sediment in September 1995 (summer) and April 1996 (spring) was 13 and 7 °C, and that of the water 14 and 6 °C, respectively. The cores were stored at 10 °C, and after 4 days the sediments were processed further.

Media. A basal bicarbonate buffered medium with a composition as described by Huser *et al.* (17) was used for the most-probable-number (MPN) counts. To one liter of medium 1 ml of a vitamin solution (46) and 1 ml of an acid and alkaline trace elements solution (39) were added. The vitamin solution was filter sterilized separately. The gas phase above the medium was 172 kPa N_2/CO_2 (80%/20%) resulting in a pH of the medium of 6.8-6.9. Acetate, sulfate and nitrate were added from 1M heat-sterilized stock solutions.

Quantification of functional groups of bacteria. All handlings were done under anaerobic conditions in a glovebox. The 0-10 cm layer of the sediment was homogenized, and 15 ml was transferred to a 250-ml serum bottle containing 135 ml of medium. The bottle was closed with a butylrubber stopper, evacuated and gassed with N_2/CO_2 (80%/20%). After shaking the bottle for 5 min, the sediment slurry (15 ml) was serially diluted to the 10^{-10} dilution. A three-tube MPN series was prepared by transferring 5-ml samples to 120-ml serum bottles containing 45 ml of medium. The MPN-tests for acetate-utilizing bacteria were performed with 10 mM acetate with or without sulfate or nitrate (10 mM). Incubations were carried out in the dark at 20 °C. Tubes were checked weekly and final scores were determined after 6-12 months of incubation. In positive tubes the concentration of the substrate and products was determined. Routinely, growth in the highest positive dilution was checked by transfer to fresh medium. MPN, deviance and 95% confidence intervals

were determined using a Basic computer program described by Hurley and Roscoe (16). Enumerations in the summer and spring samples were evaluated with the Student's *t*-test ($\alpha<0.05$) using the logarithm of the bacterial numbers. The populations were expressed as cells per cm^3 of sediment.

Set up of incubation experiments. Where necessary handlings were done under anaerobic conditions in a glovebox. Homogenized sediment from the 0-10 cm layer was distributed in 10-ml portions into 26-ml tubes and closed with butylrubber stoppers. The tubes were repeatedly evacuated and gassed with N_2 (152 kPa), and stored at 10 °C. The next day [2-^{13}C] acetate and electron acceptors were added. Controls without acetate were made as well. Inactivated controls were made by the addition of formaldehyde to a concentration of 3.75%. The total recovery of labeled acetate from these tubes was higher than 90%. Incubation experiments were done with a set of 21 tubes. For the controls a set of 18 tubes was used. The tubes were incubated in the dark at 17 °C. Tubes were sacrificed for gas and liquid analysis. Gas samples were taken by syringe from the headspace of the tubes which had been acidified with 3 M HCl and analyzed by gas chromatography (GC) for the accumulation of CH_4 and CO_2. For analysis of the consumption of [2-^{13}C] acetate, anions and other dissolved intermediates, the contents of the tubes were centrifuged two times at 17,380 × g. Supernatants were stored at –20 °C and analyzed later by gas chromatography and high performance liquid chromatography (HPLC). The pH was checked at the end of the experiment and was always between 6.8 and 7.0.

Calculation of turnover rates. Turnover rates (T_R : mol×l^{-1}×h^{-1}) were calculated from the changes in measured concentrations of labeled acetate. Rate constants were calculated directly by regression of the natural logarithm of the concentration of labeled acetate (11). The rate constant of the acetate consumption by methanogens was calculated assuming that the acetate concentration was equal to the amount of labeled CH_4 ([$^{13}CH_4$]) formed:

$$[^{13}\text{C-Ac}^{m}]_{(t=x)} = [^{13}CH_4]_{(t=x)} \quad (1)$$

where $[^{13}\text{C-Ac}^{m}]_{t=x}$ is the amount of ^{13}C-acetate consumed by the aceticlastic methanogens. The rate constant of the acetate consumption by sulfate reducers ([$^{13}\text{C-Ac}^{s}$]) may be described as :

$$[^{13}\text{C-Ac}^{s}]_{(t=x)} = [^{13}\text{C-Ac}]_{(t=0)} - [^{13}\text{C-Ac}]_{(t=x)} - [^{13}CH_4]_{(t=x)} \quad (2)$$

where $[^{13}\text{C-Ac}]_{(t=0)}$ is the acetate concentration at time zero and [^{13}C-Ac] the actual concentration of labeled acetate.

Calculation of cell numbers based on turnover rates. The numbers of acetate-utilizing sulfate reducers and methanogens were calculated from their actual activities. In kinetic studies where bacterial growth can be neglected Michaelis-Menten kinetics may be used to describe the substrate utilization of whole cells:

$$V = V_{max} \times S / K_m + S \quad (3)$$

where V_{max} and V are given in moles per gram of cellular dry weight per hour (mol×g dw^{-1}×h^{-1}). The actual activity of the sulfate reducers or methanogens can be obtained from the turnover rates of acetate divided by the total cell mass of each population (X_i : g dw×L^{-1}):

$$V = T_r/X_i \quad (4)$$

The dry mass of a microorganism may be described as:

$$X_i = N_i \times x_i \quad (5)$$

where N_i is the number of cells in the sediment (cells$\times L^{-1}$) and x_i is the specific cell mass (g dw$\times$cell^{-1}). Because V_{max} , K_m values and specific cell masses for sulfate reducers and methanogens have been reported (29,38) the number of cells can be calculated according to:

$$N = \{T_R \times [(K_m+S)/V_m \times S]\}/x_i \quad (6)$$

These values based upon activity measurements can be compared with the numbers obtained by MPN counts. For the calculation of the number of methanogens the following values were used: V_m: 2.28 mmol$\times h^{-1}\times$g dw^{-1}, K_m: 0.4 mM (28) and x_i: 0.85 pg (38). For the sulfate reducers these values were: V_m: 1.29 mmol$\times h^{-1}\times$g dw^{-1}, K_m: 0.6 mM (29) and x_i: 0.18 pg (38).

Analytical techniques. Determination of ^{13}C-labeled acetate, methane and carbon dioxide was carried out by GC (Hewlett Packard model 5890/5971A) equipped with a mass selective detector (MS). Samples were acidified with formic acid (Suprapur; Merck) to 0.3 % (vol./vol.) before injecting into the apparatus. Acetate and its stable isotopes were analyzed with a capillary column (innowax, 30 m × 0.25 mm (df=0.5 µm), Hewlett Packard, the Netherlands). Methane, carbon dioxide and their stable isotopes were separated on a capillary plot fused silica column (coating Poraplot Q, 25 m × 0.32 mm (df=0.32 mm), Chrompack, the Netherlands) with helium as the carrier gas. Samples for acetate (*m/z* 61) determination were acidified with formic acid (Suprapur; Merck) and [U-^{13}C] acetate (*m/z* 62) was added as an internal standard (final concentration: 100 µM) prior to injection into the apparatus. Samples (2 µl) were introduced via a splitless injection port (300 °C) at a column temperature of 160 °C. Gas samples (200 µl) were injected in a split injector (inlet pressure 1 kPa; split ratio 25:1) at a column temperature of 35 °C. Detection was performed with a mass-selective detector (ionization energy, 2970 eV). Spectral data were processed with a computer. Acetate and its stable isotopes were monitored at *m/z* 60 to 62. Methane and carbon dioxide and their stable isotopes were monitored at *m/z* 16 and 17, and 44 and 45, respectively. Total methane was measured on a 417 Packard chromatograph equipped with a flame ionization detector (FID) and a molecular sieve 5A column (110 cm × 2.1 mm, Chrompack). The column temperature was 70 °C and the carrier gas was nitrogen at a flow rate of 20 ml.min^{-1}. Anions were analyzed by HPLC as previously described (34). The HCO_3^- concentration in the porewater was calculated from the amount of CO_2 which accumulated in the gas phase after acidification. Sulfide was determined as described by Trüper and Schlegel (42). Samples for the analysis of sulfide were kept on ice in closed eppendorf tubes and determined at the end of the experiment. Determination of methane, hydrogen, and volatile fatty acids in the MPN incubations were done as previously described (33).

Thermodynamic calculations. The Gibbs free energy changes (ΔG) of the individual reactions were calculated from the standard Gibbs free energy changes at pH 7 ($\Delta G^{0'}$) and the actual concentration of

reactants and products. $\Delta G^{0'}$ data were obtained from Thauer *et al* (41).

RESULTS

The effect of sulfate and nitrate on the biotransformation of ^{13}C-labeled acetate in a freshwater sediment was studied. Addition of [2-^{13}C] acetate to sediment collected in summer stimulated the production of methane (Fig. 1a and 1b). Almost no methane was produced in the controls without acetate during the 6 hours of incubation (Table 1). This suggests that the methanogens were limited by the availability of electron donor(s). Methanogenesis accounted for more than 60% of the acetate conversion. Sulfate reduction was not simulated by the addition of labeled acetate, although 50-70 μM sulfate was present in the porewater. Furthermore, analysis of the porewater revealed that the natural pool of acetate was below detection limit (<20 μM). When sulfate was added to the sediment the sulfate reducing activity (SRA) was stimulated directly (Fig 2a). Addition of labeled acetate in combination with sulfate inhibited the production of labeled methane (Fig 2b). The ratio CH_4/acetate was lower compared to the incubations where only labeled acetate was added, and the total amount of acetate consumed was not affected by the extra sulfate although more sulfate was reduced in 6 hours (Table 1). These results indicated that acetate-utilizing sulfate reducers were competing successfully with the methanogens for the available acetate provided that sufficient sulfate was present. This suggests that in the summer incubations both the methanogens and the sulfate reducers were limited by the availability of electron donor(s). Furthermore, it appeared that the sulfate-reducing community was limited by the availability of sulfate as well. Addition of nitrate led to the accumulation of sulfate in the sediment incubations (Fig. 3a). When nitrate was added in combination with labeled acetate, the formation of methane was almost completely inhibited and sulfate accumulated (Fig. 3b). Methanogenesis was inhibited by the activity of nitrate-reducing bacteria but due to the accumulation of SO_4^{2-} it remained unclear if the SRA was inhibited as well. The rate of nitrate reduction was not significantly affected by the presence of acetate. Also the amount of acetate consumed within 6 hours of incubation was not significantly different compared to the other incubations. These results suggest that acetate was not an important substrate for nitrate-reducing bacteria. However, the accumulation of sulfate indicates that reduced sulfur compounds served as electron donors for these organisms.

In sediment collected in spring the natural pool of sulfate was between 500-800 μM, which is significantly higher than the sulfate concentration found in summer. Almost no sulfate was reduced or methane was produced in the control incubations indicating that methanogens and sulfate reducers were both limited by substrate availability (Table 1). The addition of labeled acetate stimulated sulfate reduction instantaneously. Sulfate reduction accounted for 90% of the acetate conversion. The initial production of labeled methane was low but started after 4 hours of incubation (data not shown). Nevertheless, the production of labeled CH_4 showed that aceticlastic methanogens did compete with the sulfate reducers for the available acetate

Table 1. The amounts of ^{13}C-acetate, sulfate, nitrate and ^{13}C-methane which were consumed or formed during incubation of freshwater sediment samples for 6 hours (September 1995) and 7 hours at 17°C (April 1996).

Incubation	Degradation			Formation	
	^{13}C-acetate (μM)	SO_4^{2-} (μM)	NO_3^- (μM)	^{13}C-CH_4 ($\mu mol.\ L^{-1}$)	SO_4^{2-} (μM)
September sample					
acetate	131 ± 18	20 ± 17	-	82 ± 12	-
control	-	14 ± 4	-	-	-
acetate+SO_4^{2-}	124 ± 23	83 ± 18	-	43 ± 8	-
control	-	55 ± 13	-	-	-
Acetate+NO_3^-	100 ± 27	-	180 ± 12	1 ± 5	56 ± 24
control	-	-	163 ± 34	-	34 ± 14[1]
April sample					
acetate+SO_4^{2-}	126 ± 12	144 ± 48	-	34 ± 1	-
control	-	26 ± 28	-	-	-
acetate+NO_3^-	65 ± 6	99 ± 78	215 ± 25	0	-
control	-	60 ± 47	210 ± 27	-	-

[1]measured but no degradation or formation

(Table 1). The consumption of acetate was significantly reduced by the addition of nitrate, indicating that nitrate reduction affects other processes like sulfate reduction as well. In the summer and spring incubations, the amount of $^{13}CO_2$ in the samples where labeled acetate was added did not significantly differ from the amount of $^{13}CO_2$ in the incubations without acetate. Therefore, determination of the contribution of sulfate reduction and nitrate reduction to the consumption of labeled acetate ($^{13}CO_2$ formed/ SO_4^{2-} reduced and $^{13}CO_2$ formed/ NO_3^- reduced) was not possible.

The reactions which possibly are involved in the intermediary metabolism in the sediment are summarized in Table 2. In situ concentrations of [2-^{13}C] acetate, sulfate and nitrate as described in Figure 1, 2 and 3 were used to calculate the actual Gibbs free energy changes for the different reactions under methanogenic, sulfate-reducing or nitrate-reducing conditions (Table 3). The results show that the Gibbs free energy change of aceticlastic acetate conversion was only slightly affected by the addition of sulfate or nitrate. The ΔG-value of acetate oxidation by sulfate reducers was in the same range as that of acetate cleavage by methanogens. The Gibbs free energy change calculations of the two types of dissimilatory nitrate reduction, where nitrate is reduced to dinitrogen or to ammonium, showed that under the experimental conditions acetate oxidation coupled to nitrate reduction is energetically more favorable than sulfate reduction or methanogenesis.

Table 2. Reactions possibly involved in the degradation of acetate in a freshwater sediment from Zegveld. $\Delta G^{o'}$ values obtained from Thauer *et al.* (41).

Reaction	$\Delta G^{o'}$ (kJ /reaction)
Methanogenic reactions	
$Acetate^- + H_2O \rightarrow CH_4 + HCO_3^-$	-31.0
Sulfate reducing reactions	
$Acetate^- + SO_4^{2-} \rightarrow 2\ HCO_3^- + HS^- + H^+$	-47.3
Nitrate reducing reactions	
$5/4\ Acetate^- + 2\ NO_3^- + 3/4\ H^+ \rightarrow 21/2\ HCO_3^- + N_2 + H_2O$	-990.1
$Acetate^- + NO_3^- + H^+ + H_2O \rightarrow 2\ HCO_3^- + NH_4^+$	-495.4
$5/4\ HS^- + 2\ NO_3^- + 3/4\ H^+ \rightarrow 5/4\ SO_4^{2-} + N_2 + H_2O$	-932.4
$HS^- + NO_3^- + H^+ + H_2O \rightarrow SO_4^{2-} + NH_4^+$	-450.5

Quantification of methanogenic, sulfate reducing and denitrifying microorganisms. Populations of methanogenic, sulfate-reducing and nitrate reducing bacteria which used acetate as a substrate were enumerated with sediments sampled in summer and spring (Table 4). No significant differences in the summer and spring samples were observed. Differences in the required incubation times of the different

types of microorganisms were observed. For example, in MPN-dilutions used to enumerate acetate-utilizing nitrate reducing bacteria (ANRB), the maximum cell number was already reached after 4-6 weeks, whereas in the dilution series used to enumerate acetate-utilizing sulfate-reducing bacteria (ASRB) and acetate-consuming methanogens (AMPB) final cell numbers were reached after 9-10 months. The estimation of the number of acetate-utilizing methanogens and sulfate reducers based on turnover rates are given in Table 4. The calculated number of acetate-utilizing methanogens and sulfate reducers were in the same order of magnitude as the numbers obtained with the MPN-counts.

Table 3. Gibbs free energies (ΔG) available for different acetate-consuming reactions in freshwater sediment from Zegveld at 17°C.

Reaction		ΔG (kJ mol^{-1}/reaction) at incubation time (h)				
		0	2	4	6	ΔG change
September 1995						
Acetate	AM	-38	-36	-33	-32	-6
	SR	-34	-32	-29	-28	-6
Acetate+SO_4^{2-}	AM	-37	-36	-34	-31	-6
	SR	-37	-36	-34	-31	-6
Acetate+NO_3^-	AM	-37	-36	-34	-31	-4
	SR	-35	-35	-32	-32	-3
	NR^1	-970	-968	-960	-949	-21
	NR^2	-500	-488	-482	-472	-28

ΔG values of reactions in Table 2 were calculated using the concentrations shown in Figs. 1, 2 and 3. The pH used was 7.0. The following measured concentrations were used: HCO_3^-, 2 mM; HS^-, 0.5 to 0.2 mM and NH_4^+, 0.001 to 0.2 mM. AM: aceticlastic methanogenesis, SR: sulfate reduction (acetate as electron donor), NR^1: nitrate reduction (acetate as electron donor) and NR^2: nitrate reduction (HS^- as electron donor).

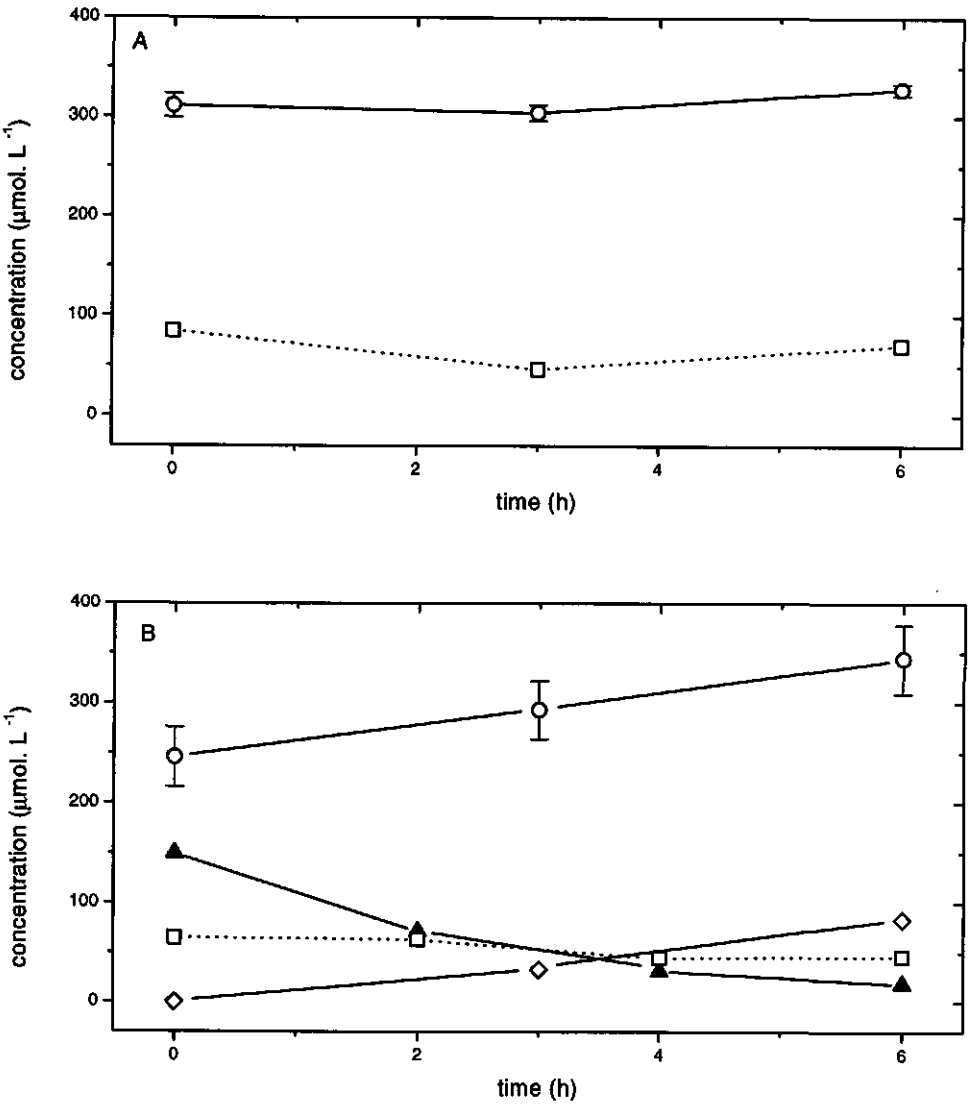

Figure 1. The [2-^{13}C] acetate and sulfate utilization, and formation of labeled and total methane in freshwater sediment (Zegveld, 4 September 1995) incubated at 17°C (A) and with the addition of [2-^{13}C] acetate (B). Symbols: ○: total CH_4, ◊: $^{13}CH_4$, ▲: [2-^{13}C] acetate and □: sulfate. Error bars represent standard error of mean (n=3).

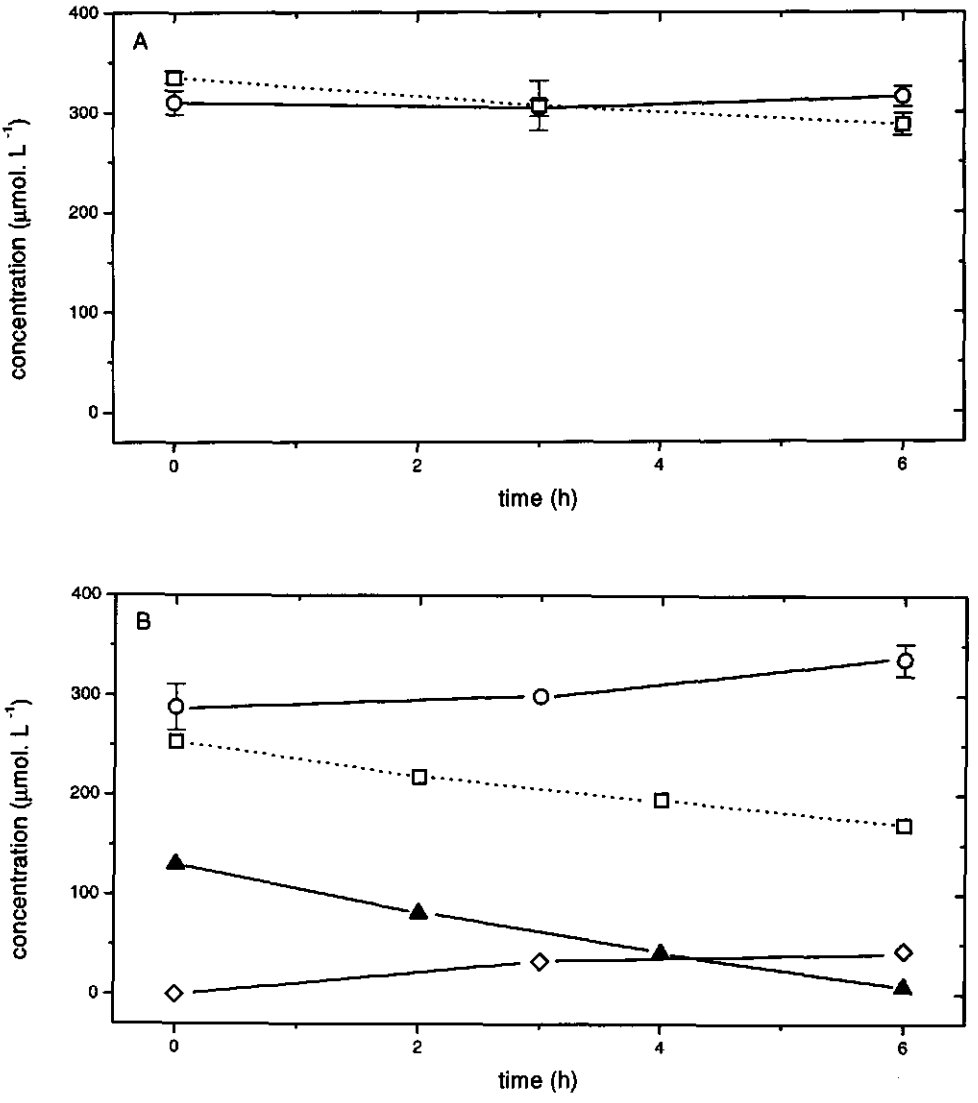

Figure 2. The [2-^{13}C] acetate and sulfate utilization, and formation of labeled and total methane in freshwater sediment (Zegveld, 4 September 1995) incubated at 17°C with the addition of sulfate (A) and with the addition of [2-^{13}C] acetate and sulfate (B). Symbols: ○: total CH_4, ◊: $^{13}CH_4$, ▲: [2-^{13}C] acetate and □: sulfate. Error bars represent standard error of mean (n=3).

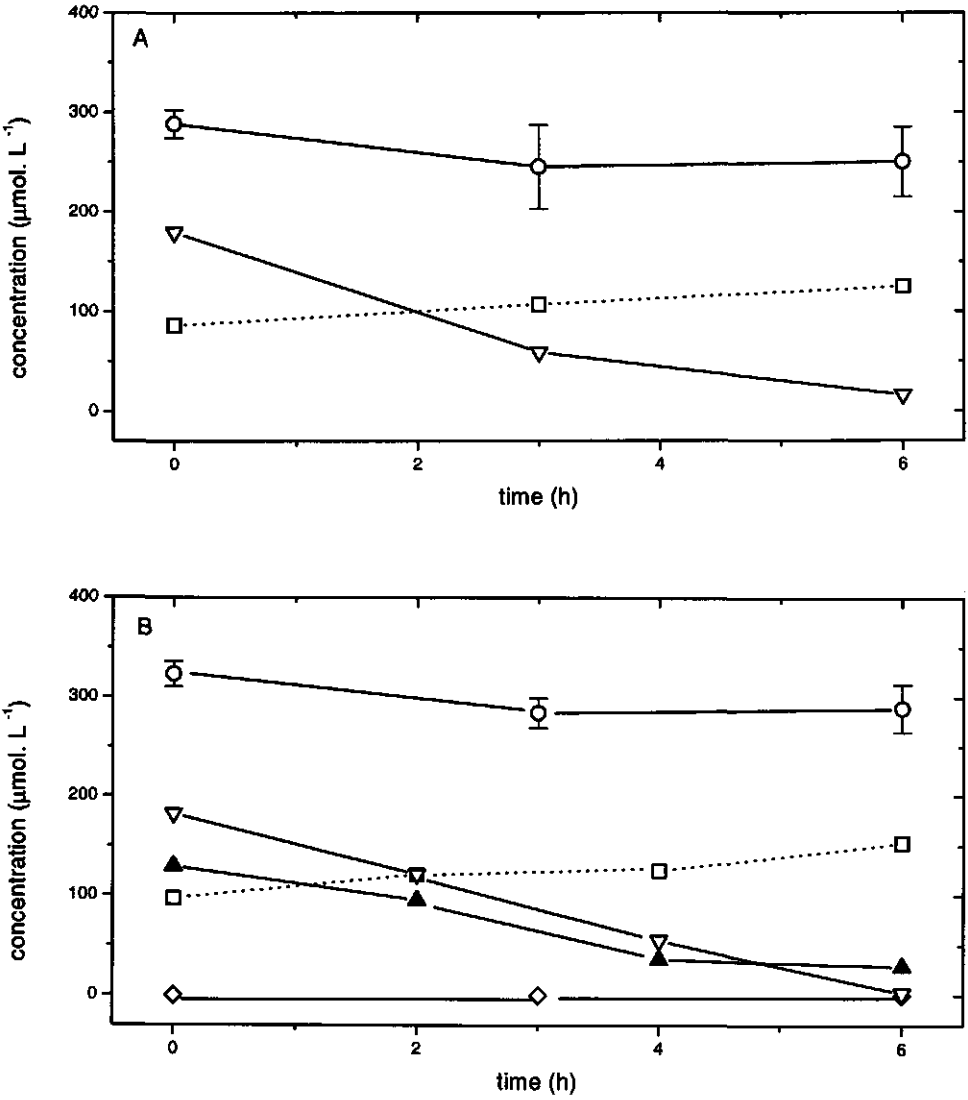

Figure 3. The [2-^{13}C] acetate and nitrate utilization, and formation of sulfate, labeled and total methane in freshwater sediment (Zegveld, 4 September 1995) incubated at 17°C with the addition of nitrate (A) and with the addition of [2-^{13}C] acetate and nitrate (B). Symbols: ○: total CH_4, ◊: $^{13}CH_4$, ▲: [2-^{13}C] acetate, □: sulfate and ∇: nitrate. Error bars represent standard error of mean (n=3).

Table 4. The results of MPN experiments and the estimated number of cells based up on turnover rates of ^{13}C-labeled acetate in sediment from Zegveld sampled in summer (September 1995) and spring (April 1996) performed at 17°C, respectively.

Acetate-utilizing microorganisms	Cells cm^{-3} sediment			
	MPN counts[a]		Estimates from turnover rate	
	September sample	April sample	September sample	April sample
Methanogens	2×10^8 (1-8)	2×10^8 (1-8)	8×10^7	7×10^7
Sulfate reducers	2×10^8 (1-8)	9×10^7 (3-42)	7×10^8	1×10^9
Nitrate reducers	5×10^5 (2-28)	9×10^4 (3-43)	ND[b]	ND

[a]95% confidence interval in parentheses.

[b]ND: not done.

DISCUSSION

Our experiments show that in summer aceticlastic methanogenesis is a dominant process in the consumption of acetate in the freshwater sediment from Zegveld. The stimulation of the methane production upon the addition of traces of acetate indicates that the methanogens are limited by the availability of acetate. The quantitative importance of acetate to methanogenesis could not be judged in these experiments, but inhibition studies revealed that about 70-80% of the total carbon flow to CH_4 was through acetate (33). This relative high contribution of acetate to methanogenesis was also observed by other researchers in paddy soil slurries and lake sediments (32,36). In mesophilic environments generally 60-70% of the methane is formed from acetate (15). Differences in threshold concentrations may give an indication which population of acetate-utilizing methanogens dominated the production of CH_4. Jetten *et al* (20) reported relatively low threshold concentrations for *Methanosaeta spp.* compared to *Methanosarcina spp.*, 7-70 μM versus 0.2-1.2 mM, respectively. Since in situ acetate concentrations were below 20 μM, it seems likely that acetate turnover was mainly due to the activity of *Methanosaeta spp.* The main acetate-utilizing methanogens obtained from the highest dilutions in the MPN counts indeed were *Methanosaeta*-like microorganisms. This strongly suggests

that these methanogens are the most important acetate-utilizing methanogens in the sediment.

The almost complete inhibition of methane formation due to the presence of sulfate is in accordance with previous studies (4,44). Stimulation of both processes by the addition of acetate shows that these two groups of microorganisms are competing directly for the available acetate. Generally, sulfate reducers have a higher affinity for acetate, a higher maximum rate of acetate utilization per unit biomass and a higher growth yield than methanogens (18,28,35,45). Because of these properties sulfate reducers outcompete methanogens for the available acetate. Lovley and Klug (26) described a model to predict the relative importance of CH_4-production and sulfate reduction in different lakes. According to this model no methane will occur when sulfate reducers are able to maintain the acetate concentrations below 22 μM. The sulfate reducers could only maintain such a low acetate concentration when steady state sulfate concentrations were above 30 μM. In our experiments, methanogenesis seems to account for more than 60% of the acetate mineralization in the sediment when sulfate concentrations were between 50-70 μM. The reduction of sulfate was not stimulated by the presence of acetate at these low sulfate concentrations. However, addition of sulfate stimulated sulfate reduction suggesting that the sulfate concentration for acetate-utilizing sulfate reducers should be above 70 μM, otherwise they are sulfate-limited. Sulfate reduction was the process dominating in the consumption of acetate (>90%) when sulfate concentrations were higher than 500 μM. Nevertheless, aceticlastic methanogens were able to compete with the acetate-utilizing sulfate reducers for the available acetate at sulfate concentrations between 20-200 μM. Our results suggest that methane production and sulfate reduction from acetate occurred simultaneously in the sediment. This in contrast to the results of Ward and Winfrey (43) who mentioned that these processes occur sequentially in time and space. The seasonal change in sulfate concentration (34) may be a major factor controlling the formation of methane in these sediments. However, up to now it is unclear why such high sulfate concentrations are observed in sediments from Zegveld, but high sulfate concentrations were confirmed by others (12).

Nitrate reduction was more affected by electron acceptor availability than by the addition of acetate. Nitrate-reducing bacteria hardly compete with methanogens and sulfate reducers for the available acetate at the in situ nitrate concentrations The electron-acceptor/acetate ratio indicated that besides acetate other electron donors for nitrate reducers were present. Our observation of nitrate reduction coupled to the oxidation reduced sulfur compounds has been reported in studies of natural environments (13,14,40). Colorless sulfur bacteria are able to carry out denitrification using reduced compounds of sulfur as electron donors (30). However, the oxidation of inorganic sulfur compounds coupled to reduction of nitrate/nitrite may also be done by sulfate-reducing bacteria (10).

The addition of nitrate inhibited methanogenesis completely and sulfate reduction partially which is in accordance with data from others (4,44). From our experiments, the Gibbs free energy change could not explain the inhibition of the methanogens and sulfate reducers because the ΔG-values calculated for both processes in the presence of NO_3^- were almost similar to the incubations without nitrate (Table 3). On the basis of the ΔG-values calculations both nitrate reduction to dinitrogen and nitrate reduction to ammonium,

are feasible. Brunet and Garcia-Gil (7) mentioned that the initial concentration of free sulfide determines the type of nitrate reduction. At very low concentrations of free sulfide nitrate was reduced to N_2 whereas at high sulfide (>1 mM) ammonium was formed and incomplete reduction to nitrogen oxides took place. If we take into account that the sulfide concentrations were between 0.1-0.5 mM, inhibition of the methane production may be explained by an inhibition of methanogens by gaseous N-oxides although the formation of nitrogen oxides was not measured in our experiments (2,3,47,48).

The acetate-utilizing microorganisms in the sediment were quantified by the most probable number method in liquid media. Our incubations showed that up to 9 months were needed to obtain true numbers. Bak and Pfennig (1) already mentioned that prolonged incubation times may positively influence the counting efficiency. Also other limitations of the MPN technique have been reported which could result in the underestimation of the number of cells (6). However, the calculated number of acetate-utilizing methanogens and sulfate reducers based on turnover rates was in the same order of magnitude as the number obtained with the MPN counts. Because of the independent approach of the *turnover* method, our results show that the MPN method with a prolonged incubation time seems to estimate the active *in situ* populations of sulfate reducers and methanogens quite well.

Our experiments indicate a coupling between the sulfur and nitrogen cycles. The oxidation of reduced sulfur-compounds to sulfate may cause the seasonal fluctuation of sulfate. Therefore, the sulfur cycle controls the formation of methane in the sediments. Further comparative studies between the denitrification with organic matter and reduced S-compounds as mentioned by Garcia-Gil and Golterman (13), may give more insight into the coupling of the two cycles.

ACKNOWLEDGEMENTS

This study was subsidized by a grant from the Netherlands Organization for Scientific Research (NWO). We thank M. van Eekert and P. van Bodegom for useful discussions. The authors also thank Dr. Tetsuro Kono for providing the Basic computer program for the calculation of cell numbers.

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APPENDIX CHAPTER 6

MODELING OF INTERACTIONS AMONG ANAEROBICALLY RESPIRING BACTERIA AND METHANOGENS

Peter van Bodegom, Alfons J.M. Stams and Johannes C.M. Scholten

INTRODUCTION

From experiments and studies described in previous chapters, a schematic representation of the microbial community in the sediment can be made. This section describes the construction and sensitivity analysis of a mathematical model to express quantitative descriptions of the interactions between the different microbial populations in complex environments, with an emphasis on acetate. The interdependence of the microorganisms within the sediment in terms of substrate (acetate), SO_4^{2-}, NO_3^-, H_2S and NO is quantified to understand the influence of SO_4^{2-} and NO_3^- on the formation of CH_4. Results of experimental work were used for validation for freshwater sediments. The sensitivity analysis of the model can be employed to develop future laboratory experiments to understand the anaerobic processes in sediments using single species or well-defined mixtures of the relevant microorganisms.

MODEL STRUCTURE

The basis of the model is the oxidation of reduced fermentation products given in Figure 1 (chapter 1 and 2) and Figure 2 (chapter 2). In the model, five functional groups of microorganisms are considered. These groups of organisms are listed in Table 2 (chapter 2) with the reactions carried out by these groups. In the model acetate is used as the only substrate originating from organic matter and it is formed at a constant rate. The model describes the competition between methanogens and bacteria which use alternative electron acceptors. In reality, acetate is not the only substrate for which competition occurs; e.g. H_2 is also an important substrate. However, in many environments, acetate is the main product of organic matter mineralization and is the main precursor for methane formation (see chapter 2 and 5). The model was therefore simplified to this single substrate. The substrate competition was described with Michaelis-Menten kinetics. In this way, the competition can be described with two parameters: the rate of the reaction (V_{max}) and the affinity constant (K_m). The reaction is a function of the microbial biomass. However, in our model, the microbial biomass is assumed not to be the rate limiting factor. Arguments for the non-limiting biomass for alternative electron acceptors are given by Segers and Kengen (1) and for methanogens by van Bodegom and Stams (2).

Some additional inhibitory effects due to product inhibition or direct redox effects were included as well. First, the inhibitory effects of HS^-, NO and N_2O on methanogenesis and the effects of HS^- on denitrification were incorporated in the model. The product inhibition was described with two parameters, a threshold concentration below which no inhibition occurred and a maximum concentration above which complete inhibition occurred. In between these concentrations, inhibition was assumed to increase linearly. It was further more assumed that sulfate reduction and methane production were inhibited at a redox potential higher than 0 mV.

Inorganic compounds such as H_2S and $S_2O_3^{2-}$ can also be used as electron donors by facultative anaerobic chemolithoautotrophs that respire NO_3^- (3). Sulfate-reducing bacteria produce H_2S and thus provide a substrate for nitrate-reducing bacteria. Such chemolithoautotrophic denitrifiers may compete with heterotrophic microorganisms for the available NO_3^-. The values for all affinity constants (K_m) and inhibitory concentrations were derived from published values (Table 1). The potential reaction rates (V_{max}) were derived from our experimental data (chapter 5 and 6).

MODEL PERFORMANCE

The model was validated for its performance for short term incubations in a freshwater sediment described by in chapter 6. For the model conditions, only the initial concentrations of the different compounds were varied according to the experimental data. All kinetic parameters were kept constant. The results are shown in Figure 1and 2. The model was able to describe most incubations properly.

An exception is the description of the system when both nitrate and sulfate are present. In that case, we could not get the balance of the different compounds complete. This is clearest at high acetate concentrations. The best description was obtained if a significant part of the nitrate reduction was coupled to the oxidation of sulfide rather than to the oxidation of acetate. The calculated maximum conversion rate of the nitrate reduction-sulfide oxidation reaction was high (Table 1). In combination with the incomplete balance, this indicates that conversions of acetate and sulfurous compounds in the presence of nitrate are missed by the model. A sensitivity analysis was performed to understand the possible influence of coupled nitrogen reduction-sulfur oxidation processes (Figure 3). Nitrate concentrations could more or less be described by the model, but at all conditions sulfate concentrations were underestimated by the model. Sulfate must thus have been formed from sulfide (or another reduced sulfurous compound that was not incorporated in the model) with an inorganic electron acceptor other than nitrate. Acetate concentrations were overestimated, even when the maximum conversion rate of autotrophic denitrification was so low that this reaction could not compete for nitrate (and thus all nitrate was reduced with acetate). Thus, acetate must thus have been converted by processes other than described in the model.

The influence of product inhibition was also tested by sensitivity analysis. Nitrate or sulfate was added to a model freshwater sediment slurry. A pulse of sulfate (up to 15 mM, a concentration which is not reached in Dutch ground- and surface water (4)) did not lead to product inhibition by sulfide within 48 hours. Sulfide concentrations remained below the inhibiting concentrations for nitrate reduction and

Table 1. Parameters derived from literature data used for the modelling [chapter 5 and 6].

Reaction	K_m electron donor (mM)	K_m electron acceptor (mM)	V_{max} (mM s^{-1})
$Acetate^- + H_2O \rightarrow CH_4 + HCO_3^-$	0.94		$8.0 * 10^{-5}$
$Acetate^- + SO_4^{2-} \rightarrow 2\ HCO_3^- + HS^-$	0.32	0.31	$4.0 * 10^{-5}$
$Acetate^- + 8\ Fe^{3+} + 4\ H_2O \rightarrow 2\ HCO_3^- + 8\ Fe^{2+} + 9\ H^+$	0.80	$6.1 * 10^{1}$	$2.6 * 10^{-4}$
$5/4\ Acetate^- + 2\ NO_3^- + 3/4\ H^+ \rightarrow 21/2\ HCO_3^- + N_2 + H_2O$	0.71	0.12	$3.5 * 10^{-4}$
$5/4\ HS^- + 2\ NO_3^- + 3/4\ H^+ \rightarrow 5/4\ SO_4^{2-} + N_2 + H_2O$	1.90	1.75	$1.0 * 10^{-4}$
Mineralisation rate[a]			$0.7 * 10^{-6}$

product inhibition	Threshold concentration (mM)	maximum inhibitory concentration (mM)
HS^- inhibition on NO_3^- reduction	1.0	10.0
HS^- inhibition on methanogenesis	7.79	22.1
NO inhibition on methanogenesis	$1.8 * 10^{-3}$	$1.8 * 10^{-2}$

[a] Constant mineralisation rates are assumed

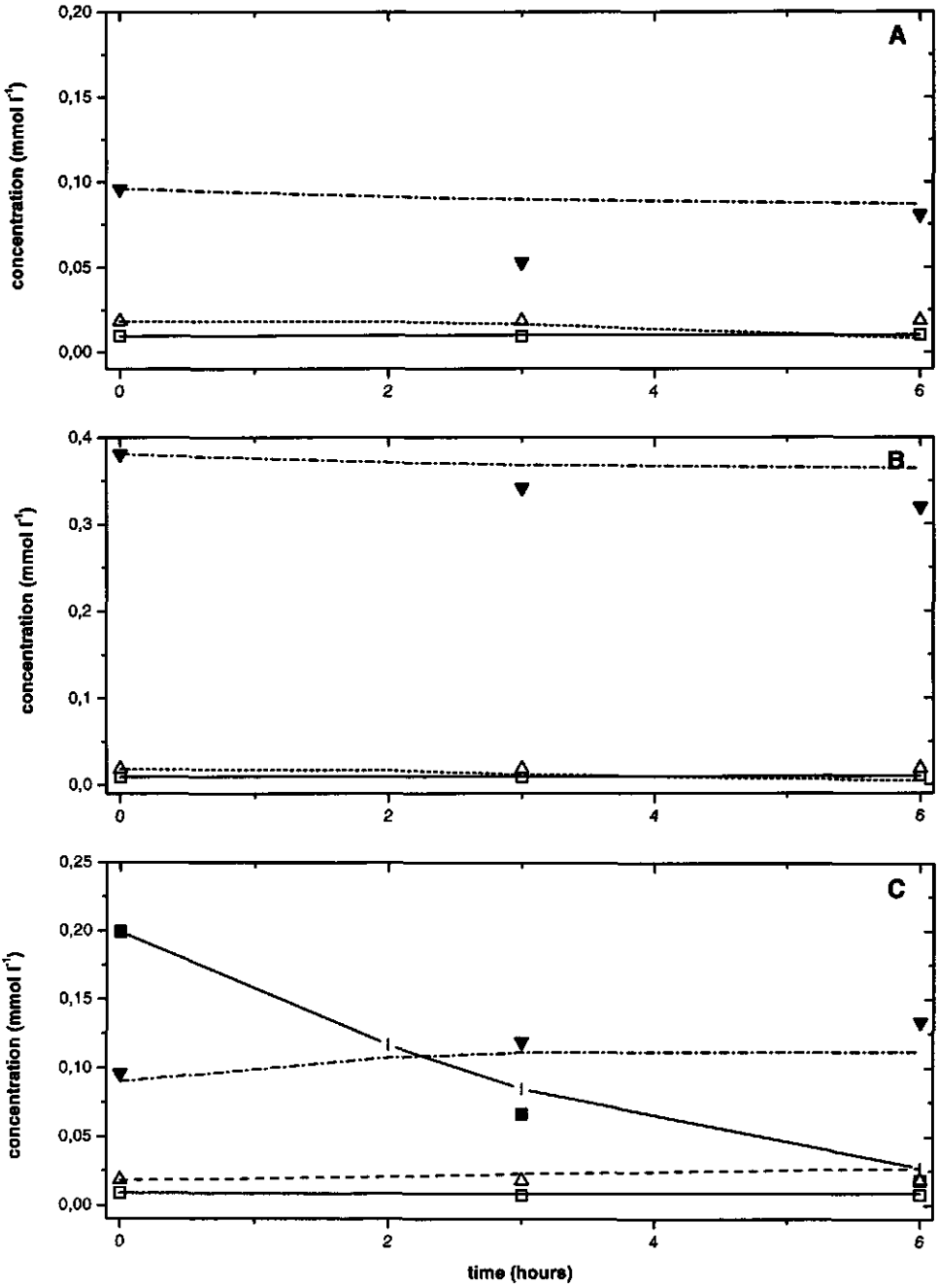

Figure 1. The degradation of acetate in an anaerobic freshwater sediment in the absence and presence of sulfate or nitrate; incubation without acetate (a-c). Measured (symbols) and modelled (lines) concentrations: Δ/ short dash : acetate, ▼/ dot dash : sulfate, / closed : methane and ■ / closed with marker: nitrate.

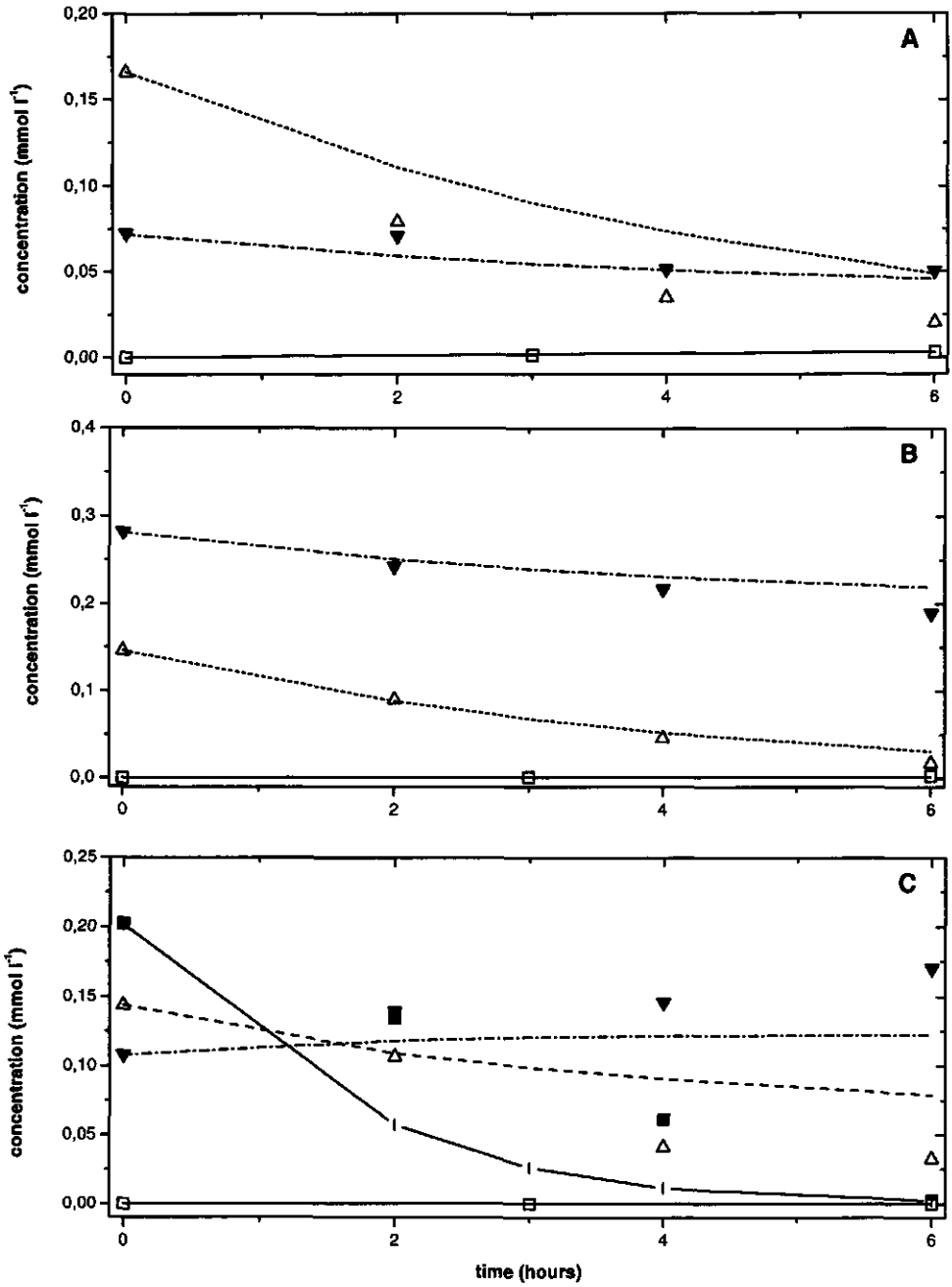

Figure 2. The degradation of acetate in an anaerobic freshwater sediment in the absence and presence of sulfate or nitrate; incubation with acetate (a-c). Measured (symbols) and modelled (lines) concentrations: Δ/ short dash : acetate, ▼/ dot dash : sulfate, / closed : methane and ■ closed with marker: nitrate.

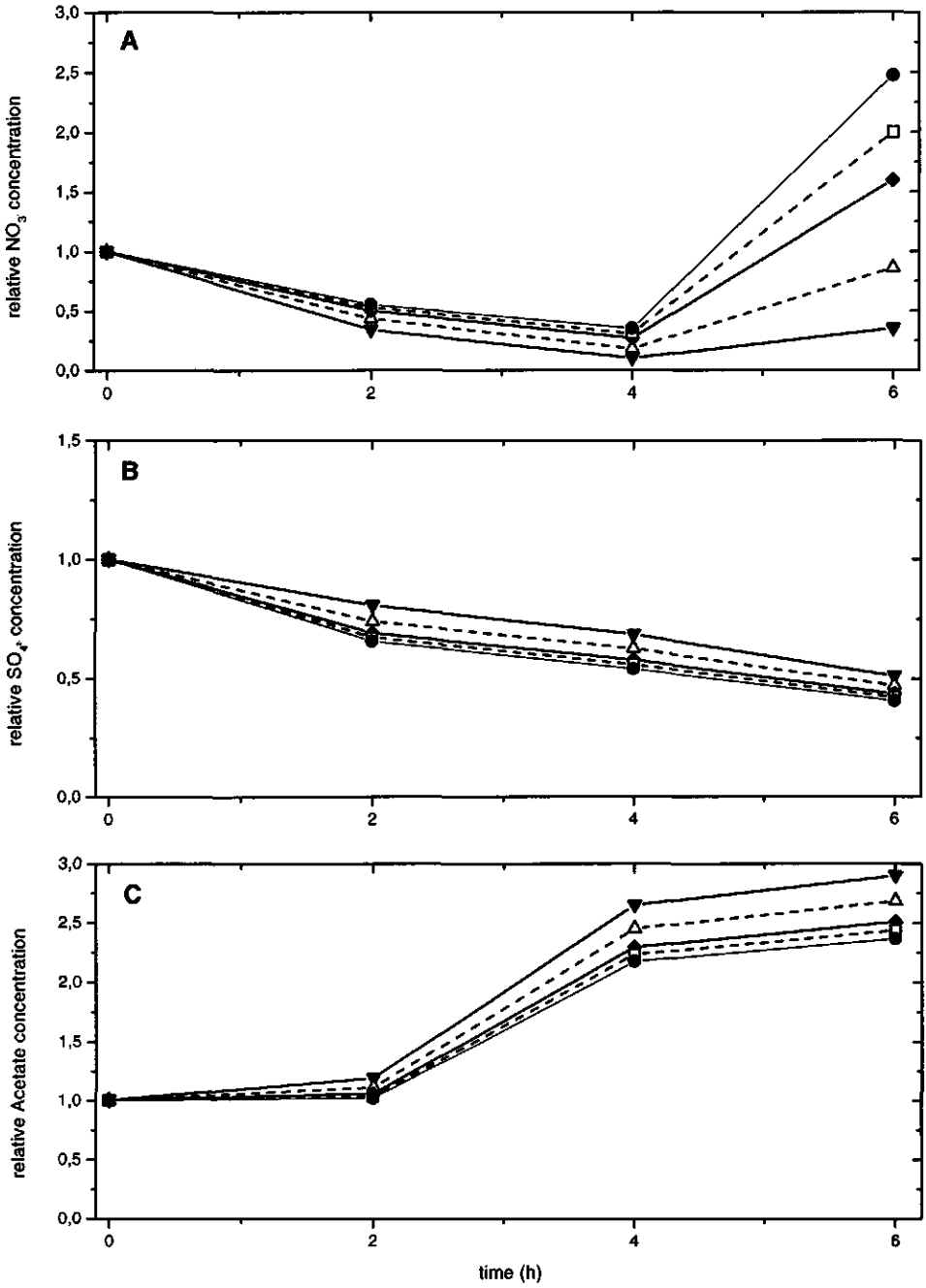

Figure 3. Influence of different maximum conversion rates of autotrophic denitrification on (a) nitrate, (b) sulfate and (c) acetate concentrations during a short term incubation with freshwater sediment. Modelled concentrations are presented relative to measured concentrations. Conversion rates ($mM\ s^{-1}$): ●:10^{-5}; □:10^{-4}; ◆:2×10^{-4}; Δ:5×10^{-4} and ▼:10^{-3}.

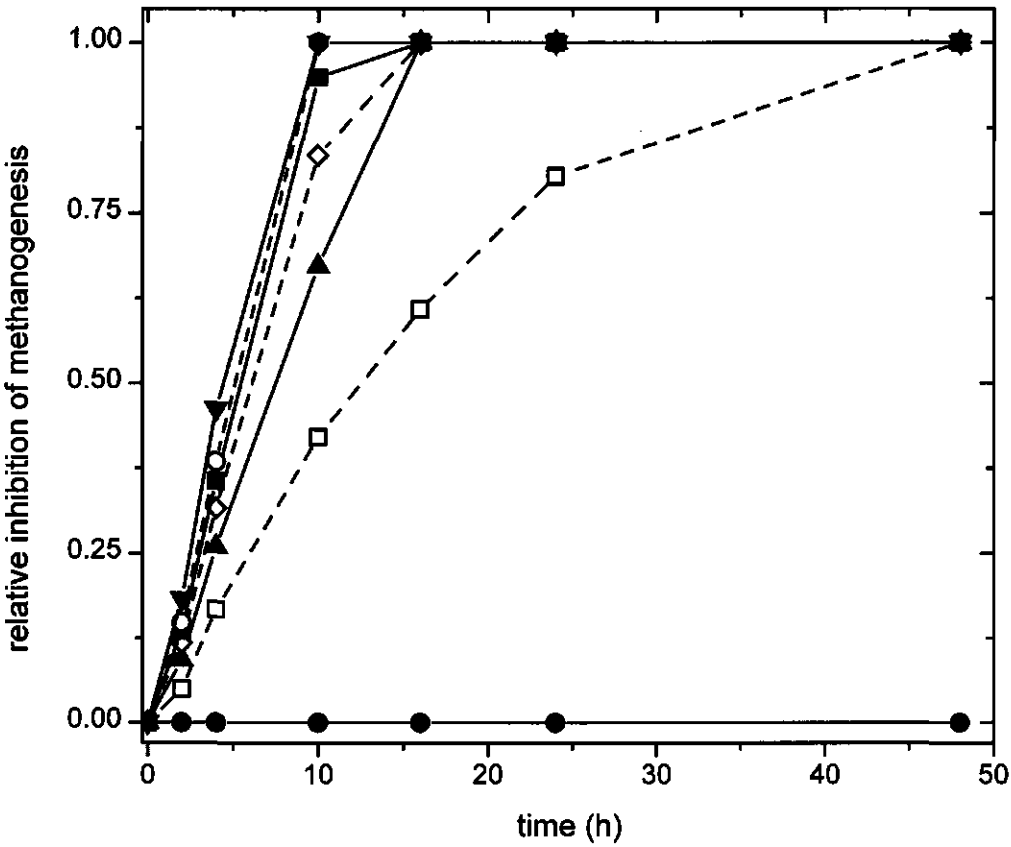

Figure 4. Relative inhibition of methanogenesis by nitric oxide after different additions of nitrate to a freshwater system. Initial nitrate concentration (mM): ●: 0; □: 0.5; ▲: 1; ◇: 1.5; ■: 2; ○: 2.5 and ▼: 5.

methanogenesis. However, addition of nitrate did inhibit methanogenesis due to accumulated nitric oxide (Figure 4). Concentrations up to 0.5 mM nitrate occur in groundwater near fertilizer agricultural fields (5), but concentrations up to 10-15 mM may occur in rice paddy fields by fertilization.

The kinetic model after incorporation of some additional feedbacks between the different microorganisms in freshwater systems could describe the real system in these sediment reasonably well. However, at high nitrate and acetate concentrations the conversions could not be described properly by the model as indicated by a sensitivity analysis. However, such high acetate concentrations do not occur in a sediment system at a steady state. Contrary to sulfide, nitric oxide (accumulated after addition of

moderately high nitrate concentrations) can inhibit other reduction processes temporarily. This is an important feedback that deserves further attention.

CONCLUSIONS

Mathematical modelling showed to be very helpful to understand the fate of acetate under different redox conditions in a freshwater sediment. The model could describe most of the data on the basis of competition, and the incorporation of an inhibitory mechanism by toxic intermediates. In this way an important feedback was revealed that deserves further attention in future experiments. Therefore, mathematical modelling might be used to expose other interactions between microbial populations in complex environments.

ACKNOWLEDGEMENTS

This research was made possible by a grant from the Netherlands Organization for Scientific Research (NWO).

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CHAPTER 7

ISOLATION AND CHARACTERIZATION OF ACETATE-UTILIZING ANAEROBES FROM A FRESHWATER SEDIMENT

Johannes C.M. Scholten and Alfons J.M. Stams

ABSTRACT

From the highest dilutions of a most probable number counting on acetate of a freshwater sediment a methanogen, a sulfate-reducing and a nitrate-reducing bacterium were isolated with acetate as sole carbon and energy source. The methanogen (culture AMPB-Zg) was non-motile, rod-shaped with blunted ends (0.5-1 μm × 3-4 μm long). Optimum growth with acetate occurred around 30-35 °C (doubling times: 5.6–8.1 days). The methanogen grew only on acetate. Phylogenetically (16S rRNA sequence), AMPB-Zg is closely related to *Methanosaeta concilii*. The isolated sulfate-reducing bacterium (strain ASRB-Zg) was rod-shaped with pointed ends (0.5-0.7 μm × 1.5-3 μm long), weakly motile, spore-forming and gram positive. Optimum growth with acetate occurred around 30 °C (doubling times: 3.9–5.3 days). The bacterium grew on a range of organic acids, such as acetate, butyrate, fumarate and benzoate but did not grow autotrophically with H_2, CO_2, and sulfate. Strain ASRB-Zg closest relatives were *Desulfotomaculum nigrificans* and *Desulfotomaculum thermosapovorans*. The nitrate-reducing bacterium (strain ANRB-Zg) was rod-shaped (0.5-0.7 μm × 0.7-1 μm long), weakly motile and gram negative. Optimum growth with acetate occurred at 20-25 °C. The bacterium grew on a range of organic substrates, such as acetate, butyrate, lactate and glucose but did not grow autotrophically with H_2, CO_2, and nitrate. In the presence of acetate and nitrate, thiosulfate was oxidized to sulfate. Phylogenetically, strain ANRB-Zg closest relative is *Variovorax paradoxus*.

INTRODUCTION

Acetate is the most important intermediate in the degradation of organic matter in anaerobic freshwater environments (1-4). Many anaerobic microorganisms capable of growth on acetate as energy source have been described. Acetate may even be the sole substrate for some methanogenic and sulfate reducing microorganisms (2,5). Other anaerobic microorganisms are generalists which can grow on other substrates as well (6,7). The methanogens which grow on acetate are *Methanosarcina* and *Methanosaeta* (formerly *Methanothrix*). *Methanosarcina* is metabolically versatile. It is able to grow on several substrates including, H_2/ CO_2, methanol, methylamines and acetate (2,8). *Methanosaeta* uses only acetate as energy source.

Different genera of sulfate-reducing bacteria can grow on acetate. Growth on acetate was demonstrated for *Desulfobacca acetoxidans*, *Desulforhabdus amnigenus*, and *Desulfobacterium*, *Desulfotomaculum* and *Desulfobacter* species (5, 9-16). *Desulfobacca acetoxidans* and most *Desulfobacter* species are specialized in growth on acetate (10,15). *Desulfobacter* strains are mostly isolated from brackish and marine sediments, and may be enriched from freshwater environments using brackish water or marine media (14). However, these sulfate reducers probably are not important in the conversion of acetate in freshwater environments. In contrast, *Desulfobacca acetoxidans* shows best growth in freshwater media. *Desulforhabdus amnigenus*,

Desulfobacterium and *Desulfotomaculum* species are generalists which use besides acetate a wide variety of substrates such as propionate, hydrogen and ethanol (9,11,12). Some sulfate reducers, e.g. *Desulfovibrio baarsii*, *Desulfosarcina variablis*, *Desulfococcus* and *Desulfobacterium* species show very poor growth on acetate, despite the fact that an acetate-degrading pathway is present (15). The reason for the marginal capacity or inability to use acetate as a growth substrate is not clearly understood. Furthermore, these sulfate reducers generally prefer other substrates than acetate. The utilization of mixed substrates was studied with the generalist *D. amnigemus* (17). Cells growing on acetate immediately stopped to use acetate when ethanol, lactate or propionate was added. However, addition of hydrogen did not affect acetate oxidation. Hydrogen and acetate were used simultaneously and this may increase the competitive advantage of *D. amnigenus* over other acetate degrading microorganisms.

Acetate is a common substrate for nitrate reducing bacteria. Most nitrate reducers are regarded as generalists and they often are able to grow with O_2 as an electron acceptor (6). So far, not much attention has been paid to growth of nitrate-reducing bacteria with acetate as electron donor. Therefore, little is known about the role of nitrate reducers as anaerobic acetate-degraders in natural environments.

Recently, we have described studies with a freshwater sediment in which labeled acetate was used to examine the influence of sulfate and nitrate on methane production (Chapter 6). In the presence of sulfate, addition of acetate stimulated both sulfate reduction and methanogenesis indicating that both populations were competing for the available acetate. The influence of nitrate on the formation of methane was not clear-cut, as denitrifying bacteria were using other substrates as well. To understand the impact of inorganic electron acceptors on methanogens, we also quantified the different groups of bacteria involved in acetate metabolism in the freshwater environment. In this study we determined the physiological properties of an acetate-utilizing methanogen, sulfate-reducing and nitrate reducing bacterium obtained by direct serial dilution of freshwater sediment.

MATERIALS AND METHODS

Source of organisms. The acetate-utilizing methanogen (culture AMPB-Zg), sulfate-reducing (strain ASRB-Zg) and nitrate reducing bacterium reducing (strain ANRB-Zg), were isolated from a freshwater sediment taken from ditches near Zegveld, the Netherlands.

Media and cultivation. A basal bicarbonate buffered medium with a composition as described by Huser *et al.* (1982) was used. To one liter of medium 1 ml of a vitamin solution (Wolin *et al.* 1963), and 1 ml of an acid and alkaline trace elements solution was added (Stams *et al.* 1993). The vitamin solution was filter sterilized separately. The gas phase above the medium was 180 kPa N_2/CO_2 (80%/20%) or H_2/CO_2 (80%/20%) and the pH of the medium was 6.8-6.9. Electron donors and acceptors were added from 1-M sterile, anoxic stock solutions. Except for some heat-labile substrates that were filter-sterilized, all substrates were sterilized by heat (20 min, 120 °C).

Isolation. All manipulations were done under anaerobic conditions in a glove box. The 0-10 cm layer of the sediment was homogenized, and 15 ml was transferred to a 250-ml serum bottle containing 135 ml of medium. The bottle was closed with a butylrubber stopper, evacuated and gassed with N_2/CO_2 (80%/20%). After shaking the bottle for 5 min, the sediment slurry (15 ml) was serially diluted to the 10^{-10} dilution. A three-tube MPN series was prepared by transferring 5-ml samples to 120-ml serum bottles containing 45 ml of medium. The MPN-tests for acetate-utilizing bacteria were performed with 10 mM acetate with or without sulfate or nitrate (10 mM). Incubations were carried out in the dark at 20 °C. The highest dilutions that showed growth were used for further isolation. Pure cultures were obtained for the sulfate-reducing bacterium by pasteurizing a full grown culture for 10 min at 80 °C. The spores were transferred to fresh medium and the application of pasteurization was repeated twice. Pure cultures were obtained for the nitrate-reducing bacterium by repeated application of the agar roll-tube-dilution method as described by Hungate (1969). To check purity, isolates were inoculated into medium with 0.2% yeast extract (BBL-Becton Dickinson), lactate, pyruvate, or glucose as substrates. After incubation, the cultures were examined microscopically.

Physiological tests. Utilization of carbon sources, energy sources, and electron acceptors was tested using a concentrations of 10 mM. These test were performed in 120 ml serum bottles containing 45-ml of medium. The substrates and electron acceptors consumed, and the products formed were measured.

Sequence analysis and phylogenetic tree. Nucleic acids from strain ASRB-Zg was isolated by sonification followed by phenol extraction and ethanol precipitation as previously described (22). The 16S rRNA gene was amplified by PCR using a set of primers corresponding to positions 8-27 [5'-CACGGATCCAGACTT-TGAT(C/T)(A/C)TGGCTCAG-3'] and 1492-1513 [5'-GTGCTGCAGTACGG(T/C)TACCTTGTTACG-ACTT-3'] of *Escherichia coli*. PCR amplification, purification, and sequencing of the PCR product were performed as previously described (10). The 16S rDNA sequencing for culture AMPB-Zg and strain ANRB-Zg was carried out by Dr. W. Liesack and H. Lüdemann (Max-Planck-Institute für terrestrische Mikrobiologie, Marburg) as previously described (23). The phylogenetic tree for culture AMPB-Zg and strain ANRB-Zg were constructed by H. Lüdemann. The 16S rDNA of the isolates were integrated in an alignment of about 8000 full and partial primary structures using the respective tools of the ARB software (24). Only almost complete sequenced 16S rDNA references were used to calculate the dendrogramm. The phylogenetic trees were constructed from dissimilarity matrices by the neighbor-joining method implemented in the ARB software package. The phylogenetic tree for strain ASRB-Zg was constructed by Dr. H.J.M. Harmsen (Rijksuniversiteit Groningen, Groningen, The Netherlands).

Analytical techniques. Methane and hydrogen were measured as described in Chapter 4. The utilization of acetate and production of acetate and other fatty acids was analyzed on a CP9001 gas chromatograph (Chrompack) equipped with a FID as described in Chapter 5. The accumulation of non-volatile organic acids was analyzed by high pressure liquid chromatography (HPLC) on a Merck-column (Polyspher OA HY). The

mobile phase was 0.01 N H_2SO_4 at a flow of 0.6 ml/min at 60 °C. Anions were analyzed by HPLC as described in Chapter 4.

RESULTS

Isolation and morphological characterization. The methanogenic culture AMPB-Zg was obtained from 1 × 10^8-fold diluted, freshwater sediment inoculated with acetate, and was obtained by repeated application of dilution method. Formation of methane and consumption of acetate was detected in the highest sediment dilutions. Cells of the isolated methanogen were non-motile, rod-shaped with blunted ends (0.5-1 μm wide and 3-4 μm long) (Fig. 1). The purity check with medium containing 0.2% yeast extract showed that AMPB-Zg still contained a contaminating bacterium.

The sulfate-reducing strain ASRB-Zg was obtained from 1 × 10^7-fold diluted sediment inoculated with acetate and sulfate, and was obtained by pasteurizing a full grown culture for 10 min at 80 °C. The isolated sulfate-reducing bacterium was weakly motile, rod-shaped with pointed ends (0.5-0.7 μm wide and 1.5-3 μm long) occurring single or in pairs (Fig. 1). The formation of bright spores was observed occasionally, which were spherical and central. Cells stained Gram negative but Gram positive cells were observed occasionally.

The nitrate-reducing strain ANRB-Zg was obtained from 1 × 10^6-fold diluted sediment inoculated with acetate and nitrate, and was isolated by repeated application of the agar roll-tube-dilution method. The isolated nitrate-reducing bacterium was weakly motile, rod-shaped (0.5-0.7 μm wide and 0.7-1 μm long), and occurring single or in pairs (Fig. 1). Cells stained Gram negative.

Growth and substrate utilization. The culture AMPB-Zg grew on acetate at 20 °C to 40 °C, the optimum temperature for the methanogen was around 30-35 °C. In the presence of acetate methane was produced. The isolate did not grow on H_2/ CO_2 or formate. An average growth yield of 0.75 g cell protein was obtained per mol of acetate consumed. With acetate, doubling times of 5.6–8.1 days were measured at 30 °C.

The optimum growth temperature for strain ASRB-Zg on acetate and sulfate was around 30 °C. No or little growth was observed below 15 °C or above 35 °C. Strain ASRB-Zg used sodium sulfate (10 mM) or sodium thiosulfate (10 mM) as electron acceptors. However, growth was stimulated significantly when $FeSO_4$ was used as electron acceptor. Sulfur and nitrate could not be used as electron acceptor with acetate as electron donor. The sulfate-reducing bacterium did not grow chemolithoautotrophically with H_2 and sulfate as energy substrate and CO_2 as sole carbon source. It grew chemoorganotrophically with a large number of organic compounds (Table 1). All substrates were oxidized completely to CO_2. The complete oxidation of acetate (19 mM) led to the consumption of 19 mM sulfate. With sulfate, an average growth yield of 2.6 g cell protein was obtained per mol of acetate consumed. With acetate, doubling times of 3.9–5.3 days were measured at 30 °C.

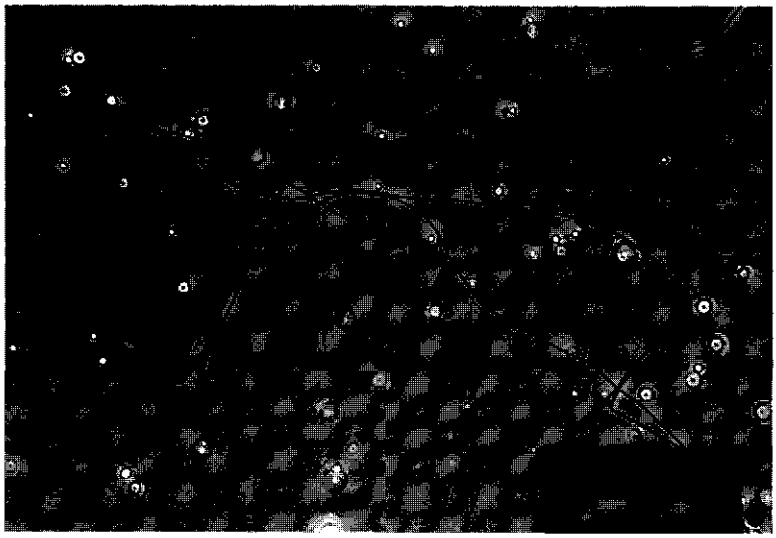

Figure 1a. Phase contrast photomicrograph of culture AMPB-Zg, grown on acetate. *Bar* 10μM.

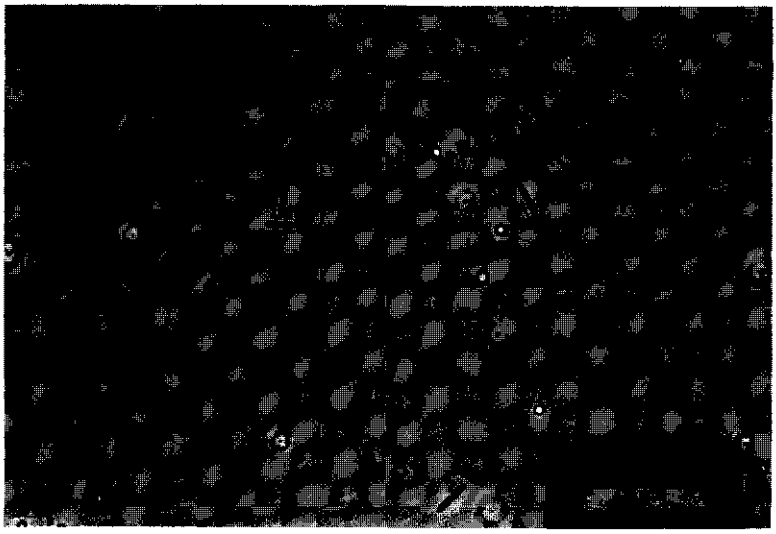

Figure 1b: Phase contrast photomicrograph of strain ASRB-Zg, grown on acetate and sulfate. *Bar* 10μM.

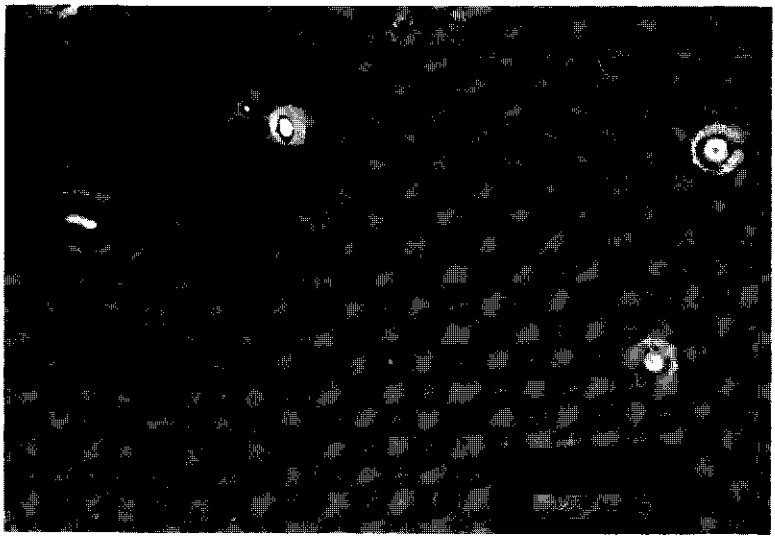

Figure 1c. Phase contrast photomicrograph of strain ANRB-Zg, grown on acetate and nitrate. *Bar* 10μM.

Table 1. Organic compounds tested as electron donors and carbon sources for strain ASRB-Z in the presence of 10 mM sulfate. The substrate concentrations are given in mM in parentheses.

Utilized:
Formate (10), acetate (10), butyrate (10), iso-butyrate (10), methanol (10), ethanol (10), succinate (10), fumarate (10), benzoate (10)
Tested, but not utilized:
H_2-CO_2 (80:20), lactate (10), propanol (10), iso-propanol (10), butanol (10), propionate (10), valerate (10), glucose (10), fructose (10), xylose (10)
Tested, but not utilized in the absence of sulfate:
Ethanol (10), lactate (10), pyruvate (10)

Strain ANRB-Zg grew on acetate and nitrate at 4 °C to 30 °C, the optimum temperature for the nitrate-reducing bacterium was around 20-25 °C. In the presence of acetate, strain ANRB-Zg used nitrate (10 mM) or oxygen. Sulfur, thiosulfate and sulfate could not be used as electron acceptor with acetate as electron donor.

In the presence of acetate and nitrate, thiosulfate (5 mM) was oxidized to sulfate (5 mM) showing that thiosulfate was a suitable electron donor for the isolate. No growth was observed in the presence of thiosulfate and nitrate alone. The nitrate-reducing bacterium did not grow chemolithoautotrophically with H_2 and nitrate as energy substrate and CO_2 as sole carbon source, but it was able to grow chemoorganoheterotrophically with a large number of organic compounds (Table 2). All substrates were oxidized completely to CO_2, unless stated otherwise. The complete oxidation of acetate (17 mM) led to the consumption of 17 mM nitrate.

Phylogenetic analysis.

Comparative 16S rRNA sequence analysis revealed a relationship of culture AMPB-Zg to *Methanosaeta concilii* (sequence similarity 99%). The phylogenetic relationships of culture AMPB-Zg derived from 16S rRNA sequence analysis are depicted in Fig. 2. Strain ASRB-Zg displayed after sequence analysis (ASRB-Zg fragment: 598 bp) a relationship to *Desulfotomaculum nigrificans* and *Desulfotomaculum thermosapovorans* (sequence similarity 59.7% and 62.7%, respectively). The phylogenetic tree depicted in Fig. 3 reflects the phylogenetic relationship of strain ASRB-Zg to its next relatives. Comparative 16S rRNA sequence analysis revealed a relationship of strain ANRB-Zg with *Variovorax paradoxus*. The phylogenetic relationships of strain ANRB-Zg derived from 16S rRNA sequence analysis are depicted in Fig. 4.

Table 2. Organic compounds tested as electron donors and carbon sources for strain ANRB-Z in the presence of 10 mM nitrate. The substrate concentrations are given in mM in parentheses.

Utilized:
Formate (10), acetate (10), propionate (10), butyrate (10), lactate (10), methanol (10), ethanol (10), propanol (10), glucose (10), fructose (10)
Tested, but not utilized:
H_2-CO_2 (80:20), xylose (10)
Tested, and utilized in the absence of nitrate:
glucose (10)), fructose (10)
Tested, but not utilized in the absence of nitrate:
Ethanol (10), pyruvate (10)

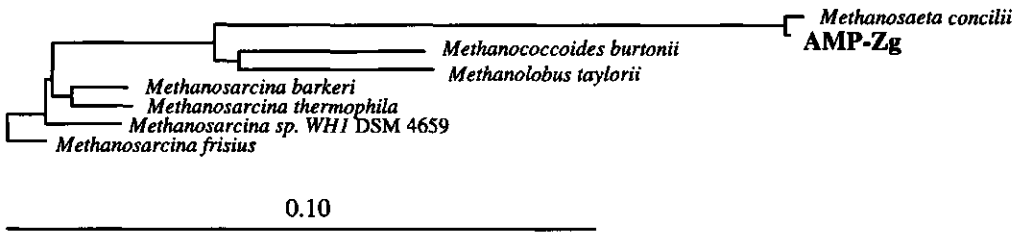

Figure 2. Phylogenetic tree inferred from 16S rRNA sequence data, showing the relationship of the methanogenic culture AMPB-Zg to its closest relatives. Distance matrices were constructed from aligned sequences and corrected for multiple base changes at single positions by the method of Jukes and Cantor (25), and a phylogenetic tree was constructed by the neighbor-joining method of Saitou and Nei (26) by using the ARB software package (24). The scale bar represents 10 nucleotide substitutions per 100 nucleotides. All nucleotide positions were used for the construction of the phylogenetic tree.

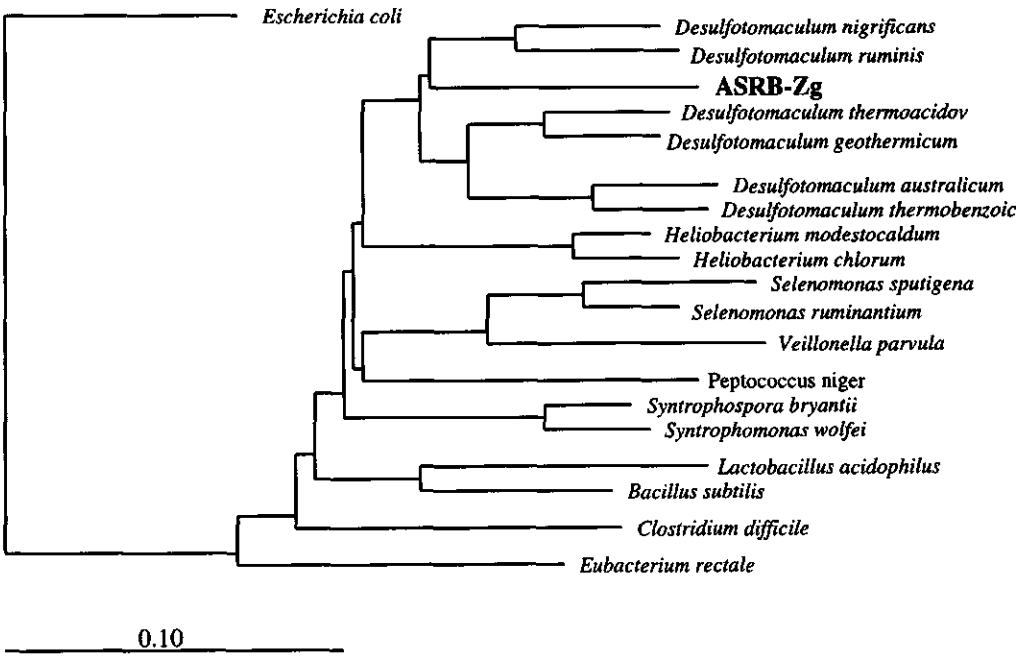

Figure 3. Phylogenetic tree inferred from 16S rRNA sequence data, showing the relationship of the isolated sulfate-reducing bacterium ASRB-Zg to its closest relatives. Distance matrices were constructed from aligned sequences and corrected for multiple base changes at single positions by the method of Jukes and Cantor (25), and a phylogenetic tree was constructed by the neighbor-joining method of Saitou and Nei (26) by using the ARB software package (24). The scale bar represents 10 nucleotide substitutions per 100 nucleotides.

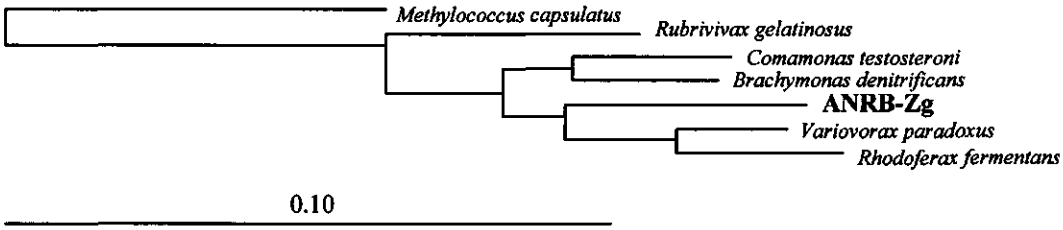

Figure 4. Phylogenetic tree inferred from 16S rRNA sequence data, showing the relationship of the isolated nitrate-reducing bacterium ANRB-Zg and cultured members of the beta subclass of proteobacteria. Distance matrices were constructed from aligned sequences and corrected for multiple base changes at single positions by the method of Jukes and Cantor (25), and a phylogenetic tree was constructed by the neighbor-joining method of Saitou and Nei (26) by using the ARB software package (24). The scale bar represents 10 nucleotide substitutions per 100 nucleotides. Only nucleotide positions which are in more than 50 % of full sequenced beta proteobacteria in the database of the ARB software package were used to calculate the phylogenetic tree. *Metylococcus capsulatus* was used to root the tree.

DISCUSSION

Acetate was shown to be an important intermediate in the degradation of organic matter in a freshwater sediment (Chapter 5). It became clear that methanogens and sulfate reducers were competing for the available acetate. However, nitrate-reducing bacteria hardly competed with methanogens and sulfate reducers for the available acetate when sufficient nitrate was present (Chapter 6). To get insight into the importance of these inorganic electron acceptors on aceticlastic methanogenesis *in situ*, the different groups of microorganisms involved in the acetate metabolism in the freshwater sediment were quantified (Chapter 6). The dominant microorganisms were acetate-utilizing methanogens (2×10^8 cells cm^{-3} sediment) and sulfate reducers (2×10^8 cells cm^{-3} sediment). Acetate-utilizing nitrate reducers (5×10^5 cells cm^{-3} sediment) were clearly outnumbered by the methanogens and sulfate reducers. These results indicate that acetate-utilizing nitrate reducers indeed play a minor role in the degradation of acetate in the

sediment. The acetate-utilizing anaerobes obtained from the dilution series are described below and their properties are summarized in Table 3.

Table 3. Properties of the isolated acetate-utilizing anaerobes.

Species	Morphology	Width (μm)	Length (μm)	Gram staining	Spores	Temperature optimum (°C)	Oxidation of organic substrates
AMPB-Zg	Rod	0.5-1	3-4	ND	NO	30-35	
ASRB-Zg	Rod	0.5-0.7	1.5-3	+[a]	+	30	Complete
ANRB-Zg	Rod	0.5-0.7	0.7-1	-	NO	20-25	Complete

ND, not determined.

NO, not observed

Symbols: +, positive; +[a], positive and negative; -, negative.

The aceticlastic methanogen, culture AMPB-Zg, grew only on acetate, which is the characteristic property for all *Methanosaeta* species (8). On the basis of the 16S rRNA sequence analysis, it became clear that the isolate is closely related if not identical to *Methanosaeta concilii*. Other acetate-utilizing methanogens are *Methanosarcina* species but these were only observed in the lower dilutions of the serial dilution ($<10^{-3}$; based on morphology and autofluorescence). The isolation of culture AMPB-Zg from freshwater sediment using the highest positive dilution of a serial dilution on acetate strongly indicates that culture AMPB-Zg is the most abundant acetate-degrading methanogen in this sediment. It was shown that *Methanosaeta* species have an advantage over *Methanosarcina* species in ecosystems with acetate concentrations below 1 mM (27). In the sediment, in situ acetate concentrations were in general below <20 μM. This observation supports the assumption that culture AMPB-Zg was indeed the major acetate-utilizing methanogen in the sediment.

Strain ASRB-Zg is a sulfate-reducing bacterium which forms heat-resistent endospores. The isolate grew on a variety of organic compounds that are formed during anaerobic degradation of organic matter, such as acetate, butyrate and alcohols. From analysis of the 16S rRNA sequence it became clear that strain ASRB-Zg is closely related to *Desulfotomaculum* species. The specific growth rate of ASRB-Zg (μ_{max} = 0.13-0.18 day^{-1}) is slightly higher than that of AMPB-Zg (μ_{max} = 0.09-0.12 day^{-1}). Strain ASRB-Zg and AMPB-Zg are the most abundant acetate-degrading microorganisms in the sediment and both organisms are probably competing for the available acetate when sufficient sulfate is present. Strain ASRB-Zg is a generalist and it is possible that acetate degradation is not the only activity of the strain in the sediment. It might prefer other substrates than acetate. This ability to use other substrates besides acetate can give strain ASRB-Zg a

competitive advantage over the methanogen under conditions of high sulfate concentrations. However, at low sulfate concentrations versatile acetate degrading sulfate reducers may prefer other substrates than acetate (17). Unfortunately, no studies were done to elucidate the outcome of competition for acetate between methanogens and sulfate reducers and the influence of mixed substrate utilization on this competition.

The nitrate-reducing bacterium, strain ANRB-Zg, is a facultative anaerobe which grows chemoorganotrophically. The physiological property of growth on reduced sulfur compound like thiosulfate in the presence of nitrate is a common feature of colorless sulfur bacteria (28). In an earlier study, we described that the addition of acetate and nitrate to sediment from Zegveld led to the oxidation of reduced sulfur compounds to sulfate. The isolation of an acetate-utilizing nitrate reducer which is capable of oxidizing thiosulfate to sulfate supports these observations. Strain ANRB-Zg might be involved in the oxidation of reduced sulfur compounds to sulfate in the sediment. However, analysis of the 16S rRNA sequence revealed that strain ANRB-Zg is related to the genera *Variovorax*. It is not known if these bacteria are capable of growth on reduced sulfur compounds. At the moment too little information is available to understand the role of strain ANRB-Zg in the sulfur and carbon cycle of the sediment.

ACKNOWLEDGMENTS

This study is subsidized by a grant from the Netherlands Organization for Scientific Research (NWO). We thank W.A. Akkermans-van Vliet for her help with sequencing and H.J.M. Harmsen for constructing the dendrogram for strain ASRB-Zg. We also like to thank W. Liesack and H. Lüdemann for sequencing and constructing the dendrograms for strains AMPB-Zg and ANRB-Zg.

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CHAPTER 8

ENRICHMENT OF METHANOGENIC AND SULFATE-REDUCING COMMUNITIES FROM A FRESHWATER SEDIMENT IN ACETATE LIMITED CHEMOSTATS

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ABSTRACT

The conversion of acetate by methanogenic and sulfidogenic communities under acetate-limited conditions was studied in the chemostat. Label studies with [2-^{13}C] acetate showed that aceticlastic methanogens were the dominant acetate-utilizers in the methanogenic chemostat. After 347 days of operation the community in the methanogenic chemostat consisted mainly of aceticlastic methanogens (9×10^6 cells ml^{-1}) and homoacetogenic bacteria (2×10^6 cells ml^{-1}). The presence of homoacetogens is explained by assuming that these bacteria were feeding on excretion or hydrolysis products of acetate-degrading microorganisms. The composition of the sulfate-reducing community was more complex containing homoacetogenic bacteria (5×10^5 cells ml^{-1}), H_2-utilizing sulfate reducing bacteria (3×10^7 cells ml^{-1}), aceticlastic methanogens (5×10^4 cells ml^{-1}) and sulfate reducers (3×10^7 cells ml^{-1}). Acetate-utilizing methanogens were able to compete efficiently with the sulfate reducers for the available acetate. The presence of homoacetogenic and H_2-utilizing sulfate-reducing bacteria suggested the syntrophic degradation of acetate. Unfortunately, the labelling experiment was not suited to elucidate if acetate was oxidized by a sulfidogenic syntrophic consortium or a single sulfate-reducing bacterium.

INTRODUCTION

Acetate is an important metabolite in anoxic freshwater sediments (1). In methanogenic habitats acetate is cleaved by methanogens to CH_4 and CO_2. In the presence of sulfate, sulfate-reducing bacteria are able to compete with acetate-utilizing methanogens for the available acetate. When two types of microorganisms are competing for the same growth-limiting substrate and no other interactions occur, the result of the competition can be predicted from the relation between their specific growth rates and the concentration of the growth-limiting substrate (2). Table 1 (Chapter 2) shows the physiological properties of some acetate-utilizing methanogens and sulfate reducers. The influence of kinetic parameters (μ_m, K_s and threshold) on the growth rate of *Methanosaeta* sp., *Methanosarcina* sp., *Desulfobacca acetoxidans* and *Desulforhabdus amnigenus* at different substrate concentrations is depicted in Fig. 4 (Chapter 2). In general, sulfate reducers have a higher affinity for acetate and a lower threshold concentration of acetate compared with aceticlastic methanogens (Chapter 2). This lower threshold concentration for sulfate reducers can be explained thermodynamically. A threshold concentration exists below which the overall change in free energy of the degradation reaction is too low to couple it to the formation of metabolic energy (3-5). Because the energy yield of acetate conversion is higher for sulfate reducers than for methanogens, sulfate reducers are able to carry out the degradation of acetate at lower concentrations. From Fig 4. (Chapter 2) it is clear that the sulfate reducer *Desulfobacca acetoxidans* (specialist) is preferred in environments with acetate concentrations below 4 mM and where sulfate is not limiting.

However, *Methanosarcina* should be favored in the ecosystem when acetate concentrations are above 4 mM. The kinetic properties of *Methanosaeta* sp. are slightly better than those of the generalist *Desulforhabdus amnigenus*. On the basis of these parameters one would expect that *Methanosaeta* sp. outcompete the sulfate reducer (see Chapter 2, Table 3 and Fig. 4). However, the generalist *D. amnigenus* outcompeted acetate-degrading methanogens in a bioreactor treating complex wastewater. Oude Elferink et al. mentioned that mixed substrate utilization by generalists might play a role in the competition for acetate (6). Thus, the ability to use other substrates besides acetate gives *D. amnigenus* a competitive advantage over *Methanosaeta* sp. This shows that differences in the kinetic parameters can only partly explain the competition between sulfate-reducing bacteria and methanogens.

Recently, we have described studies with samples of a freshwater sediment in which ^{13}C-labelled acetate was used to investigate the fate of acetate under different redox conditions (7). Together with most-probable-number (MPN) counts it became clear that aceticlastic methanogens and sulfate-reducing bacteria were responsible for the consumption of acetate. The objective of this study was to get insight into the conversion of acetate by methanogenic and sulfidogenic communities under acetate-limiting conditions in the chemostat. We used ^{13}C-labelled substrates to investigate the fate of acetate in these enrichments. In addition, we quantified the different groups of microorganisms present in the two continuous cultures.

MATERIALS AND METHODS

Source of inoculum for the chemostats. Freshwater sediment was collected on the 6 th of June 1995 from a ditch of a peat grassland near Zegveld (Province of Utrecht, The Netherlands). The sediment surface of the sampling site was overlaid with 30 cm of water. The temperature of the sediment was 13 °C and that of the water 17 °C. Sampling of the sediment was done with a sediment corer (acrylic glass tubes, 50 cm in length and 6.5 cm I.D.). After transport the cores were stored at 10 °C. After 10 days the sediment was processed further.

Media composition. A basal bicarbonate buffered medium with a composition as described by Hüser et al. was used (8). To one litre of medium 1 ml of a vitamin solution (9) and 1 ml of an acid and an alkaline trace element solution were added (10). The vitamin solution was filter sterilized separately. The gas phase above the medium was N_2/CO_2 (80%/20%) and the pH of the medium was 6.8-6.9. Acetate and sulfate were added from 1-M heat-sterilized stock solutions. In the most probable number (MPN) countings and batch cultures the bacteria were cultivated in 120-ml serum bottles containing 50 ml medium. For the continuous cultivation culture experiments the same medium was used.

Continuous cultivation culture experiments. Two 2000-ml chemostats (Applikon Dependable Instruments b.v., Schiedam, The Netherlands) were inoculated with 500 ml of sediment. Continuous cultivation was performed at 20 °C in the dark. The working volume was 1000 ml. Both continuous cultures were run at a

dilution rate of 0.0036 ± 0.0003 h^{-1}. The dilution rate was 80% of the maximum growth rate, estimated from the acetate consumption rate in the sediment, in separate batch experiments. One chemostat was run under methanogenic conditions and the other under sulfate-reducing conditions. The acetate concentration was 3 mM. In the sulfate-reducing fermentor the sulfate concentration was 5 mM. The pH was not controlled but checked during the cultivation, it varied between 6.8 and 7.1. A continuous stream of N_2/CO_2 (80%/20%) at a flow of 100 ml per hour was led over the cultures. The cultures were stirred at 50 rpm.

Quantification of functional groups of bacteria. Samples were taken aseptically from the two chemostats after 8 and 30 volume changes. From each sample 15 ml was transferred to a 250-ml serum bottle containing 135 ml of medium. After mixing the bottle for 5 min, the sample (15 ml) was serially diluted to the 10^{-10} dilution. A three-tube MPN series was prepared by transferring 5.0-ml samples to 120-ml serum bottles containing 45 ml of medium. The tubes were sealed with butyl rubber stoppers and aluminum caps. The MPN-tests for acetate-utilizing bacteria were performed with 10 mmol/l acetate with or without sulfate (10 mmol/l). Hydrogen-consuming bacteria were enumerated in bottles containing medium with or without sulfate (10 mmol/l) under H_2/CO_2 (80%/20%). Incubation was carried out in the dark at 20 °C. Growth was judged from turbidity. Tubes were checked weekly and final scores were determined in three replicates after 6-12 months of incubation. In positive tubes the concentrations of substrates and products were determined. Routinely, growth in the highest positive dilution was checked by transfer to fresh medium. Most probable numbers, deviance and 95% confidence intervals were determined using a Basic computer program described by Hurley and Roscoe (11). The populations were expressed as cells per ml of chemostat content. Direct counts were performed with a Bürker Türk counting chamber using a phase contrast microscope.

Determination of dry weight of single cells. Bacterial dry mass was determined in batch cultures for the aceticlastic methanogen and sulfate-reducing bacterium obtained from the highest positive dilution of the different MPN series. Bacterial growth was followed by protein determination. Cell pellets of 6 ml cultures were resuspended in 1 ml 0.5 M NaOH. After heating at 100 °C for 14 min the samples were treated further according to the method of Bradford (13). Bovine serum albumin was used as a standard. The specific cell mass ($m_{cell\ dw}$: dry weight × $cell^{-1}$) of both microorganisms was calculated by dividing the total cell mass by the number of cells. The number of cells was determined by direct counting.

Calculation of maintenance coefficients based on acetate consumption rates during growth in chemostats. The substrate consumption rate (r_s: mol × l^{-1} × h^{-1}) shows a linear relation (equation 1) with the biomass production rate (r_x: g × l^{-1} × h^{-1}) and the biomass (B_x: g × l^{-1}).

$$r_s = r_x/Y_{xs} + m_s \times B_x \qquad (1)$$

With yield value Y_{xs} (g × mol^{-1} substrate) and the substrate maintenance coefficient m_s (mol substrate × g $biomass^{-1}$ × h^{-1}) (13). The maintenance coefficient is assumed to be growth rate independent (14). The substrate consumption rate (r_s) can be obtained from the influent concentration of the substrate (C_{So}: mol × l^{-1}), effluent concentration (C_{Si}: mol × l^{-1}) and dilution rate (D: h^{-1}). This can be written as:

$$r_s = (C_{So}-C_{Si}) \times D \qquad (2)$$

Growth rate in the chemostat is equal to the dilution rate. Furthermore, the growth can be expressed with the specific growth rate μ. Thus, the biomass production rate can be expressed as:

$$r_x = \mu \times B_x = D \times B_x \quad (3)$$

The biomass can be written as:

$$B_x = N \times m_{cell\ dw} \quad (4)$$

Here, is N the number of cells (cells $\times$ l^{-1}) and $m_{cell\ dw}$ the specific cell mass (g dw $\times$ l^{-1}). Using equations (2), (3), and (4), equation (1) can be rewritten as:

$$m_s = D \times ((C_{So}-C_{Si}/ N \times m_{cell\ dw})-(1/Y_{xs})) \quad (5)$$

Y_{xs} values for the acetate-utilizing sulfate reducers and methanogens isolated from the chemostats were determined in batch incubations (Table 3). The number of cells (N) are obtained by MPN-incubations and the specific cell mass ($m_{cell\ dw}$) was known (see above). The maintenance coefficient (m_s: mol $\times$ g dw^{-1} $\times$ h^{-1}) is used to calculate the Gibbs free energy for maintenance m_e (J $\times$ g dw^{-1} $\times$ h^{-1}) according to (15,16):

$$m_e = m_s \times \Delta G^{0'} \quad (6)$$

With $\Delta G^{0'}$ the Gibbs energy of the acetate mineralization reaction under methanogenic (MP) and sulfate-reducing (SR) conditions. $\Delta G^{0'}$ values were calculated as -31.0 J $\times$ $mmol^{-1}$ (MP) and -47.3 J $\times$ $mmol^{-1}$ (SR) (17).

Labelling experiments. All handlings were done under anaerobic conditions. From the methanogenic chemostat 600 ml was taken after 33 volume changes. The sample was centrifuged at 13,000 rpm and the pellet was resuspended in 20 ml of medium. An incubation series in duplicate was prepared by transferring 5.0-ml samples to 120-ml serum bottles containing 45 ml of medium. The incubations were performed with 10 mmol/l unlabelled and labelled acetate ([2-^{13}C]-acetate) in combination with unlabelled and labelled bicarbonate (40 mM). Controls with a pure culture of *Methanosaeta concilii* GP6 (DSM 3671) and sterile media were included. The bottles with the chemostat enrichment and *Methanosaeta concilii* GP6 were incubated at 20 and 37 °C, respectively. Gas samples were taken by syringe from the headspace and analyzed by GC-MS for the accumulation of labelled and unlabelled CH_4. For analysis of the consumption of labeled and unlabelled acetate liquid samples were taken and centrifuged at 13,000 rpm. Supernatants were stored at -20 °C and analyzed later by gas chromatography.

Sequence analysis. Nucleic acids from an acetate-utilizing sulfate reducer isolated by sonification followed by phenol extraction and ethanol precipitation as previously described (see Chapter 7). PCR amplification, purification, and sequencing of the PCR product were performed as previously described (see Chapter 7). DNA sequencing for an acetate-utilizing methanogen was carried out by Dr. W. Liesack (Max-Planck-Institute für terrestrische Mikrobiologie, Marburg) (see Chapter 7).

Analytical techniques. Determination of ^{13}C-acetate, methane and carbon dioxide was carried out by GC-mass-selective detection (GC-MS) as described in chapter 6. Samples for acetate (*m/z* 61) determination were acidified with formic acid (Suprapur; Merck) with *m/z* 74 propionate as internal standard (final concentrati-

on: 100 μM) prior to injection into the apparatus. Acetic acid and its stable isotopes were monitored at *m/z* 60 to 62. Total acetate was analyzed on a CP9001 gas chromatograph (Chrompack) equipped with a FID as described in Chapter 4. Methane and carbon dioxide and their stable isotopes were monitored at *m/z* 16, 17, 44 and 45, respectively. Total methane was measured on a 417 Packard chromatograph equipped with a flame ionization detector (FID) and a molecular sieve 5A column (110 cm long by 2.1 mm [internal diameter (i.d.)], Chrompack). The column temperature was 70 °C and the carrier gas was nitrogen at a flow rate of 20 ml/min.

RESULTS

Continuous culture experiments. Degradation of acetate occurred in the chemostats in the presence and absence of sulfate (Fig. 1). Following the acetate concentration in the continuously fed methanogenic chemostat, more than 98% of the incoming acetate was degraded after 50 days of operation. The acetate concentration in the chemostat was below the detection limit (<50 μM). In the sulfate-reducing chemostat a similar pattern was observed, but the acetate concentration did not decrease as fast as in the methanogenic chemostat. It took more than 150 days (13 volume changes) before more than 98% of the incoming acetate was also degraded. When the acetate concentration in the chemostat was below the detection limit about 50% of the incoming sulfate was consumed. In the sulfate-reducing chemostat anaerobic flagellates were observed after 121 days of operation. This led to a decrease in the number of microorganisms ($< 10^4$ cells $\times$ ml^{-1}) in the chemostat (direct count). On the 238th day (20 volume changes) both the acetate and sulfate concentration increased in the sulfate-reducing chemostat due to a pump failure, and bacteria and flagellates were washed out. However, after another 62 days (5 volume changes) the culture functioned as before but the flagellates were not observed any longer.

Quantification of methanogenic, sulfate reducing and acetogenic bacteria. Methanogenic, sulfate-reducing and acetogenic bacteria which used acetate or H_2/CO_2 as substrates were quantified after 8 and 30 volume changes (92 and 347 days) (Table 1). After 8 volume changes there were no obvious differences in the community composition in the two chemostats. However, differences became clear after 30 volume changes. In the methanogenic chemostat aceticlastic methanogens were most abundant in the microbial community. Relative high numbers of acetogenic bacteria were counted, and a small population of acetate-utilizing sulfate reducers was still present. H_2-utilizing methanogens were not detected in the chemostat samples obtained after 8 and 30 volume changes. Acetate-utilizing sulfate-reducing bacteria were present in high numbers in the sulfate-reducing chemostat. Also, a dominant group of H_2-utilizing sulfate reducers and acetogenic bacteria was present. Only a small population of aceticlastic methanogens remained present in the chemostat. The cell shapes of the acetate-utilizing sulfate-reducing (oval), H_2-utilizing sulfate-reducing (vibrio's) and acetogenic (rods) bacteria were sufficiently distinct to identify them as separate groups. In all

cases the direct counts were in the same order of magnitude as the results obtained with the MPN-counts (Table 1).

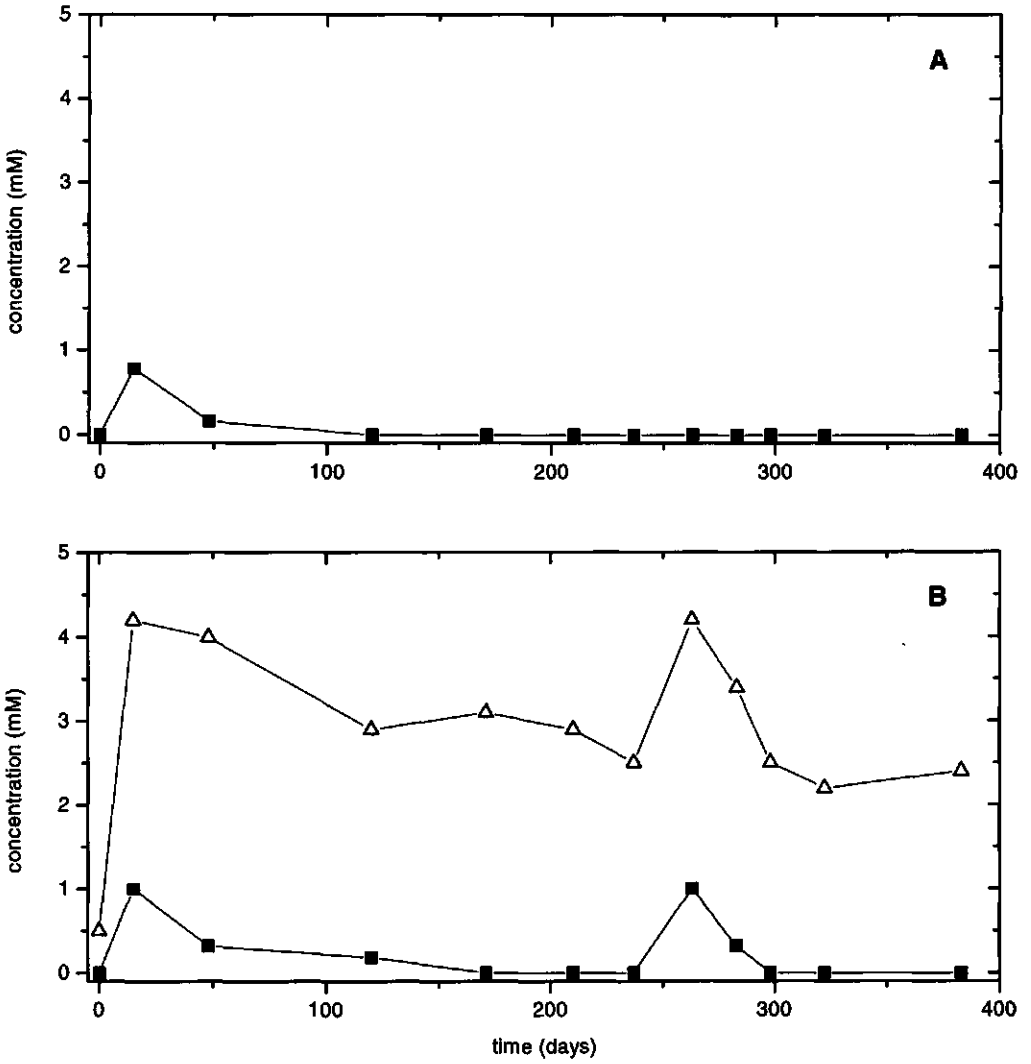

Figure 1. The consumption of acetate in chemostats operated at 20 °C in the absence (A) and presence of sulfate (B). Symbols: □: acetate and Δ: sulfate.

Table 1. Most probable number estimates of microorganisms growing in acetate-limited chemostats[a].

volume changes in chemostat	methanogens		sulfate reducers		Acetogenic bacteria	Total count	Direct count
	H_2-utilizing	acetate-utilizing	H_2-utilizing	acetate-utilizing			
Methanogenic							
8	n.d.[b]	9×10^6 (3-43)	2×10^5 (1-8)	3×10^5 (1-10)	5×10^6 (2-28)	1.5×10^7	1×10^7
30	n.d.	9×10^6 (3-43)	n.d.	3×10^3 (1-10)	2×10^6 (1-8)	1.1×10^7	9×10^6
Sulfate-reducing							
8	n.d.	9×10^6 (3-43)	5×10^5 (2-25)	3×10^5 (1-10)	5×10^4 (2-25)	9.9×10^6	1×10^7
30	n.d.	5×10^4 (2-26)	3×10^7 (1-10)	5×10^6 (2-28)	5×10^5 (2-25)	3.6×10^7	1×10^7

[a]Estimates in number of cells per ml (95% confidence interval).
[b]Not detected.

Table 2. The calculated maintenance coefficients (m_s and m_e) of the acetate-utilizing methanogens and sulfate reducing bacteria based up on acetate consumption rates.

microorganism	C_0 (mM)	C_i (mM)	D (h^{-1})	$mass_{cell\ dw}$ (pg)	Y_{batch} ($g \times mol^{-1}$)	N ($cells \times l^{-1}$)	m_s ($mmol \times g\ dw^{-1} \times h^{-1}$)	m_e ($J \times g\ dw^{-1} \times h^{-1}$)
aceticlastic methanogens	3.0	0.05	3.6×10^{-3}	0.20	1.5	9×10^{9}	3.5	109
aceticlastic sulfate reducers	3.1	0.1	3.8×10^{-3}	0.24	4.4	5×10^{9}	8.6	405

Determination of dry weight of single cells. The dry weight of a single cell and the yield for the different microorganisms determined in batch cultures are given in Table 2. For the calculation of the specific cell mass the assumption was used that 1 g of dry cells corresponds to 0.5 g of protein. The growth yield was determined from the total protein content of the culture. Also the observed yield of the acetate-utilizing methanogens and sulfate-reducing bacteria in the chemostats was calculated (Table 2).

Calculation of maintenance coefficient based on acetate consumption rates. The calculated maintenance coefficient m_s and the Gibbs energy for maintenance m_e of the acetate-utilizing methanogens and sulfate reducing bacteria based on acetate consumption rates are given in Table 2. The acetate maintenance coefficient for the methanogens is about 2 times lower than that of the sulfate reducers. Also the energy based maintenance coefficient of the acetate-utilizing methanogens was lower.

Labelling experiments. The fates of the methyl group of ^{12}C- and ^{13}C-acetate in the different incubations in combination with labelled and unlabelled bicarbonate are shown in Table 3. In the methanogenic enrichment culture the observed label distribution in CH_4 was in agreement with the distribution one would expect when aceticlastic methanogens are responsible for the consumption of acetate. This was confirmed by the distribution of the label in the *Methanosaeta concilii* GP6 incubations (Table 4). The amount of ^{13}C-CH_4 produced in the incubations with 2-^{12}C-acetate and ^{12}C-HCO_3^- was 1.3±0.2% of the total amount of CH_4 formed (data not shown). This was somewhat higher in the incubations with 2-^{12}C-acetate and ^{13}C-HCO_3^- where 2.1±0.2% consisted of ^{13}C-CH_4. The average isotope recovery in the duplicate bottles of the samples was above 90%.

Table 3. Fate of $^{12/13}$C-acetate (10 mM) and bicarbonate (40 mM) in a pure culture of *Methanosaeta concilii* GP6 and a methanogenic acetate-degrading enrichment culture.

Substrate combination	$^{12}CH_3COO^-$ (mM)	$^{13}CH_3COO^-$ (mM)	$^{12}CH_4$ (mM)	$^{13}CH_4$ (mM)
Methanosaeta concilii GP6				
$^{12}CH_3COO^- + H^{12}CO_3^-$	6.7 ± 0.6	-	5.9 ± 0.1	-
$^{12}CH_3COO^- + H^{13}CO_3^-$	7.2 ± 0.6	-	6.5 ± 0.3	-
$^{13}CH_3COO^- + H^{12}CO_3^-$	-	14.2 ± 0.3	-	14.1 ± 0.1
$^{13}CH_3COO^- + H^{13}CO_3^-$	-	12.2 ± 0.7	-	12.8 ± 0.7
Chemostat culture				
$^{12}CH_3COO^- + H^{12}CO_3^-$	11.6 ± 0.4	-	10.7 ± 0.2	-
$^{12}CH_3COO^- + H^{13}CO_3^-$	8.5 ± 0.4	-	7.4 ± 0.3	-
$^{13}CH_3COO^- + H^{12}CO_3^-$	-	13.1 ± 0.1	-	12.4 ± 0.1
$^{13}CH_3COO^- + H^{13}CO_3^-$	-	11.1 ± 0.3	-	11.5 ± 0.2

Table 4. Fate of $^{12/13}C$-acetate (10 mM) and bicarbonate (40 mM) in a culture of an aceticlastic methanogen and an acetate-degrading syntrophic culture.

Substrate combination	$^{12}CH_4$ (mM)	$^{13}CH_4$ (mM)	$^{12}CO_2$ (mM)	$^{13}CO_2$ (mM)
Aceticlastic methanogen				
$^{12}CH_3COO^- + H^{12}CO_3^-$	10	-	50	-
$^{12}CH_3COO^- + H^{13}CO_3^-$	10	-	10	40
$^{13}CH_3COO^- + H^{12}CO_3^-$	-	10	50	-
$^{13}CH_3COO^- + H^{13}CO_3^-$	-	10	10	40
Syntrophic culture				
$^{12}CH_3COO^- + H^{12}CO_3^-$	10	-	50	-
$^{12}CH_3COO^- + H^{13}CO_3^-$	-	10	10	40
$^{13}CH_3COO^- + H^{12}CO_3^-$	10	-	50	-
$^{13}CH_3COO^- + H^{13}CO_3^-$	-	10	10	40

Sequence analysis. Comparative 16S rRNA sequence analysis of an acetate-utilizing methanogen and sulfate reducers revealed a relationship with strain AMPB-Zg (fragment: 350 bp; sequence similarity of PCR products: 95%) and strain ASRB-Zg (fragment: 400 bp; sequence similarity of PCR products: 97%) (see Chapter 7). The acetate-utilizing methanogen was obtained from a 1×10^4-fold diluted, sulfidogenic chemostat sample (after 30 volume changes) inoculated with acetate. The sulfate reducer was obtained from the same chemostat but from a 1×10^5-fold dilution inoculated with acetate and sulfate.

DISCUSSION

To investigate if the fate of acetate in a freshwater sediment can be predicted by Monod kinetics the microbial population enriched in acetate-limited chemostats operated under methanogenic and sulfate-reducing conditions was examined. In the methanogenic continuous culture the community consisted mainly of methanogens and acetogens. While the sulfate-reducing community contained methanogens, sulfate reducing and acetogenic bacteria. The role of homoacetogenic bacteria in acetate degradation is not clear. Other researchers described methanogenesis from acetate in enrichment cultures in which the presence of two or three organisms rather than a single aceticlastic methanogen was required. Zinder and Koch obtained such a culture; acetate was oxidized to H_2 and CO_2 by one organism, while H_2 was subsequently used by a H_2-utilizing methanogen to reduce CO_2 to CH_4 [18]. It was shown that the acetate-oxidizing bacterium was a homoacetogen. To get evidence for this metabolic interaction ^{13}C-labelling studies were done with material

from the methanogenic chemostat. These label studies showed that syntrophic oxidation of acetate is not of quantitative importance. This is further confirmed by the absence of H_2-utilizing methanogens in the chemostat. The presence of H_2-utilizing methanogens is essential for syntrophic acetate oxidation. The presence of homoacetogenic bacteria can not be explained by this metabolic interaction. Therefore, it is likely to assume that these bacteria are feeding on excretion or hydrolysis products of acetate-degrading microorganisms.

The population obtained in the sulfate-reducing chemostat was different than expected. Next to acetate-utilizing sulfate-reducing bacteria also aceticlastic methanogens and homoacetogens were present. Our results showed that the methanogens were able to compete efficiently with the sulfate reducers for the available acetate. This in contrast with results obtained in short-term incubations where acetate-utilizing methanogens were outcompeted by the sulfate reducers for the available acetate (Chapter 5 and 6). Previous studies have shown that acetate is mainly consumed by sulfate reducers when sufficient sulfate is present (19-21). It became clear that sulfate reducers were outcompeting the methanogens for the available acetate. In general, sulfate reducers conserve more per mole of acetate and have better enzyme and growth kinetic properties than methanogens (22,23). A simulation of the competition between methanogens and sulfate reducers in bioreactors revealed that the outcome can be predicted by Monod kinetics (24). The simulation model included affinities for acetate, and sulfate, decay rates and growth yields. Affinities for acetate, growth rates and decay rates for acetate-utilizing methanogens and sulfate reducers appeared to be in the same range. However, the biomass yield on acetate was two times higher for sulfate reducers than for methanogens. A small difference in the growth rate between sulfate reducers and methanogens, resulted in a very long time before methanogens were outcompeted by sulfate reducers. This may explain why aceticlastic methanogens were still present in the sulfidogenic chemostat. Additionally, it suggests that the methanogens and sulfate reducers have similar kinetic properties and acetate affinities. Sequence analysis revealed that the dominant acetate-utilizing methanogen and sulfate reducer in the sulfidogenic chemostat were closely related or identical strains as strain AMPB-Zg and strain ASRB which were isolated from the sediment (Chapter 7). The two isolated strains, strain AMPB-Zg and strain ASRB, are therefore suitable to study the competition between acetate-degrading methanogens and sulfate reducers in new chemostat experiments. Our results also showed that H_2-utilizing sulfate reducers and homoacetogenic bacteria were a significant part of this community. Galouchko and Rozanova described an acetate-oxidizing syntrophic association which consisted of an acetogenic bacterium and a H_2-utilizing sulfate-reducing bacterium (25). For thermodynamical reasons such a consortium is more capable of oxidizing acetate than a consortium consisting of a methanogen and a homoacetogen (Chapter 2, Fig. 5). It cannot be excluded that the syntrophic oxidation of acetate took place in the enrichment culture obtained in the sulfate-reducing chemostat. Unfortunately, the labeling experiment was not suited to elucidate if acetate was oxidized by a sulfidogenic syntrophic consortium or a single sulfate-reducing bacterium. However, in view of the fact that high numbers of homoacetogens were also

present in the methanogenic reactor, it is more likely to assume that also in this case homoacetogens grow on excretion products in the sulfate-reducing chemostat.

Assuming that the maintenance coefficient is growth rate independent, a theoretical maintenance requirement of Gibbs energy (m_e) for an anaerobic growth system at 20 °C (293 K) can be calculated (16,17). By comparing the energy normalized maintenance coefficients (m_e) calculated for the methanogenic and sulfate-reducing chemostat with the theoretical value of 88 $J \times g\ dw^{-1} \times h^{-1}$, our data can be validated. In case of the sulfate-reducing system the m_e was 7 times higher than expected and therefore the substrate maintenance rate (m_s) was overestimated as well. This can be explained by the fact that the amount of acetate consumed by the acetate-utilizing sulfate reducers was overestimated (Table 2), i.e. the number of sulfate reducers present consume less acetate than assumed. When the theoretical maximal population size (equation 5 and 6) of the acetate-utilizing sulfate reducers is calculated this number can be compared with the actual number of sulfate reducers. The calculated theoretical maximal population of sulfate reducers is 2×10^{10} cells l^{-1} which is 4 times higher than observed in the chemostat (Table 1). Therefore, acetate had to be utilized by other organisms as well to explain the acetate consumption rate in the sulfidogenic chemostat. This was confirmed by our results obtained by the MPN counts (see above). The maintenance coefficient (m_e) calculated for the methanogenic system was higher than the theoretical value but lies within the given uncertainty range of 32% (16). This indicates that the determined substrate maintenance rate (m_s) is a credible value. The theoretical maximal population size of the methanogens is 1×10^{10} cells l^{-1} which corresponds with the number observed in the chemostat (Table 1). This finding supports our "saprophytic commensalism" hypothesis because it confirms (again) that the number of methanogens present are responsible for all the acetate consumption in the methanogenic chemostat.

Intriguingly, the observations of flagellates in one of the chemostats shows that predation of the anaerobic microorganisms does occur as well. These results indicate that this type of metabolic interaction is of importance in the freshwater sediment. It is not known if the flagellates have a preference for one of the microorganisms.

ACKNOWLEDGEMENTS

This study is subsidized by a grant from the Netherlands Organization for Scientific Research (NWO). We thank K.A. Ennik-Maarsen for providing the *Methanosaeta concilii* GP6 cultures and Steef Biesterveld for technical support.

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CHAPTER 9

GENERAL DISCUSSION AND SUMMARY

INTRODUCTION

Methane (CH_4) in an important greenhouse gas. It has been reported that atmospheric methane is increasing at a rate of about 1-2% per year for at least the last decades (1,2). This increase of atmospheric methane is of great concern because of its potential role in climate change and atmospheric chemistry. About 60 % of the methane in the earth's atmosphere is of biological origin. Natural wetlands, paddy fields, gastro-intestinal track of ruminants and insects and landfills are major natural and anthropogenic sources of CH_4.

This research was part of a NWO/NOP project "Disturbance of Earth Systems" (Verstoring van Aardsystemen). It was the aim of this project to study how changes in environmental conditions, especially those caused by anthropogenic activities, may alter the microbial interactions and processes involved in the conversion of organic matter to methane. In this thesis the effect of inorganic electron acceptors (sulfate and nitrate) on methane emission from freshwater sediments in the Netherlands was investigated. The chosen study area was a polder located between Leiden and Utrecht, and is representative for similar polders in The Netherlands (Chapter 3). The polder contains peat grasslands in which ditches are lying used for maintaining stable water levels. The ditches contain sediment which is a potential source of CH_4. In freshwater environments, sulfate can be introduced by infiltration water, supply water or due to the oxidation of S-rich organic matter and iron sulfide (3,4). Also high nitrate concentrations can occur in the groundwater as a result of intensive agricultural activities. Therefore, in The Netherlands, sulfate and nitrate concentrations in the water may control the methane emission from methanogenic environments.

THE INFLUENCE OF SULFATE AND NITRATE ON METHANOGENESIS

Methane is produced by methanogenic archaea (methanogenesis) living in syntrophic association with fermentative and acetogenic bacteria (5-7). In presence of sulfate and nitrate, sulfate- and nitrate-reducing populations may successfully compete with these methanogenic consortia. In Chapter 4 the sediment was investigated for its potential methanogenic and syntrophic activity and the influence of sulfate and nitrate on these potential activities. Addition of acetate stimulated both methane formation and sulfate reduction, indicating that an active acetate-utilizing population of methanogens and sulfate reducers was present in the sediment. When inorganic electron acceptors were absent, substrates like propionate and butyrate were converted by syntrophic methanogenic consortia. However, addition of sulfate or nitrate resulted in the complete inhibition of these consortia. Our results show that propionate and butyrate were directly used by the sulfate and nitrate reducers. This indicated that the syntrophic methanogenic consortia could not compete with nitrate and sulfate reducers.

ACETATE, A KEY INTERMEDIATE IN THE ANAEROBIC DEGRADATION OF ORGANIC MATTER

In Chapter 5 the importance of methanogenesis and sulfate reduction in a freshwater sediment was investigated by using (non) specific inhibitors. Only the combined inhibition of methanogenesis and sulfate reduction resulted in the accumulation of intermediates (acetate, propionate and valerate). Acetate was the most important compound in the accumulation (93 mole %) and thereby confirming its role as a key intermediate in the terminal step of organic matter mineralization. Furthermore, the inhibition studies show that about 70-80% of the total carbon flow to CH_4 was through acetate. This clearly demonstrates that acetate was quantitatively the most important substrate for methanogens in the sediment. Addition of chloroform ($CHCl_3$) inhibited methanogens and acetate-utilizing sulfate reducers in the sediment. It is known that $CHCl_3$ inhibits several functions of enzymes in the acetylCoA-pathway of methanogens (8-10). So it is possible that other microorganisms with a similar pathway were inhibited as well. Therefore, we studied the inhibitory effect of $CHCl_3$ on homoacetogenic bacteria (*Acetobacterium woodii* and *Sporomusa acidovorans*), sulfate-reducing bacteria (*Desulfotomaculum acetoxidans, Desulfobacter postgatei* and *Desulfovibrio vulgaris*), a syntrophic bacterium (*Syntrophobacter fumaroxidans*) and methanogens (*Methanosaeta concilii, Methanosarcina barkeri, Methanospirillum hungatei* and *Methanobacterium bryantii*). The results show that $CHCl_3$ was an inhibitor of growth and product formation by methanogenic archaea, homoacetogenic bacteria, the syntrophic and the sulfate-reducing bacterium (*Desulfotomaculum acetoxidans*) operating the acetylCoA-pathway. The acetate-utilizing sulfate reducer *Desulfobacter acetoxidans* and the H_2 or lactate grown cultures of *Desulfovibrio vulgaris* were not inhibited by $CHCl_3$. These organisms do not possess the acetylCoA-pathway but utilize other biochemical routes for growth on these substrates. This is also the case for homoacetogenic bacteria when grown on fructose. However, the conversion rate of fructose by the homoacetogens was lower in the presence of $CHCl_3$ and hydrogen and formate accumulated. During fructose-dependent growth the main function of the acetylCoA-pathway is the recycling of reduced electron carriers (11). The recycling of these carriers was probably inhibited by $CHCl_3$. Thus the inhibition of microorganisms by $CHCl_3$ appears to be correlated with microorganisms which operate the acetylCoA-pathway and this supports our hypothesis that the population of acetate-utilizing sulfate reducers in the sediment operated the acetylCoA-pathway. Furthermore, the use of chloroform might allow a better elucidation of the role of different metabolic types of sulfate reducers to sulfate reduction in natural environments.

In most methanogenic environments acetate is quantitatively the most important substrate for methanogens. Therefore, the anaerobic conversion of [2-^{13}C] acetate in the presence of sulfate or nitrate was investigated (Chapter 6). Aceticlastic methanogenesis was the dominant acetate-utilizing process when the sulfate concentration was below 70 μM. At higher sulfate concentrations the formation of ^{13}C-

labeled CH_4 decreased significantly, indicating that methanogens and sulfate reducers were competing for the same substrate. When sufficient sulfate (>500 μM) was present the outcome of the competition was in favor of the sulfate reducers. Unexpectedly, nitrate-reducing bacteria hardly competed with methanogens and sulfate reducers for the available acetate. The electron-acceptor/acetate ratio indicated that denitrification was coupled to the oxidation of reduced sulfur compounds or other electron donors rather than to the oxidation of acetate. Furthermore, nitrate reduction seemed to have a direct inhibitory effect on methanogenesis, and an indirect effect as a consequence of the oxidation of reduced sulfur-compounds to sulfate. It was shown that acetate-utilizing methanogens are inhibited by reduced nitrogen forms during denitrification (12,13). This was confirmed by studies were the inhibitory effects of nitrate, nitrite, NO and N_2O on pure cultures of methanogens was investigated (14,15). Therefore, it may be speculated that the inhibition of methanogenesis by nitrate is not the result of competition for substrate but is due to the formation of toxic intermediates of the denitrification processes. The fact that acetate-utilizing nitrate reducers were outnumbered by the methanogens and sulfate reducers and hardly competed with these types of microorganisms for the available acetate indicate that acetate-utilizing nitrate reducers played a minor role in the degradation of acetate in the sediment.

ANAEROBIC ACETATE-UTILIZING MICROORGANISMS

Enumeration of acetate-utilizing anaerobes gave insight into the different groups of microorganisms involved in the acetate metabolism in the sediment (Chapter 6). In Chapter 7 the physiological properties of the acetate-utilizing anaerobes obtained by direct serial dilution of freshwater sediment are described. An acetate-utilizing methanogen (culture AMPB-Zg) was enriched and appeared to be closely related to *Methanosaeta concilii*. The most dominant acetate-utilizing sulfate reducer (strain ASRB-Zg) in the sediment was related to *Desulfotomaculum nigrificans* and *Desulfotomaculum thermosapovorans*. This result supports our hypothesis that acetate is a competitive substrate for methanogens and sulfate reducers in the sediment (Chapter 5 and 6). Oude Elferink et al. (16) mentioned that mixed substrate utilization by generalists may play a role in the competition for acetate. The kinetic properties of *Methanosaeta* sp. are slightly better than those of the generalist *Desulforhabdus amnigenus*. On basis of these parameters one would expect that *Methanosaeta* sp. outcompete the sulfate reducer (Chapter 2). However, *D. amnigenus* outcompeted acetate-degrading methanogens in a bioreactor treating complex wastewater. This indicates that the ability to use other substrates besides acetate gives *D. amnigenus* a competitive advantage over *Methanosaeta* sp. Strain ASRB-Zg turned out to be a generalist and this physiological characteristic may give the strain a competitive advantage over strain AMPB-Zg. Furthermore, the fact that strain ASRB-Zg belongs to the genus *Desulfotomaculum* confirmed our hypothesis that the acetate-utilizing sulfate reducers in the sediment metabolize acetate via the acetylCoA-pathway (Chapter 5). An acetate-utilizing nitrate reducer (strain ANRB-Zg) was isolated which showed to be related to *Variovorax paradoxus*. In the presence of acetate and

nitrate, strain ANRB-Zg was capable of oxidizing reduced sulfur compounds to sulfate. Strain ANRB-Zg may have been involved in the oxidation of reduced sulfur compounds to sulfate in the sediment (Chapter 6). However, at this moment too little information is available to understand the exact role of strain ANRB-Zg in the sulfur and carbon cycle of the sediment. The degradation of acetate in the absence and presence of SO_4^{2-} and NO_3^- is depicted in Fig. 1. The dominant acetate-utilizing anaerobes and their metabolic interactions are given as well.

Finally, the conversion of acetate by methanogenic and sulfidogenic communities under acetate-limited conditions was studied in Chapter 8. Our results show that the acetate-utilizing methanogens were able to compete efficiently with the sulfate reducers for the available acetate in an acetate-limited chemostat with sulfate in excess during a long-term experiment (1 year). This in contrast with results obtained in short-term incubations (6-120 hours) where acetate-utilizing methanogens were outcompeted by the sulfate reducers for the available acetate (Chapter 5 and 6). Carbon limited conditions prevailed in both the sediment incubations and chemostat experiments. However, in the chemostat experiments only acetate was available as carbon source. This in contradiction to the sediment incubations where other carbon sources were present as result of the degradation of organic matter (Chapter 5). It is known that under carbon limited conditions generalists might utilize different carbon substrates simultaneously (Chapter 2). Therefore, generalists might have had a advantage in the competition for acetate in the sediment incubations but this benefit was lacking in the chemostat experiment. This could partly explain why both acetate-utilizing methanogens and sulfate reducers were present in the sulfidogenic chemostat. Sequence analysis of the dominant acetate-utilizing sulfate reducer in the sulfidogenic chemostat supports this hypothesis because the partial 16S rDNA sequence was identical to that of the generalist strain ASRB-Zg (Chapter 7). Furthermore, the kinetic properties of the acetate-utilizing methanogens and sulfate reducers must have been almost similar (Chapter 8). Unfortunately, the kinetic properties of the dominant acetate-utilizing methanogens and sulfate reducers are still lacking. Therefore predictions based on these parameters about the outcome of the competition for acetate cannot be made yet. An overview of the most important interactions and processes is given in Chapter 2.

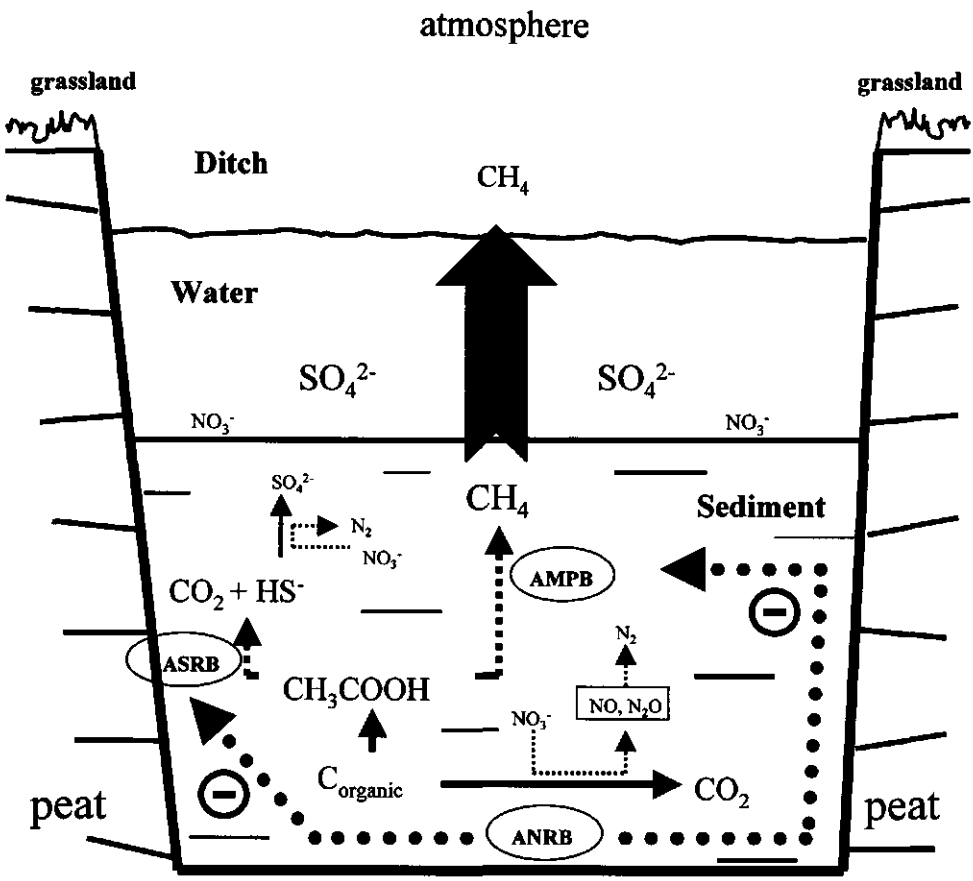

Figure 1: The influence of sulfate and nitrate on aceticlastic methanogenesis in freshwater sediment. AMPB: aceticlastic methanogen, ASRB: acetate-utilizing sulfate reducer, ANRB: acetate-utilizing nitrate reducer. Thick stripped lines represent competition for acetate between AMPB and ASRB. Thick dotted lines represent inhibition caused by toxic intermediates.

CONCLUDING REMARKS

The results which are presented in this thesis advanced our knowledge of the effect of sulfate and nitrate on methane formation in sediments which are found in a typical Dutch polder. The sediment is a potential source of methane but it remains unclear if the sediment emits high quantities of methane. It is assumed that the methane emission is in the same order of magnitude (42-225 kg CH_4 ha^{-1} yr^{-1}) as reported for a similar but not the same sediment (17). The presence of sulfate appeared to be a major factor in controlling the

formation of methane. This is due to the competition between acetate-utilizing methanogens and sulfate reducers. Nevertheless, the origin of sulfate and its effect on methane emission on the long-term is not fully understood. The inhibitory effect of nitrate on methanogenesis appears to be the result of the formation of toxic intermediates of the denitrification processes but tangible proof is still lacking at this moment. Also the physiology and ecophysiology of some of the dominant acetate-utilizing anaerobes, and the metabolic interactions among them are not completely resolved. Further investigations of these topics are needed to get a better understanding of the environment as a source of methane and the emission from it. Intriguingly, measurements of CH_4 emissions from grasslands near the location of the sediments have shown that a net methane consumption in the area is possible (18). They found that when ever an oxic top layer in the grassland is present, the grassland acts as a sink for atmospheric methane. These results indicate that methane produced in the ditches and originating from other sources may be oxidized again by the grassland soils. To determine a methane budget for Dutch polders the potential sink and/or source capacity of the grasslands should be included to get insight in the contribution to the emission of methane to the atmosphere.

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SAMENVATTING

INTRODUCTIE

Methaan (CH_4) is een belangrijk broeikasgas. In de afgelopen jaren is het duidelijk geworden dat de atmosferische methaan concentratie aan het stijgen is met een snelheid van ongeveer 1-2% per jaar. Deze toename is verontrustend omdat ze kan leiden tot een verhoging van de gemiddelde temperatuur op aarde als gevolg van het zogenaamde broeikaseffect. Belangrijke biogene bronnen van CH_4 zijn wetlands, rijstvelden, maag- en darmkanaal van runderen en insekten en landfills.

Dit onderzoek maakte deel uit van het NWO/NOP project "Verstoring van Aardsystemen". Het doel van dit onderzoek was om inzicht te krijgen in hoe veranderingen in de milieucondities, met name die veroorzaakt worden door menselijke activiteiten, de microbiële interacties en processen beïnvloeden die betrokken zijn bij de afbraak van organisch materiaal in methaan. In dit proefschrift werd het effect van inorganische electron acceptoren (sulfaat en nitraat) op de methaanproduktie in zoetwater sedimenten in Nederland onderzocht. In deze studie werd de invloed van sulfaat en nitraat op de methaanproduktie in zoetwater sedimenten afkomstig uit een polder gelegen tussen Leiden en Utrecht onderzocht (Hoofdstuk 3). In de polder bevinden zich graslanden met daartussen sloten die gebruikt worden om de grondwaterstand stabiel te houden. Het sediment in de sloten vormt een potentiele bron van methaan. In een zoetwater milieu is de sulfaat-concentratie meestal laag maar door infiltratie-water, toevoer-water of door de oxidatie van zwavel-rijk organisch materiaal en ijzersulfide kan de concentratie verhoogd worden. Ook hoge nitraat concentraties kunnen in het grondwater voorkomen als het resultaat van intensieve agrarische activiteiten. De aanwezigheid van sulfaat en/ of nitraat beïnvloedt de methaanproduktie en daarmee de uiteindelijke emissie van methaan.

DE INVLOED VAN SULFAAT EN NITRAAT OP DE METHANOGENESE

Methaan wordt geproduceerd door methanogene archaea (methanogenese) die in een syntrofe associatie leven met fermentatieve en acetogene bacteriën. In de aanwezigheid van sulfaat en nitraat, kunnen sulfaat- en nitraat-reducerende populaties succesvol competeren met deze syntrofe methanogene consortia. In Hoofdstuk 4 werd het sediment onderzocht op de potentiele activiteit van methanogene archaea en syntrofe consortia, en de invloed van sulfaat en nitraat op deze potentiele activiteiten. De toevoeging van acetaat stimuleerde zowel de methaanproduktie als de sulfaatreductie. Dit betekende dat er een actieve acetaat-afbrekende populatie van methanogenen en sulfaatreduceerders aanwezig was in het sediment. In de afwezigheid van sulfaat en nitraat werden de substraten propionaat en butyraat omgezet door syntrofe methanogene consortia. Echter, de toevoeging van sulfaat of nitraat resulteerde in de complete inhibitie van deze consortia. Sulfaat- en nitraatreduceerders consumeerde propionaat en butyraat direct. Dit gaf aan dat de syntrofe methanogene consortia niet succesvol waren in de competitie om deze substraten, met nitraat- en sulfaatreduceerders.

ACETAAT, EEN SLEUTEL INTERMEDIAIR IN DE ANAEROBE AFBRAAK VAN ORGANISCH MATERIAAL

Om het inzicht in het belang van methanogenese en sulfaatreductie in het sediment te vergroten werd er gebruik gemaakt van (niet) specifieke remmers (hoofdstuk 5). Alleen de gecombineerde specifieke inhibitie van de methanogenese en sulfaatreduceerders resulteerde in de accumulatie van intermediairen (acetaat, propionaat en valeraat). Van alle componenten die zich ophoopten was acetaat de belangrijkste (93 mol %) en bevestigde daarmee zijn rol als een sleutel intermediair in de afbraak van organische materiaal. De methaanproduktie kon voor 70-80% worden toegeschreven aan de afbraak van acetaat. Dit demonstreerde duidelijk dat acetaat het belangrijkste substraat is voor de methaanvorming in het sediment. De toevoeging van chloroform ($CHCl_3$) aan het sediment remde zowel methanogenen als acetaat-consumerende sulfaatreduceerders. Methanogenen bezitten de zogenaamde acetylCoA-stofwisselingsroute en het is bekend dat $CHCl_3$ verschillende functies van enzymen in deze route remt. Het is goed mogelijk dat andere microorganisms met een vergelijkbare route ook geremd worden door de toevoeging van $CHCl_3$. Om hier meer inzicht in te krijgen werd het effect van $CHCl_3$ op homoacetogene bacteriën (*Acetobacterium woodii* en *Sporomusa acidovorans*), sulfaat-reducerende bacteriën (*Desulfotomaculum acetoxidans, Desulfobacter postgatei* en *Desulfovibrio vulgaris*) en methanogenen (*Methanosaeta concilii, Methanosarcina barkeri, Methanospirillum hungatei* en *Methanobacterium bryantii*) onderzocht. De resulten lieten zien dat $CHCl_3$ de groei en produktvorming remt van methanogene archaea, homoacetogene bacteriën en de sulfaat-reducerende bacterie (*Desulfotomaculum acetoxidans*). De bacteriën hebben een stofwisselingsroute die vergelijkbaar is met de acetylCoA-route van de methanogenen. De acetaat-consumerende sulfaatreduceerder *Desulfobacter acetoxidans* en de op H_2 of lactaat gekweekte culturen van *Desulfovibrio vulgaris* werden niet geremd door $CHCl_3$. Deze organismen gebruiken namelijk niet de acetylCoA-route maar een andere biochemische route voor de groei op deze substraten. Dit is ook het geval voor homoacetogene bacteriën wanneer deze op fructose groeien. Echter, de omzettingssnelheid van fructose door homoacetogenen was lager in de aanwezigheid van $CHCl_3$. Ook de produktvorming was gewijzigd want behalve acetaat werd er ook waterstof en formiaat gevormd. Gedurende de groei op fructose is het belangrijk dat de gevormde gereduceerde electronen carriers gerecycled worden. Dit is de belangrijkste taak van de acetylCoA-route gedurende de groei op fructose. De recycling van de electronen carriers werd hoogst waarschijnlijk geremd door $CHCl_3$. De inhibitie van microorganismen door $CHCl_3$ blijkt dus gecorreleerd te zijn met microorganismen die een acetylCoA-route bezitten. Dit ondersteunt de hypothese dat de populatie acetaat-consumerende sulfaatreduceerders in het sediment inderdaad de acetylCoA-route gebruiken om acetaat te oxideren. Het gebruik van chloroform kan misschien een beter onderscheid maken tussen de rol van verschillende metabole typen van sulfaatreduceerders met betrekking tot sulfaatreductie in het milieu.

In de meeste methanogene milieu's is acetaat kwantitatief het belangrijkste substraat voor methanogenen. Daarom werd de anaerobe omzetting van [2-^{13}C] acetaat in de aanwezigheid van sulfaat of nitraat onderzocht (Hoofdstuk 6). Aceticlastische methanogenese was het belangrijkste acetaat-consumerende proces wanneer de concentratie van sulfaat beneden de 70 μM was. Bij een hogere sulfaat-concentratie werd er significant minder ^{13}C-gelabeld methaan gevormd. Dit gaf aan dat methanogenen en sulfaatreduceerders voor hetzelfde substraat competeerde namelijk acetaat. In de aanwezigheid van voldoende sulfaat (>500 μM) was de uitkomst van de competitie in het voordeel van de sulfaatreduceerders. Nitraat-reducerende bacteriën bleken nauwelijks met methanogenen en sulfaatreduceerders te competeren voor het beschikbare acetaat. Dit was onverwacht maar de electron-acceptor/acetaat ratio gaf aan dat denitrificatie eerder gekoppeld was aan de oxidatie van gereduceerde zwavel-componenten of andere electronen donoren dan aan de oxidatie van acetaat. De nitraatreductie had echter wel een directe remmende werking op de methaanproduktie en een indirect effect als de consequentie van de oxidatie van gereduceerde zwavel-componenten naar sulfaat. Het is bekend dat acetaat-consumerende methanogenen eerder geremd worden door gereduceerde vormen van stikstof, gevormd tijdens denitrificatie. Dit werd bevestigd in studies met reinculturen van methanogenen waarin de remming van nitraat, nitriet, NO en N_2O op de methaanvorming werd onderzocht. Het mag daarom worden aangenomen dat ook in het zoetwater sediment de inhibitie van de methanogenese toe te schrijven is aan de remmende werking van toxische intermediairen gevormd tijdens het denitrificatie proces. Het feit dat acetaat-consumerende nitraatreduceerders in veel lagere aantallen in het sediment voorkomen dan methanogenen en sulfaatreduceerders, en nauwelijk competeerden met deze microorganismen voor het aanwezige acetaat geeft aan dat acetaat-consumerende nitraatreduceerders een ondergeschikte rol speelden in de afbraak van acetaat in het sediment.

ANAEROBE ACETAAT-CONSUMERENDE MICROORGANISMEN

Om een beter inzicht te krijgen in de verschillende groepen van microorganismen die verantwoordelijk waren voor de afbraak van acetaat in het sediment werden er tellingen uitgevoerd (Hoofdstuk 6). In Hoofdstuk 7 zijn de fysiologische eigenschappen van de meest dominante anaerobe acetaat-consumerende microorganismen beschreven. Een acetaat-consumerende methanogeen (cultuur AMPB-Zg) werd opgehoopt en bleek nauw verwant te zijn met *Methanosaeta concilii*. De dominante acetate-consumerende sulfaatreduceerder (stam ASRB-Zg) was nauw verwant aan *Desulfotomaculum nigrificans* en *Desulfotomaculum thermosapovorans*. Dit gegeven ondersteunt de hypothese dat acetaat een competitief substraat voor acetaat-afbrekende methanogenen en sulfaatreduceerders is in het sediment (Hoofdstukken 5 en 6). Gemengd substraat gebruik door generalisten kan een rol spelen in de competitie voor acetaat. De kinetische eigenschappen van *Methanosaeta* sp. zijn net iets beter dan die van de generalist *Desulforhabdus amnigenus*. Op basis van deze parameters zou men verwachten dat *Methanosaeta* sp. de competitie om

aceaat zou winnen van de sulfaat reduceerder (Hoofdstuk 2). Echter, *D. amnigenus* competeerde succesvol met acetaat-afbrekende methanogenen in een bioreactor gebruikt voor de zuivering van complex afvalwater. Dit geeft aan dat de eigenschap om op andere substraten te kunnen groeien, *D. amnigenus* een competitief voordeel geeft over *Methanosaeta* sp. Stam ASRB-Zg is een generalist en misschien geeft deze fysiologische eigenschap de sulfaatreduceerder een competitief voordeel over de methanogeen (cultuur AMPB-Zg). Het feit dat stam ASRB-Zg tot het genus *Desulfotomaculum* behoort, bevestigt onze hypothese dat de acetaat-consumerende sulfaatreduceerders in het sediment acetate metaboliseren via de acetylCoA-route (Chapter 5). Een acetaat-consumerende nitraatreduceerder bacterie (stam ANRB-Zg) werd geïsoleerd en bleek nauw verwant te zijn aan *Variovorax paradoxus*. In de aanwezigheid van acetaat en nitraat was stam ANRB-Zg in staat om gereduceerde zwavel-componenten te oxideren naar sulfaat. Stam ANRB-Zg is misschien betrokken bij de oxidatie van gereduceerde zwavel-componenten naar sulfaat in het sediment (Hoofdstuk 6). Op dit moment is er echter te weinig informatie beschikbaar om de daadwerkelijke rol van stam ANRB-Zg in de zwavel en koolstof kringloop van het sediment te begrijpen. De afbraak van acetaat in de afwezigheid en aanwezigheid van sulfaat en nitraat is weergegeven in Fig. 1 (Hoofdstuk 9). De dominante populaties van acetaat-consumerende microorganismen en hun metabole interacties zijn eveneens weergegeven.

Uiteindelijk is de omzetting van acetaat door methanogene en sulfidogene gemeenschapppen onder acetaat-gelimiteerde condities bestudeerd (Hoofdstuk 8). De experimenten toonde aan dat de acetaat-consumerende methanogenen instaat waren om efficient te competeren met de sulfaatreduceerders voor het beschikbare acetaat in een acetaat-gelimiteerde chemostaat, met sulfaat in overvloed, gedurende een lange periode (1 jaar). Dit was in tegenstelling met de resultaten verkregen in korte termijn incubaties (6-120 uur) waar de acetaat-consumerende methanogenen de competitie om acetaat verloren van sulfaatreduceerders (Hoofdstukken 5 en 6). Koolstof-gelimiteerde condities heerste in zowel de sediment-incubaties als in de chemostaat- experimenten. In de chemostaten was echter alleen acetaat beschikbaar als koolstofbron. Dit in tegenstelling met de sediment-incubaties waar ook andere koolstofbronnen beschikbaar waren als resultaat van de afbraak van organisch materiaal (Hoofdstukken 2 en 5). Misschien had de sulfaatreduceerder een competitief voordeel in de sediment-incubaties maar ontbrak dit voordeel in de chemostaat-experimenten. Dit kan voor een deel verklaren waarom beide acetaat-consumerende methanogenen en sulfaatreduceerders aanwezig waren in de sulfidogene chemostat. Sequentie analyse van de dominante acetaat-consumerende sulfaatreduceerder in de sulfidogene chemostaat ondersteunt deze hypothese omdat een gedeeltelijke 16S rDNA sequentie van de sulfaatreduceerder vrijwel identiek was aan die van stam ASRB-Zg, een generalist (Hoofdstuk 7). De kinetische eigenschappen van de acetaat-consumerende methanogenen en sulfaatreduceerders moeten vrijwel gelijk zijn om zolang naast elkaar te kunnen coexisteren (Hoofstuk 8). Helaas ontbreken de kinetische eigenschappen van de dominante acetaat-consumerende methanogenen en sulfaatreduceerders op dit moment. Voorspellingen omtrent de uitkomst van de competitie voor acetaat

kunnen daarom nog niet gemaakt worden. Een overzicht van de belangrijkste interacties en processen wordt in Hoofdstuk 2 gegeven.

CONCLUDERENDE OPMERKINGEN

De resulten die in dit proefschrift worden vermeld verbeterde de kennis van het effect van sulfaat en nitraat op de methaanproductie in zoetwater sedimenten afkomstig uit een typische nederlandse polder. Het sediment is een potentiele bron van methaan maar het bleef onduidelijk hoeveel methaan het sediment uitstoot. Op dit moment wordt er aangenomen dat dit in dezelfde orde van grootte is (42-225 kg CH_4 ha^{-1} jr^{-1}) als gemeten in een vergelijkbaar sediment. De aanwezigheid van sulfaat is echter een zeer belangrijke factor die de methaanproduktie en de uiteindelijke emissie beheerst. De verklaring hiervoor is dat acetaat-consumerende methanogenen en sulfaatreduceerders competeren voor het beschikbare acetaat. De herkomst van het sulfaat en zijn effect op de methaanproduktie en -emissie op de lange termijn is echter nog niet duidelijk. Nitraat remt weleenswaar de methaanproduktie maar speelt een minder belangrijke rol dan sulfaat. De remming is hoogst waarschijnlijk het resultaat van de vorming van toxische intermediairen tijdens het denitrificatie proces maar een tastbaar bewijs hiervoor ontbreekt nog op dit moment. Ook de fysiologie en ecofysiologie van sommige dominante acetaat-consumerende microorganismen, en hun metabole interacties zijn nog niet volledig opgehelderd. Meer onderzoek aan deze onderwerpen is nodig om een beter inzicht te krijgen van dit milieu als potentiele bron van methaan. Methaanemissie metingen verricht op veengraslanden lieten zien dat een netto methaan-consumptie in het gebied mogelijk is. Telkens wanneer er een oxische toplaag in de graslanden aanwezig is kunnen de omringde graslanden als een put (sink) voor atmosferische methaan dienen. Deze resulten geven aan dat methaan afkomstig uit het sediment en andere bronnen geoxideerd kan worden door de graslanden. Voor het maken van een methaan budget voor nederlandse polders moet de potentiele sink en/of bron capaciteit van de graslanden betrokken worden om inzicht te krijgen in de bijdrage van deze gebieden aan de emissie van methaan naar de atmosfeer.

Dankwoord

Op dit moment d.w.z tijdens het schrijven van het dankwoord, zit ik in sneeuw-wit Marburg met een glaasje gloeiwijn en is mijn proefschrift nog steeds een stapel papieren waarin hier en daar nog een figuur of tabel geplaatst moet worden. Ik probeer het proefschrift al als een geheel te zien maar dat valt op dit moment niet mee. In ieder geval, als u dit leest dan is de sneeuw hier al lang gesmolten, de gloeiwijn op en mijn proefschrift af. Uiteraard is dit proefschrift tot stand gekomen met de hulp van andere mensen en organisaties. Bij deze wil ik iedereen bedanken voor hun bijdrage.

Allereerst wil ik *Fons Stams* bedanken, die in 1992 aan de basis stond van dit promotie-onderzoek, dankzij het door hem ingediende en door NWO gehonoreerde onderzoekvoorstel. Uiteraard ook voor zijn inzet gedurende mijn promotie en maar ook voor de gezellige uurtjes na het werk, om onder het genot van een zelf gebrouwen biertje of wijntje de grotere vragen van het leven te ontdekken. Uiteraard wil ik *Willem de Vos* bedanken voor zijn hulp bij het afronden van het proefschrift.

Steef Biesterveld en *Caroline Plugge* wil ik bedanken voor al hun kennis overdracht en zorg voor de chemostaat-kindjes. *Stefanie Oude Elferink*, *Wim Roelofsen*, Alexander *van Ittersum*, *Jan-Willen van Borren*, *Cathrien Bakker*, *Jaap Vogelaar* en *Peter van Bodogem* voor hun bijdrage aan mijn proefschrift. Wim wil ik nog even in het bijzonder bedanken voor zijn hulp bij het ^{13}C-gebeuren. Miriam van Eekert en Hermie Harmsen voor de bezoekjes aan een drinklokaal waarvan ik de naam niet zal noemen en de lange discussies die daar gevoerd werden. De overige collega's van de anaerobe groep, *Ine van Kuijk*, *Karin Maarsen*, *Annemarie Louwerse*, *Philippe Schyns*, *Jan Weijma*, *Serve Kengen*, Miss *Dong*, alle binnen- en buitenlandse gasten en studenten. Daarnaast wil ik *Nees Slotboom*, juffrouw Jannie en *Frits Lap* bedanken voor de organisatie achter de schermen en Nees in het bijzonder als manager van het M&M voetbalteam. Uiteraard worden alle (oud)medewerkers van de vakgroep Microbiologie bedankt voor de hulp en bijdrage aan de leuke werksfeer op de vakgroep.

De mensen van het R.O.C Zegveld wil ik bedanken voor het monsteren van mijn sedimenten en de koffie op koude monsterdagen. De mensen van het Groot-Waterschap van Woerden voor het verstrekken van een aantal gegevens omtrent de waterkwaliteit in het gebied. *Kees Hordijk* en de mensen van het NIOO in Nieuwersluis voor hun hulp bij de analytische bepalingen en *Hans de Mars* voor het beschikbaar stellen van een aantal tekeningen.

Jos, *Joep* en *Dick* bedankt voor de gezellige tijd in Wageningen en metname voor de (ont)spanning na het werk. Familie en overige vrienden worden bedankt voor de belangstelling die zij toonden

gedurende mijn promotie ondanks het feit dat ik daar nog weleens vaag over deed. *Edie*, dit moment zal ook voor jou een opluchting zijn. Ik wil je bedanken voor al je steun maar vooral voor het feit dat je ondanks mijn "hobby" altijd kon blijven lachen.

Marburg, 7 december 1998.

Curriculum Vitae

Johannes Cornelis Maria (Hans) Scholten werd geboren op 3 september 1965 in Castricum. In 1988 ging hij studeren aan de Internationale Agrarische Hogeschool Larenstein te Wageningen. Na het halen van het propadeutisch jaar in 1989, koos hij de afstudeerrichting Microbiologie. In het kader van deze richting deed hij een afstudeeropdracht op het gebied van de moleculaire biologie bij de School of Biological Sciences, Macquarie University, Sydney, N.S.W., Australia. Zijn afstudeeropdracht verrichtte hij bij de vakgroep Microbiologie van de Landbouwuniversiteit, waarbij de invloed van externe electronenacceptoren op de xylose-fermentatie van *Bacteroides xylanolyticus* werd bestudeerd. Enkele maanden na het behalen van zijn diploma begon hij als onderzoeker in opleiding bij de vakgroep Microbiologie van de Landbouwuniversiteit. Hij werkte van oktober 1992 tot oktober 1996 aan de invloed van sulfaat en nitraat op de methaanvorming in zoetwater sedimenten. Het resultaat van dit onderzoek, dat werd gefinancierd door de Nederlandse Organisatie voor Wetenschappelijke Onderzoek, staat beschreven in dit proefschrift. Op dit moment (december 1998) werkt hij als post-doc op het Max-Planck-Instituut voor terrestrische microbiologie in Marburg, Duitsland.