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Determination of sulphide production rates in laboratory cultures of the sulphate reducing bacterium *Desulfovibrio aespoeensis* with lactate and H_2 as energy sources

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Determination of sulphide production rates in laboratory cultures of the sulphate reducing bacterium *Desulfovibrio aespoeensis* with lactate and H_2 as energy sources

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Abstract

Laboratory experiments with pure cultures of the sulphate-reducing bacterium *Desulfovibrio aespoeensis*, isolated from the Äspö HRL, were performed with two energy sources, lactate and H_2. The experiments were done in order to determine the growth rates with the two energy sources and by that measure the *in vitro* sulphide production rates.

The growth experiment with different lactate concentrations showed that the highest growth rate and sulphide production were found in cultures with the highest concentration of lactate, 11.3 mM. The highest sulphide concentration in this growth series was 5.3 mM or 170 mg L^{-1} and the highest sulphide production rate was calculated to 46 μM h^{-1} or 1.5 mg L^{-1} h^{-1} in the exponential growth phase.

When *D. aespoeensis* grows with H_2 as energy source, the bacterium needs acetate as carbon source for biomass synthesis. To be able to determine the optimum concentration of H_2, the optimum concentration of acetate needed to be established. This was done in an experiment where the H_2 concentration was kept at an unlimited concentration in growth cultures with different acetate concentrations. The optimum acetate concentration was 2 mM or 118 mg L^{-1}. I cultures with this concentration, the highest measured sulphide concentration was 8.9 mM or 285 mg L^{-1}. The highest sulphide production rate in these growth cultures was 43 μM h^{-1} or 1.4 mg L^{-1} h^{-1}.

To establish the growth rates and sulphide production rates with different H_2 concentrations, growth experiments with varying H_2 concentration and fixed acetate concentration at 2 mM were done. The highest measured sulphide concentration, 6.53 mM or 209 mg L^{-1}, was found in cultures with 553‰ H_2 in the gas phase of the cultures tubes. In cultures with 258 ‰ H_2, in the gas phase, the highest concentration of sulphide was 6.09 mM or 195 mg L^{-1}. The highest sulphide production rate was measured to be 8.4 μM h^{-1} or 0.27 mg L^{-1} h^{-1}. The sulphide production rate in this experiment did not reach the rates measured in the experiment with unlimited supply of H_2 described above.

The growth experiments showed that an increase in availability of energy source gave both increased total concentration of sulphide in the growth bottles and also resulted in that the sulphide production rate was proportional to the energy source concentration and was not directly, correlated to the number of sulphate reducing bacteria in the cultures.

Contents

1 Introduction

H_2S produced by microbial SO_4^{2-} reduction under anaerobic conditions may corrode the copper canisters used for final disposal of spent nuclear fuel. Microbial SO_4^{2-} reduction rate is therefore a key process for the safety assessment. A two year project (2006−2008) to study this process was initiated as a cooperation project between the companies Swedish Nuclear Fuel and Waste Management Co. in Sweden and Electric Power Development Co., Ltd. in Japan.

1.1 Background

1.1.1 Anaerobic corrosion of metals

H_2S is a compound that can mediate anaerobic Cu corrosion (Myers and Cohen 1984). The principal mechanism for anaerobic Cu corrosion is thought to be the same as known for anaerobic corrosion of Fe (Puigdomenech and Taxén 2000).

There will be steel rock support material like rock bolts and wire mesh, left in the repository at the time for closure. The oxidation of metallic Fe with SO_4^{2-} -reduction is regarded as the principal reaction in microbial anaerobic corrosion of iron. The suggested reaction mechanism for the corrosion is that the negative redox potential (Fe^{2+}/Fe, $E_0' = -0.44$ V) of iron can liberate H_2 ($2H^+/H_2$, $E_0' = -0.41$ V) and may in this way indirectly act as an electron donor for sulphate-reducing bacteria (SRB) (Cord-Ruwisch 2000). The principle of anaerobic microbial corrosion of iron is shown in Figure 1-1. Another possible mechanism in anaerobic corrosion has been proposed (Cord-Ruwisch 2000): a direct utilization of the electrons liberated during the oxidation of Fe ($Fe \rightarrow Fe^{2+} + 2e^-$). This mechanism is kinetically more favourable than consumption of the electrochemically formed H_2.

The complete mechanism of anaerobic copper corrosion is not yet fully understood but the theory is that electrons from Cu, reduce protons to H_2 in the presence of H_2S produced by sulphate reducing bacteria (SRB), see Equation 1-1.

$$xCu + HS^- + H^+ \rightarrow Cu_xS + H_2 \qquad \text{Equation 1-1}$$

The produced H_2 can be used by SRB that produce more H_2S. The H_2S may react with the Cu and produce Cu_xS. Several different copper sulphides can be formed (Little and Lee 2007). It is therefore of great importance to characterize the potential for H_2S production in groundwater in a repository for spent nuclear fuel. One set of the parameters that are crucial is the growth kinetics of SRB when grown on different energy sources.

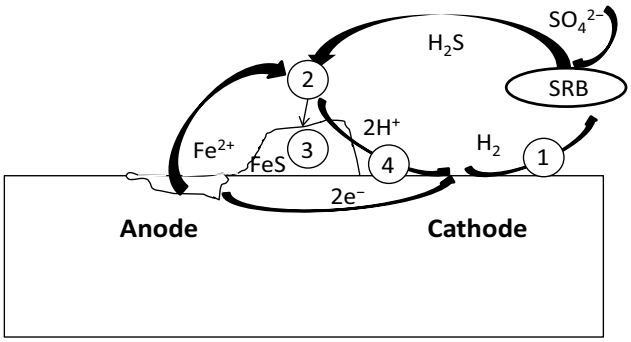

Figure 1-1. *The principle of anaerobic corrosion of metallic Fe mediated by SRB. 1. The H_2 produced by the reduction of protons by electrons from the oxidation of Fe is consumed by SRB. 2. The H_2S produced precipitates with the Fe^{2+} and is deposited as 3. FeS on the steel surface. 4. During the precipitation protons are liberated.*

1.1.2 Sulphate-reducing bacteria

Microbial SO_4^{2-} reduction to H_2S is a process that occurs in anaerobic environments. This process can for example be found in marine and lacustrine sediments, groundwater, in guts of animals and in many engineered systems. The SRB are mainly found in strict anaerobic SO_4^{2-}-rich environments even though these microorganisms can sustain O_2 (Cypionka 2000, Fareleira et al. 2003, Sass et al. 1992, Muyzer and Stams 2008). SRB are also found in environments with low SO_4^{2-} concentration like fresh-water sediments and groundwater with SO_4^{2-} concentrations well below 1 mM (Thauer et al. 2007). In such environments the produced H_2S is re-oxidized under anaerobic conditions and the SO_4^{2-} concentration is sustained.

SO_4^{2-} reduction at temperatures and pressures prevailing in deep groundwater environment is a microbiological process. The chemical reduction to H_2S at these conditions is very slow as revealed by the calculated half life for thermo-chemical SO_4^{2-} reduction in the presence of acetate and elemental sulphur at 100°C that is 372,000 years (Cross et al. 2004).

The SRB use the S atom in the SO_4^{2-} molecule as an electron-acceptor and the reduced product is H_2S. The energy and electron donor for SRB can be either organic compounds or H_2. With a general organic compound the reaction with SO_4^{2-} can be written (Equation 1-2):

$$2(CH_2O) + SO_4^{2-} + 2H^+ \rightarrow 2HCO_3^- + HS^- + 3H^+ \qquad \text{Equation 1−2}$$

Organic compounds that can be used by different types of SRB are fermentation products like short organic acids, fatty acids and higher molecular weight hydrocarbons (Widdel et al. 2007). Many SRB, especially of the genus *Desulfovibrio,* can grow on lactate. The lactate molecule is incompletely oxidized to acetate and CO_2 and the electrons are transported to electron transport enzymes in the cell membrane and then further to the SO_4^{2-} reduction enzymes in the cytoplasm (Figure 1-2). The overall reaction is written (Equation 1-3):

$$2 \text{ lactate} + SO_4^{2-} + 2H^+ \rightarrow 2 \text{ acetate} + 2CO_2 + HS^- + H^+ + 2H_2O \qquad \text{Equation 1−3}$$

In this metabolism lactate is used as an energy and electron source as well as a carbon source for biomass production.

There are also SRB that utilize H_2 as electron donor and energy source. The reaction for the reduction of SO_4^{2-} with H_2 is written (Equation 1-4):

$$4H_2 + SO_4^{2-} + 2H^+ \rightarrow H_2S + 4H_2O \qquad \text{Equation 1−4}$$

Note that there is no carbon involved in this energy transforming reaction (Equation 1-4). The carbon sources for H_2 utilizing SRB are either short organic compounds like acetate or CO_2. The carbon is used for biomass production and the biosynthesis is energy consuming. Acetate and CO_2 are incorporated into the cell metabolism via the molecule CoA to produce pyruvate in the following way (Equation 1-5) :

$$CH_3COO^-\text{-CoA} + CO_2 + H_2 \rightarrow CH_3COCOO^- + \text{CoA} + H_2O \qquad \text{Equation 1−5}$$

The pyruvate then enters the cell metabolism and will be incorporated into new biomolecules.

1.1.3 Substrate oxidation and SO_4^{2-} reduction on molecular level

The oxidation of the electron donor, either an organic molecule or H_2, and the reduction of SO_4^{2-} are separate reactions proceeding at different positions in the cell. The oxidation of H_2 takes place in the periplasmatic space or in the cell membrane via enzymes of which hydrogenase is the key enzyme (Figure 1-2) (Aubert et al. 2000). The electrons are transported via several other enzymes in the cell membrane and then released into the cytoplasm to the enzymes involved in the reduction of SO_4^{2-} to H_2S. The electron transport is coupled to the proton gradient that drives the production of ATP (adenosine tri-phosphate) from ADP (adenosine di-phosphate) and PO_4^{3-} via the enzyme ATPase. ATP is the energy carrying molecule in all cells.

The complete reduction of SO_4^{2-} to H_2S in SRB is a chain of electron transfers by different enzymes in the cytoplasm of the cell. The first step in the reduction, from SO_4^{2-} to SO_3^{2-} is an energy consuming process and requires input of energy via ATP into the activated adenosine phosphosulphate (adenylyl sulphate, APS) (Equation 1-6):

$$SO_4^{2-} + ATP + 2H^+ \rightarrow APS + PP_i \qquad \text{Equation 1−6}$$

PP_i is inorganic di-phosphate. This reaction is catalyzed by the enzyme ATP sulphurylase (Sperling et al. 1998, Taguchi et al. 2004). The electron transfer is done in two steps, one two electron transfer and one four electron transfer and ATP is produced via the proton gradient created by the H_2 oxidation.

In lactate oxidation, H_2 is produced in the oxidation step from lactate to acetate. This H_2 is then oxidized via the hydrogenase in the same manner as for H_2 oxidation without organic energy source. (Figure 1-2).

1.1.4 Sulphate-reduction in deep granitic groundwater

Presence of SRB in deep granitic groundwater has been studied at several places in Sweden and Finland. Most of the work has been done in the Äspö tunnel and includes enumeration and identification of SRB and isolation and description of one new strain of SRB, *Desulfovibrio aespoeensis* (Motamedi and Pedersen 1998). During the site investigations aiming at the selection of a candidate location for the repository for spent nuclear fuel in Sweden, enumeration of SRB was part of the full chemical characterisation of the groundwater (Hallbeck and Pedersen 2008a, b, c, 2012). In Finland enumeration of SRB has been part of the early site investigations and now in the ongoing site investigation of the ONKALO tunnel in Olkiluoto (Pedersen et al. 2008).

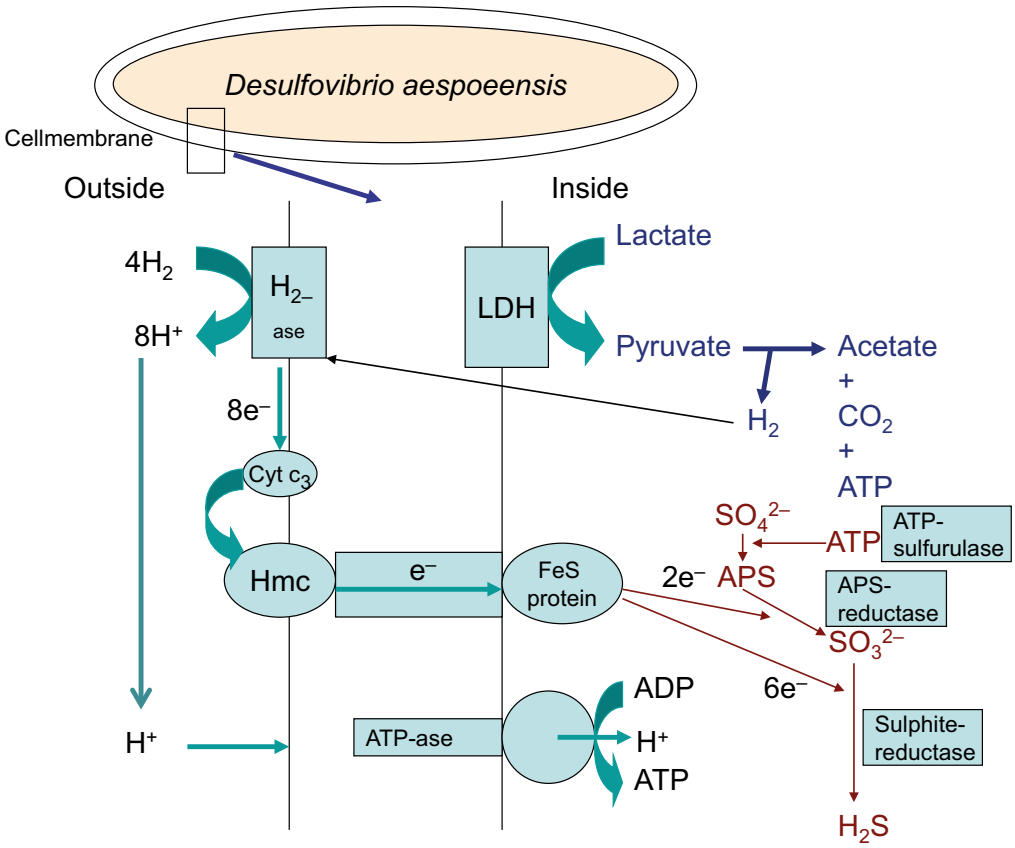

Figure 1-2. *The electron transport and energy conservation in SRB. External hydrogen (H_2) and H_2 originating from the oxidation of an organic compound such as lactate can be oxidized by the hydrogenase (H_2-ase). The electrons are then transported via other membrane bound enzymes (turquoise). From the FeS-protein on the inside of the membrane, the electrons are shuttled to a cytoplasmatic APS-reductase and the further to sulphite-reductase forming H_2S.*

The numbers of SRB found in granitic groundwater varied between the sampled boreholes and borehole sections from below the detection limit of the MPN method, < 0.2 mL^{-1}, and up to 30,000 mL^{-1}. Studies of three boreholes in the Äspö tunnel showed that the water in the boreholes are stable and the amount of SRB remain stable if there was no disturbance of the system by pumping of groundwater in the vicinity of the sampled section (Hallbeck and Pedersen 2008a). SRB are very sensitive to increased flow rates of water. This has been observed in the site investigations in Sweden and in bioreactor experiments (Hallbeck and Pedersen 2008a, Omil at al. 1996, Van den Heuvel et al. 1995, Lens et al. 2003).

The eco-physiology of SRB in granitic groundwater is not yet fully understood. One of the most important factors that still remain to be elucidated is what different types of energy sources that are used and their transport. One source that has been proposed is H_2 but its origin and transport rate are still to be clarified. Another source of energy is methane that can be used by a consortium of methanogens and SRB. This process, called anaerobic methane oxidation (AMO) was recently discovered and has mostly been studied in marine sediments (Lösekann et al. 2007). Geochemical data and microbial molecular biology data from site investigation in Olkiluoto, Finland point towards the presence of this process also in deep groundwater (Pedersen et al. 2008).

Recently a bacteriophage, a virus that attacks bacteria, specific for the strain of *D. aespoeensis* from the HRL in Äspö, was isolated (Eydal et al. 2009). This finding suggests that the SRB in deep groundwater at Äspö are metabolically active since bacteriophages only infect cells in the state of growth. It suggests that there is a predator-prey situation in the groundwater with active and growing SRB continuously predated by viruses to observed steady-state numbers. Since the SRB are active and growing they will produce H_2S as long as there is a supply of energy, presumably H_2.

2 Sulphate reduction experiments

2.1 Introduction

In order to be able to model sulphide production in natural and technical systems the sulphide production rate is needed. The aim of the experimental part of the project was to determine growth- and sulphide production rates for the electron donor in the sulphide production for a SRB isolated from groundwater. The project included three growth experiments, one with lactate for heterotrophic sulphide production and two with H_2 as energy and electron donor, for mixotrophic sulphide production. The project started with an experiment with lactate at different concentrations in order to find the optimum lactate concentration. Lactate function as organic energy source and also carbon source for the SRB. The second experiment was done with H_2 as energy source and acetate as carbon source in order to find the optimum acetate concentration. In this experiment different acetate concentrations were used and H_2 was in excess during the experiment. From the results in the second experiment the third experiment was designed. In the third experiment it was possible to measure the consumption of H_2 during growth. In this experiment different amounts of H_2 were added to the cultures with the optimal acetate concentration determined from the second experiment. Data from the three experiments are compiled in the Appendix.

- LacRate: an experiment with *D. aespoeensis*, grown on lactate as carbon and energy source.
- AcRateH2: the same organism was grown on H_2 as energy and electron source and acetate as carbon source. In this experiment different acetate concentrations were used together with an excess of H_2.
- H2RateAc: in this experiment the H_2 concentration was varied with a fixed acetate concentration.

The SRB *D. aespoeensis* was isolated from groundwater from the Äspö HRL in 1997 (Motamedi and Pedersen 1998). Later work has established that this species is present at most depths in groundwater from Äspö (Eydal et al. 2009) and it is therefore a most relevant SRB to use in H_2S production modelling work. *D. aespoeensis* can grow both heterotrophically with lactate as both carbon and energy source, see Equation 1-3, and mixotrophically, see Equation 1-4 and Equation 1-5, with H_2 as energy and electron donor and acetate and carbon dioxide as carbon sources.

2.2 Material and methods

2.2.1 Uncertainties and precision

The sulphate reduction experiments were performed with established anaerobic growth technique (Widdel and Bak 1992). The cultures were grown in 120 mL serum bottles in LacRate and AcRateH2 and in H2RateAc 26 mL anaerobic culture tubes were used. Anaerobic technique requires use of syringes and needles for additions in the medium preparation and therefore the volumes given in this report has an uncertainty of up to 5% which also gives an uncertainty in the concentrations of the measured chemical species of 5%. In addition to that the precision and uncertainty of the measurements for the methods used should be taken into account. All experiments were done at room temperature, 22 ± 3°C.

2.2.2 Defined multipurpose medium for SRB

The medium used was a modified multipurpose growth medium described by Hallbeck and Pedersen (2008a). Below, the preparation of the medium is described.

A salt medium with the following composition per litre of the finally prepared medium in the growth cultures was containing:

- 7.0 g NaCl.
- 1.0 g $CaCl_2 \times 2H_2O$.
- 0.67 g KCl.
- 1.0 g NH_4Cl.
- 0.15 g KH_2PO_4.
- 0.5 g $MgCl_2 \times 6H_2O$.
- 3 g $MgSO_4 \times 7H_2O$.

The solution was autoclaved in a special anaerobic medium device. After autoclaving, re-dissolution of oxygen was prevented by cooling under an atmosphere of N_2/CO_2 (80/20 vol%). The medium was kept under the N_2/CO_2 atmosphere while the following solutions were added (per litre of the finally prepared medium) with sterile, N_2-flushed syringes to finalize the growth medium:

- Trace element solution 10 mL.
- Vitamin solution, 10 mL.
- Thiamine solution, 1 mL.
- Vitamin B_{12} solution, 1 mL.
- Iron stock solution, 5 mL.
- Resazurin solution, 2 mL.
- Cysteine hydrochloride, 10 mL.
- $NaHCO_3$-solution, 60 mL.
- Yeast extract solution, 10 mL.

The composition of the solutions containing vitamins, thiamine, vitamin B_{12}, iron, resazurin, cysteine, yeast extract, $NaHCO_3$ and trace element solutions used in the growth medium can be found Hallbeck and Pedersen (2008a). In the medium described in Hallbeck and Pedersen (2008a) Na_2S was added to lower the redox potential in the medium. No such addition of Na_2S was made to the medium in this experiment since it would interact in the first measurements of S^{2-} in the growth experiment. After the additions, pH was adjusted to be in the range of 6.95 – 7.05 preferably 7.0 with sterile 1M NaOH or 1M HCl. During the pH adjustment the medium was kept under the gas atmosphere. After each pH adjustment small aliquots of medium were withdrawn and pH was instantly measured with a pH-meter. pH in the growth medium was around 7 both at start and at the end of the experiments as known from earlier growth studies, since the medium was buffered with hydrogen carbonate. pH 7 was chosen both because it is close to the pH optimum of the bacterium and because it is the pK_b of the S^{2-}/HS^- couple. Sodium chloride concentration was 7.0 g L^{-1} because this is the optimum concentration for *D. aespoeensis*.

2.2.3 Addition of energy and carbon source

Three sulphate reduction experiments with different energy and carbon sources were performed as mentioned above in Section 2.1. In experiment LacRate, lactate in different concentrations was added to the growth bottles. In experiment AcRateH2, acetate in different concentrations and H_2 in excess, were added to the growth bottles and, finally, in the third experiment, denoted H2RateAc, acetate was added in one fix concentration to all bottles and the addition of H_2 was done to give different H_2 gas concentrations in the different experiments.

Procedure

The experiments LacRate and AcRateH2 were performed in 120 mL sterile growth bottles at the start containing 50 mL of medium, carbon source and inoculum, and thus the bottles had at the start 70 mL of the gas mixture described below in the head space, see Section 2.2.1 for uncertainties in

volumes. The experiment H2RateAc where performed in 26 mL tubes containing 10 mL of medium and inoculum, and 16 mL head space with gas. The composition of the gas phase varied for each experiment, the gas was a mixture N_2/CO_2 (80 and 20%, respectively) with or without the addition of H_2 depending on the experiment, as described below. Gas mixture was added to a measured gas pressure of 2 bars at room temperature see Section 2.2.1 for uncertainties. All bottles and tubes were incubated in the dark at room temperature.

LacRate – varied lactate concentrations

Lactate is available as syrup of Na-lactate, approximately 50%. The lactate was sterilized by autoclaving at 121°C. Lactate solutions with different concentrations were prepared in sterile anaerobic test tubes by dilution in sterile growth medium. One mL additions from each dilution of lactate were made to the growth bottles see Section 2.2.1 for uncertainties. The measured lactate concentration was analysed in the first sample at start of the experiment (see Section 2.2.6), and Table 2-1.

AcRateH2 – varied acetate concentrations and excess H_2

The growth medium in AcRateH2 was prepared as described for LacRate with exception for energy and carbon source. The carbon source, acetate, was prepared as a dilution series of Na-acetate in sterile Hungate tubes from a sterile stock solution with a concentration of 10 g per 100 mL. The dilution was made in 10 times steps and two mL was added to the highest concentration see Section 2.2.1 for uncertainties. To the other bottles one mL of each dilution was added. Table 2-2 shows the acetate additions to the five growth series. The sodium acetate solution was sterilized by autoclaving at 121°C. Since the additions were made by means of syringes and needles, the actual acetate concentration in each growth bottle was measured with an enzymatic acetate concentration kit see Section 2.2.1.

After acetate additions and inoculations, H_2 was added to a total pressure of 2 bars in each bottle. Addition of H_2 up to a total pressure of 2 bars was done at three occasions during the growth experiments (in Series A and B at 120 h, 196 h and 266 h, see Figure 3-9), to keep the amount at a non-limiting concentration. The pressure in the bottles was 2 bar after the H_2 addition.

Table 2-1. Calculated and measured lactate concentrations in LacRate.

Experiment	Lactate concentration (calculated) (mM)	Lactate concentration (measured) (mM)
1. Lac11.3	11.3	14.5 ± 0.7 (n=2)
2. Lac1.13	1.13	1.45 ± 0.5 (n=2)
3. Lac0.11	0.11	0.05 ± 0.009 (n=2)
4. Lac0.01	0.01	n. a.
5. Lac0.001	0.001	n. a.

n.a = not analysed

Table 2-2. The dilution and concentration of acetate in the different AcRateH2 experiments.

Experiment	Calculated acetate concentration (mM)	Measured acetate concentration in growth bottle (mM)
1. Ac44	44	53.1 ± 5.4 (n=2)
2. Ac22	22	20.8 ± 0 (n=2)
3. Ac2.0	2.2	2.0 ± 0.1 (n=2)
4. Ac0.2	0.2	0.2 ± 0.01
5. Ac0.02	0.02	0.08 ± 0.007

H2RateAc – varied H_2 concentrations and fixed acetate concentration

The growth medium was the same SRB medium used for LacRate and AcRateH2 and with 0.164 g L^{-1} Na-acetate. In this experiment the gas phase was sampled and because of that every sampling had to be done in an unopened tube. Therefore 26 mL anaerobic tubes with butyl rubber stoppers (instead of 120 mL glas bottles) having 9 mL medium (containing acetate) and 1 mL of inoculum were used, and therefore each tube had 16 mL of gas phase. The sterile controls had 9 mL of medium and 17 mL of gas phase. Four experiments were done with varied concentration (Table 2-3). The H_2 was added to each tube in the experiments at room temperature, see Section 2.2.1 for uncertainties:

- H2*553, 16 mL.
- H2*258, 8 mL.
- H2*132, 4 mL.
- H2*33, 1 mL.

These amounts of hydrogen gas were added to the gas phase of 16 mL (N_2/CO_2, 80/20%). After the H_2 addition N_2/CO_2 mixture were added to the tubes to get 2 bars pressure in each tube. All gas additions were done at room temperature see Section 2.2.1 for uncertainties. In Table 2-3, the measured final amounts of H_2 in growth tubes are presented.

2.2.4 Inoculum

Growth bottles and tubes were inoculated with pre-grown *D. aespoeensis* cells in the exponential growth phase to a start concentration of approximately 1×10^4 cells mL^{-1}. The growth bottles and tubes were inoculated after addition of the carbon source lactate or acetate but before the addition of H_2.

2.2.5 Sampling

LacRate

The volume of the cultures were 50 ± 2 mL, see Section 2.2.1 for uncertainties and since all samples in one series had to be taken from the limited volume, the sample volumes had to be kept as small as possible. In Table 2-4 the times of sampling in experiment LacRate are compiled together with sample volumes and analyses made. Since the aim of this experiment was to find the optimal lactate concentration and the maximum growth rate, samples from the different parts in a batch culture growth were needed. Since the growth pattern of *D. aespoeensis* grown with lactate was unknown, samples were withdrawn at 11 occasions and from the TNC values it was decided that 7 of these samples were to be analysed for TNC and S^{2-}. The TNC and S^{2-} data were used to calculate the growth rate. Because of the limited culture volume, lactate, acetate and SO_4^{2-}, were measured at start and at the end of the experiment. Carbon dioxide production could not be measured because of too high background concentration from the carbonate-buffered growth medium and the carbon dioxide atmosphere in the cultures as shown by test measurements made at the start of the experiment. Three replicates were prepared for each series of lactate concentration. Two of them were sampled during the time of the growth experiment, see Table 2-4. All sampling were done with N_2-flushed syringes and needles and the samples were preserved until analysed.

AcRateH2

In Table 2-5, the times of sampling in experiment AcRateH2 are compiled together with sample volumes and analyses made. Since the aim of this experiment was to find the optimal acetate concentration and the maximum growth rate, samples from the different parts in a batch culture growth were needed. Since the growth pattern of *D. aespoeensis* on acetate and H_2 was unknown, samples were withdrawn at 10 occasions and from the TNC values it was decided that 8 of these samples were to be analysed for TNC and S^{2-}. The TNC and S^{2-} data were used to calculate the growth rate. Because of the limited culture volume, acetate and SO_4^{2-} were measured at the start and at the end of the experiment, sulphate in samples from the growth series with the highest sulphide production rate. Carbon dioxide production could not be measured because of too high background concentration from the carbonate buffered growth medium and the carbon dioxide atmosphere in the cultures.

Table 2-3. The amount of H_2 in the gas phase in H2RateAc experiments.

Experiments	Measured H_2 amount (‰)
H2*553	553 ± 6
H2*258	258 ± 14
H2*132	132 ± 2
H2*33	33 ± 2

Table 2-4. Sample dates and sample volumes in LacRate.

Date	Time (h)	Series A Volume (mL)	Series B Volume (mL)	Series C Volume (mL)	Analyses
2006-11-06	0	4	4	4	TNC, lactate, acetate, SO_4^{2-} and S^{2-}
2006-11-08	45	2	2	0	TNC and S^{2-}
2006-11-09	70	2	2	0	TNC and S^{2-}
2006-11-10	93	2	2	0	–
2006-11-11	119	2	2	0	–
2006-11-13	168	2	2	0	TNC and S^{2-}
2006-11-14	188	2	2	0	–
2006-11-15	219	2	2	0	TNC and S^{2-}
2006-11-17	262	2	2	0	TNC and S^{2-}
2006-11-20	334	4	4	0	TNC, lactate, acetate, SO_4^{2-} and S^{2-}
2006-11-21	358	–	–	4	TNC, lactate, acetate, SO_4^{2-} and S^{2-}

Table 2-5. Sample dates and sample volumes in AcRateH2.

Date	Time (h)	Series A Volume (mL)	Series B Volume (mL)	Series C Volume (mL)	Analyses
2006-12-01	0	4	4	4	TNC, acetate, SO_4^{2-} and S^{2-}
2006-12-04	68	2	2	0	TNC and S^{2-}
2006-12-05	92	2	2	0	TNC and S^{2-}
2006-12-06	120	2	2	0	–
2006-12-07	141	2	2	0	TNC and S^{2-}
2006-12-08	169	2	2	0	TNC and S^{2-}
2006-12-09	196	2	2	0	–
2006-12-11	236	2	2	0	TNC and S^{2-}
2006-12-12	266	2	2	0	–
2006-12-13	290	2	2	0	TNC and S^{2-}
2006-12-18	403	4	4	4	TNC, acetate, SO_4^{2-} and S^{2-}

H_2 was not measured in this experiment since the growth bottles were sampled repeatedly and by that the gas atmosphere was disturbed. To be able to keep the H_2-concentration at a non-limiting concentration during the experiment, H_2 was added to bottles in Series A and B at 120 h, 196 h and 266 h. The pressure in the bottles was 2 bar after the H_2 addition.

H2RateAc

In Table 2-6, the times of sampling in experiment H2RateAc are compiled together with analyses made. The design of this experiment was developed from the experiences found in LacRate and AcRateH2. In H2RateAC, the aim was to find the optimal H_2 concentration and the maximum growth rate for *D. aespoeensis* when grown on the optimum concentration of acetate. To be able to measure the H_2 consumption, separate tubes for each sample were prepared. In each sample, TNC, acetate, H_2 and S^{2-} were analysed. SO_4^{2-} was analysed at start and end of the experiment. Carbon dioxide production could not be measured because of too high background concentration from the carbonate buffered growth medium and the carbon dioxide atmosphere in the cultures.

2.2.6 Analytical methods

Total number of cells (TNC)

One mL samples were preserved with formaldehyde to a final concentration of 2%. The total number of cells was determined with the acridine orange stain method (AODC) according to Hobbie et al. (1977). The precision of the method is 5% (Niemelä 1983).

H_2S

One mL samples were preserved with NaOH and ZnAc according to the description of the method and stored until analysis. Total sulphide concentration was measured with the methylene blue method (Swedish Standard Method, SIS 028115).

SO_4^{2-}

One mL samples were stored frozen until analysis. SO_4^{2-} was measured with Hach Sulfate method 8051; Sulfaver 4, according to the manufacturer's instructions. The uncertainty of the measurement is 10% of a 30 mg L^{-1} sample according to the manufacturer. The sulphate concentration in the medium used had a concentration of 1.15 g L^{-1} and had to be diluted 20 times before measurements. The measured value will den be 1.15 ± 0.115 g L^{-1}.

Sulphate measurement with ISO 10304-1:2009 used by certified laboratories has an uncertainty of measurement of 15 – 20%.

Lactate

One mL samples were stored frozen until analysis. Lactate concentration was measured with D-Lactic acid/L-Lactic acid UV-method, Cat.no. 11 112 821 035, Boehringer Mannheim/R-Biopharm, Enzymatic BioAnalysis/Food (Food Diagnostics, Sweden) according to the manufacturer's instructions. The precision of the method is 10% according to the manufacturer.

Table 2-6. The time of sampling for the different growth series in H2RateAc. Note that each sampling point corresponds to one specific growth tube.

Date	Hours	Series A	Series B	Series C	Series D	Analyses
2007-04-30	0	1, 2, 3	1, 2, 3	1, 2, 3	1, 2, 3	TNC, acetate, H_2, S^{2-}, SO_4^{2-}
2007-05-03	72	1, 2, 3	1, 2, 3	1, 2, 3	1, 2, 3	TNC, acetate, H_2, S^{2-}
2007-05-04	96	1, 2, 3	1, 2, 3	1, 2, 3	1, 2, 3	TNC, acetate, H_2, S^{2-}
2007-05-07	168	1, 2, 3	1, 2, 3	1, 2, 3	1, 2, 3	TNC, acetate, H_2, S^{2-}
2007-05-08	192	1, 2, 3	1, 2, 3	1, 2, 3	1, 2, 3	TNC, acetate, H_2, S^{2-}
2007-05-10	244	1, 2, 3	1, 2, 3	1, 2, 3	1, 2, 3	TNC, acetate, H_2, S^{2-}
2007-05-15	364	1, 2, 3	1, 2, 3	1, 2, 3	1, 2, 3	TNC, acetate, H_2, S^{2-}, SO_4^{2-}
2007-05-15	364	1S, 2S, 3S	1S, 2S, 3S	1S, 2S, 3S	1S, 2S, 3S	TNC, acetate, H_2, S^{2-}, SO_4^{2-}

Acetate

One mL samples were stored frozen until analysis. Acetate concentration was measured with Acetic acid UV-method, Cat.no. 0 148 261, Boehringer Mannheim/R-Biopharm, Enzymatic BioAnalysis/Food Analysis (Food Diagnostics, Sweden) according to the manufacturer's instructions. The precision of the method is 10% according to the manufacturer. Samples with higher concentrations were diluted before analysis.

H_2

Sampling was done directly from the growth tubes and controls. One mL of the gas phase was withdrawn from the growth tubes with syringes and needles. H_2 was analysed by gas chromatography with thermal conductivity detector and Ar as carrier gas.

E_h

The oxygen-reduction potential (ORP) was measured with an ORP electrode Blue line 31 Rx from Schott Instruments. The measured potentials were corrected from the silver/silver chloride reference electrode (U_{meas}) to the standard hydrogen electrode system (U_H) by the following equation:

$$U_H = U_{meas} + U_{ref}$$

U_{ref} was set to 210 mV according to information from the manufacturer. U_H is the same as E_h.

pH

pH was measured with a pH electrode Blue Line 13, from Scott Instruments.

3 Results

3.1 LacRate – sulphate reduction with lactate

3.1.1 Sampling times for LacRate

Figure 3-1 shows the growth bottles for all LacRate SO_4^{2-} reduction experiments. For each lactate concentration a group of three replicates and a sterile control were prepared. The names of each group of replicates are the lactate concentration in µM. At the beginning of the experiment all three replicates were sampled. The intermediate samplings were done on two replicates, A and B. The third replica, C, was left growing until the last sampling occasion at 358 h. Replica A and B, were sampled the wlast time at 334 h.

The LacRate experiment was successful and the bacteria grew well at least in the two highest concentrations of lactate, see Figure 3-2 and Figure 3-3. Figure 3-4 shows a microscopic picture of *D. aespoeensis* at 334 hours. The turbidity of the growth cultures is both cells and precipitates. The LacRate11.3 culture showed the highest turbidity because of the densest growth and highest H_2S concentrations. Figure 3-3 shows the bottles of the sterile controls with clear growth medium.

3.1.2 TNC

Total number of cells (TNC) was determined with the AODC method at six occasions in replicas A and B. Figure 3-5 shows the results for all concentrations of lactate. The highest growth rates were found in series Lac11.3 and Lac1.13.

3.1.3 H_2S production

From the growth curve in Figure 3-5 appropriate times for measurement of H_2S from the preserved H_2S samples were chosen. H_2S production from replica series A and B were sampled during the experiment and the results are shown in Figure 3-6. The highest concentration was found in Lac11.3 with a maximum concentration of 170 mg L^{-1} (5.3 mM).

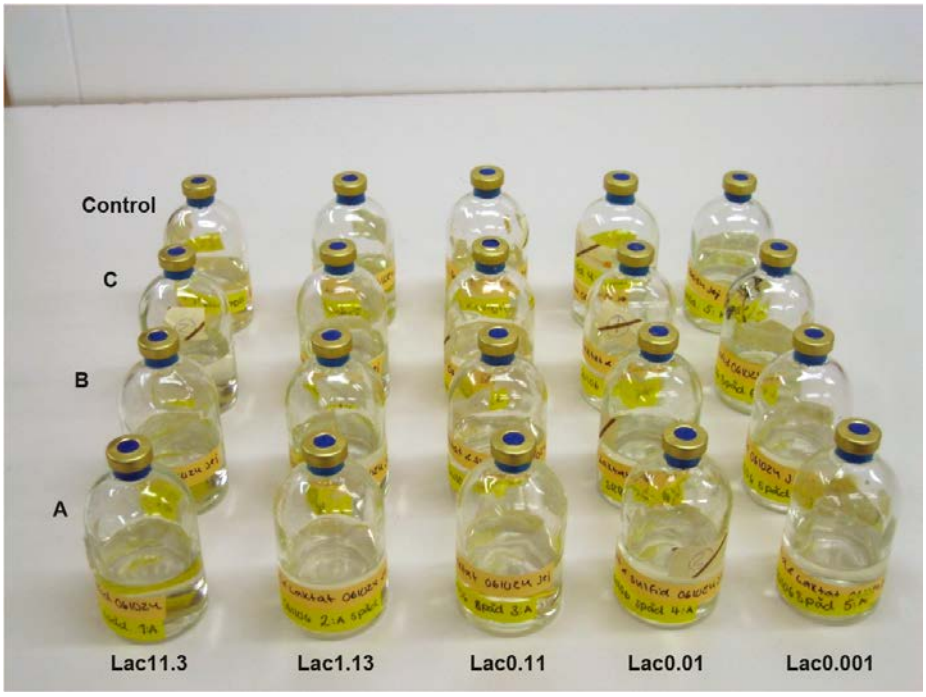

Figure 3-1. *LacRate − Experimental bottles and sterile controls 2006-11-06.*

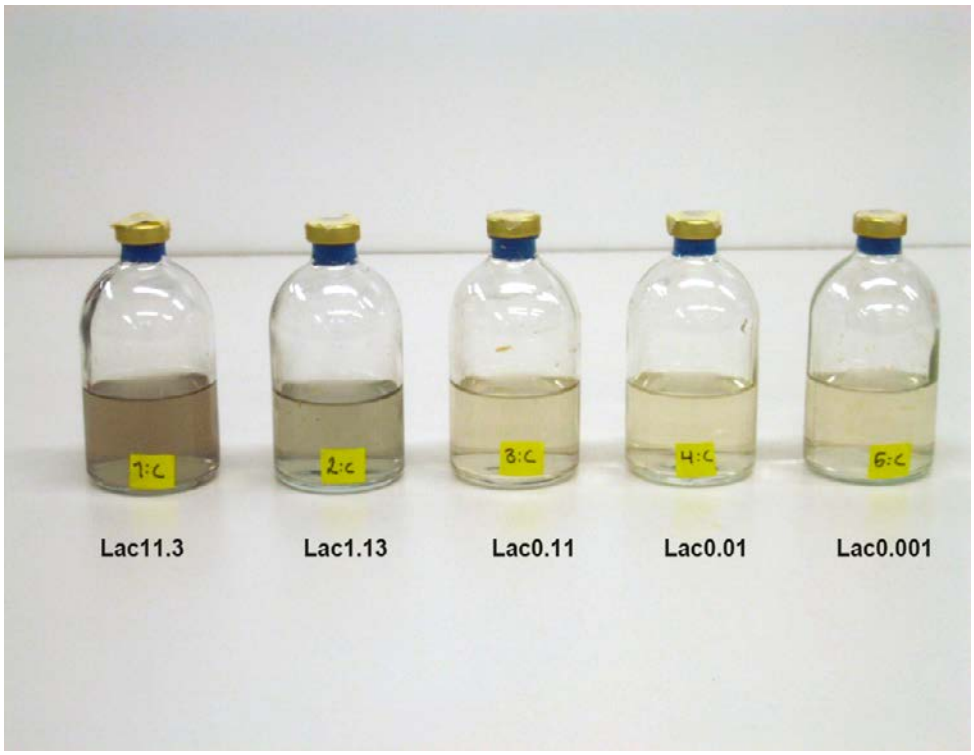

Figure 3-2. *Sulphate reduction experiment LacRate, replica C at 334 hours, not sampled during the experiment.*

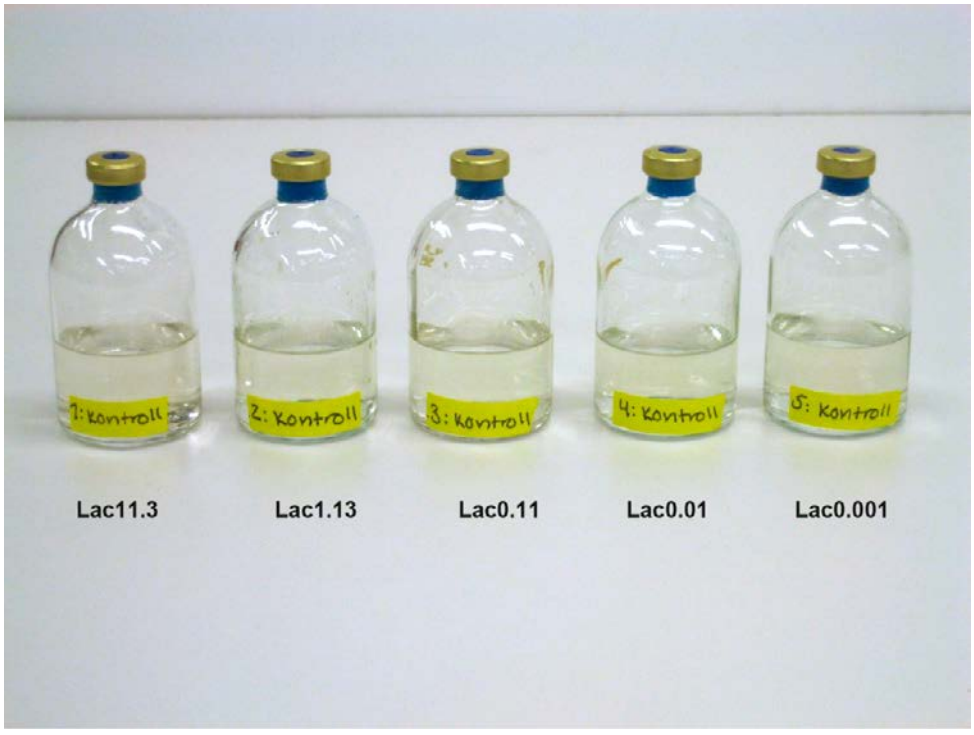

Figure 3-3. *Sulphate reduction experiment LacRate, control bottles 334 hours.*

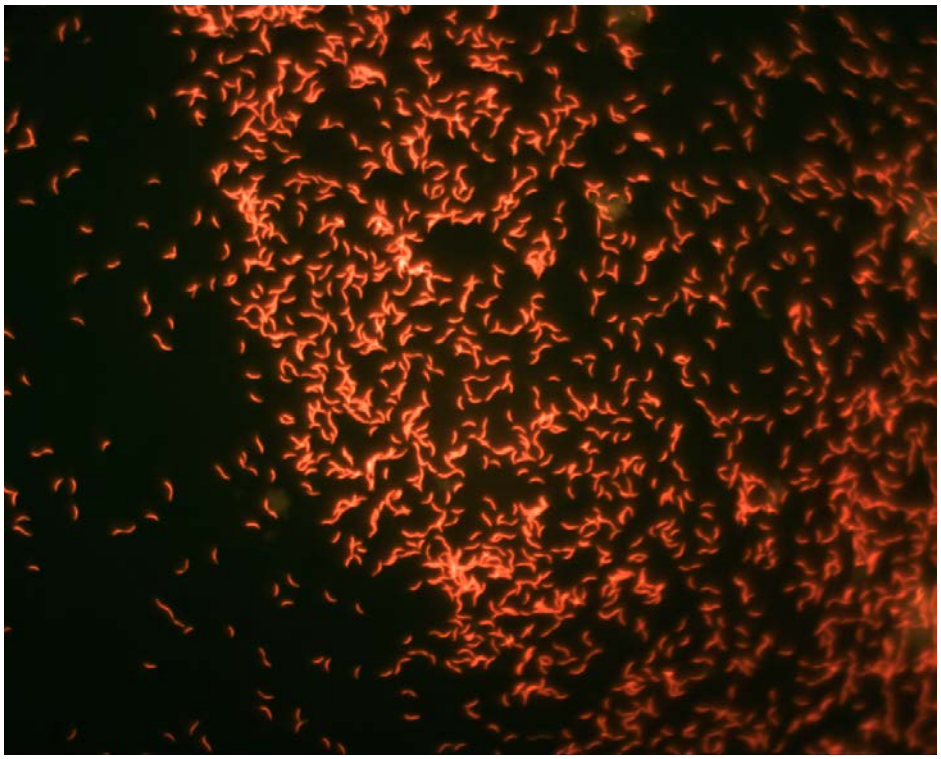

Figure 3-4. *Microscope photo of Desulfovibrio aespoeensis from one growth bottle after 334 hours in LacRate.*

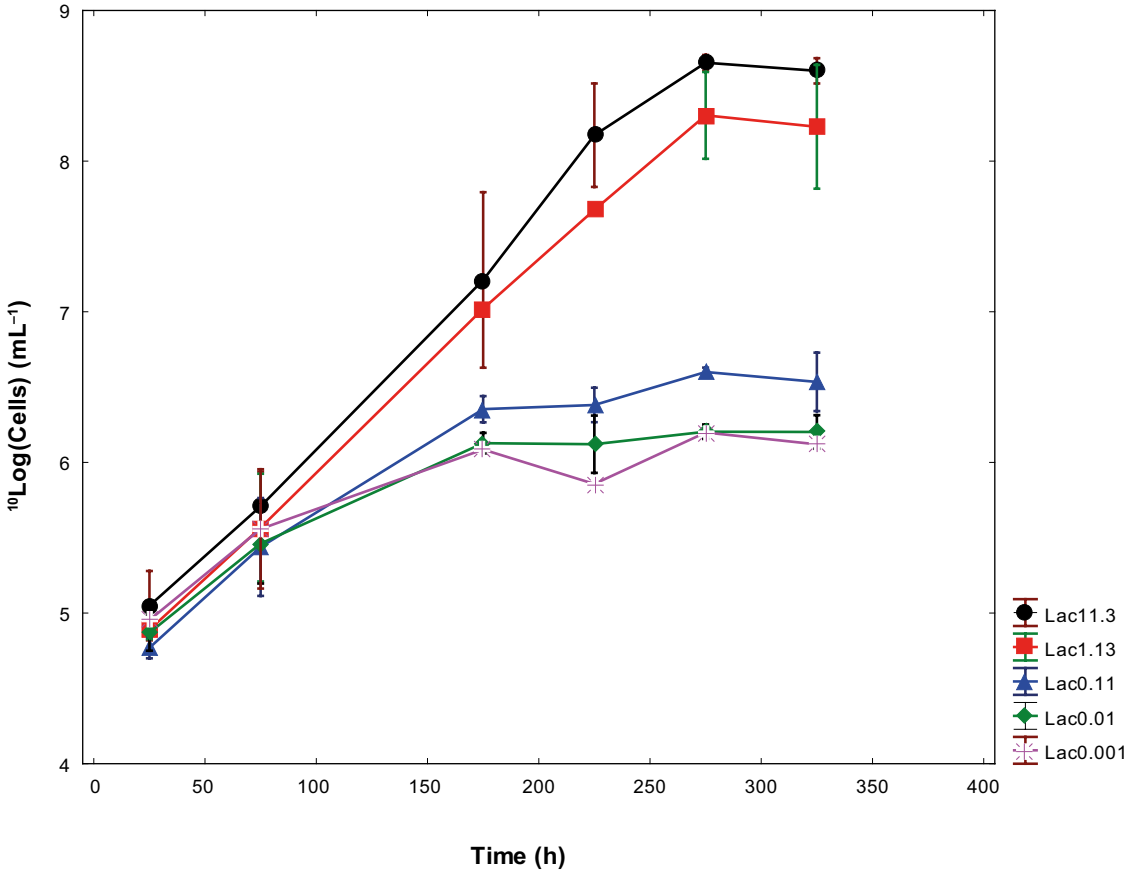

Figure 3-5. *TNC in the sulphate reduction experiment LacRate with lactate as carbon and energy source. Values are mean values of replicates A and B and the whiskers denote the sample standard deviation.*

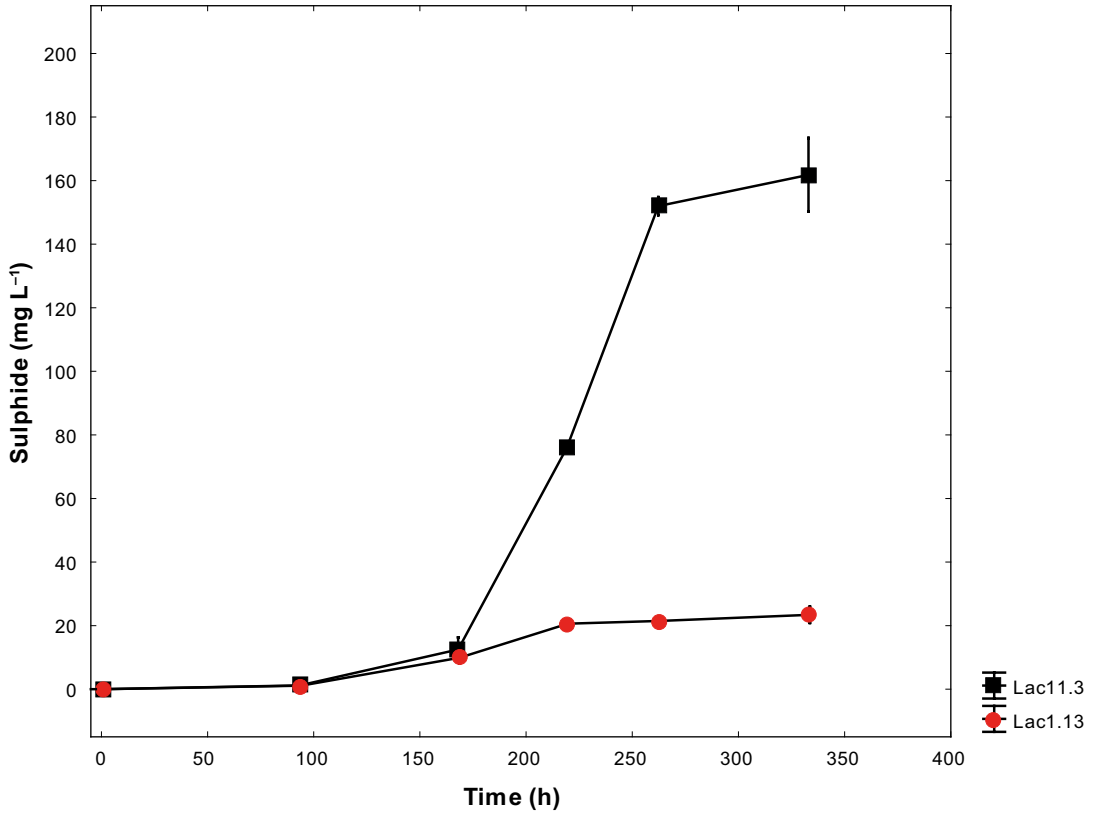

Figure 3-6. *H_2S production in sulphate reduction experiment LacRate, Lac11.3 and Lac1.13. Values are mean values of replicas A and B and the whiskers denote the sample standard deviation.*

3.1.4 Stoichiometry of the reaction

The chemical reaction of lactate oxidation by reduction of SO_4^{2-} by *D. aespoeensis* is shown in Table 3-1. In the table both the stoichiometric relation and the measured values are shown.

Anaerobic microbiology requires different sampling techniques than when working aerobically. In the anaerobic technique all samples have to be withdrawn with syringes and needles. This method is less accurate than measurements with automatic pipettes.

The difference in the theoretical and measured lactate and acetate values is less than 10%. This difference is probably due to the volume in the growth bottles that could differ with about 10% between the bottles. The discrepancy between the measured sulphate value and measured produced H_2S has two reasons that coincide, the accuracy of the sulphate analysis and that the H_2S are found both in the gas phase and in solution. For the most accurate measurement of the H_2S concentration, the sample, in this case the whole growth bottles had to be preserved with NaOH and ZnAc as in the methylene blue method (see Section 2.2.6). In this experiment the samples were continuously withdrawn from the growth bottles over the growth period and therefore subsamples had to be taken and preserved until analyses.

In LacRate the maximum H_2S production was found at a lactate concentration of 14.5 mM, which also was the maximum concentration in this experiment. Since it was the highest concentration used it is not known if an increased concentration of lactate would increase the growth rate and the produced sulphide. Too high concentrations of organic acids can inhibit growth as could be seen for the highest concentrations of acetate in AcRateH2. Lac11.3 gave 170 mg L^{-1} (5.3 mM) of H_2S after 334 hours and had a sulphide production rate of 1.5 mg L^{-1} h^{-1} (46 µM h^{-1}) in the exponential growth phase of the cultures which lasted between 93 and 168 h. The H_2S production rate for a ten times lower lactate concentration was 0.15 mg L^{-1} h^{-1} (5 µM h^{-1}) and the maximum concentration produced after 334 hours was 23.4 mg L^{-1} (0.73 mM). The tenfold increase in lactate concentration gave a tenfold increase in the sulphide production rate. The maximum H_2S concentration was almost

Table 3-1. The stoichiometry of the oxidation of lactate with sulphate. Theoretical and measured in LacRate, series Lac11.3, mean values of replicate series A and B.

	Reactants		Products			
Theory	2 lactate	SO_4^{2-}	2 acetate	2 hydrogen carbonate	H_2S	2 proton
Lac11.3	14.5 mM	7.25 mM	13.2 mM	n.a.	5.1 mM	n.a.

n.a. = not analysed

10 times higher in growth series Lac11.3 (see Figure 3-6) than in Lac1.13. The total number of cells (TNC) at 334 h in Lac11.3 and Lac1.13 did not differ significantly (Figure 3-5). This shows that the sulphide production rates and maximum concentrations in cultures grown on lactate are dependent on the available energy source and not on the TNC.

3.2 AcRateH2 – sulphate-reduction with acetate and excess H_2

3.2.1 Sampling times

The aim of this experiment was to establish the optimal acetate concentration for *D. aespoeensis*. Figure 3-7 shows the experimental set up for AcRateH2 at start. The names of the experiments are the acetate concentrations in mM. Samples for acetate analysis were taken from all growth bottles at start. The intermediate samplings were done on replicas A and B. The series of replicas C was left growing until the last sampling at 403 h.

3.2.2 TNC

The bacteria grew in all concentrations of acetate in the AcRateH2 experiment, see Figure 3-8. The turbidity of the growth cultures is cells but also precipitates. Growth series Ac2.0 had a slightly higher growth rate than the other series. The lowest growth rate was found in Ac44 followed by Ac22. Ac0.02 had produced the lowest amount of cells at the end of the experiment followed by Ac44.

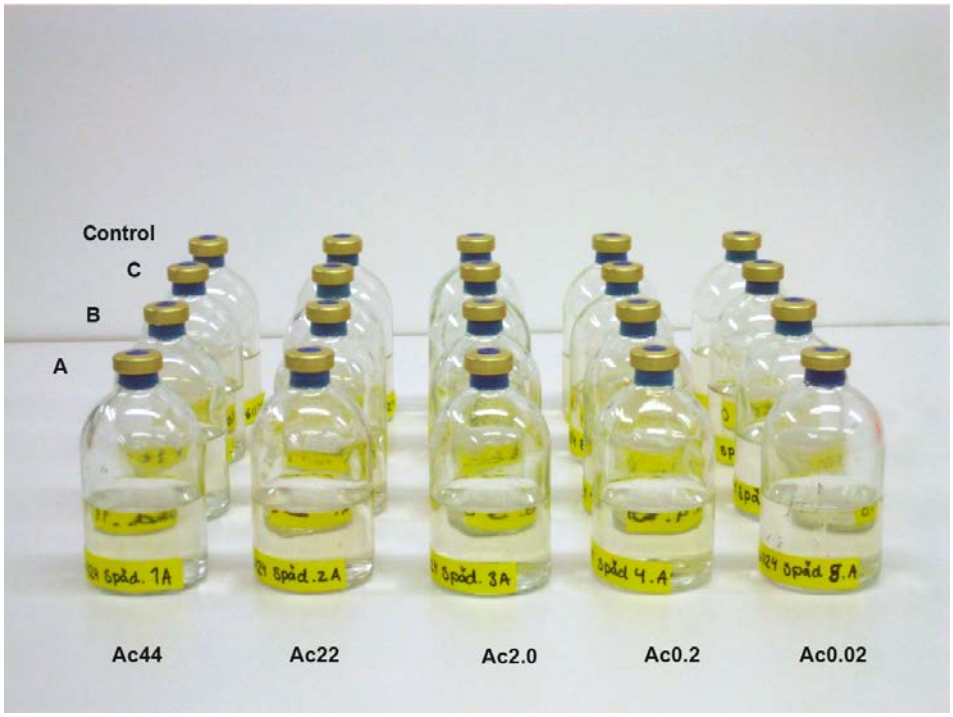

Figure 3-7. *AcRateH2 – Experiment bottles and sterile controls 2006-12-01.*

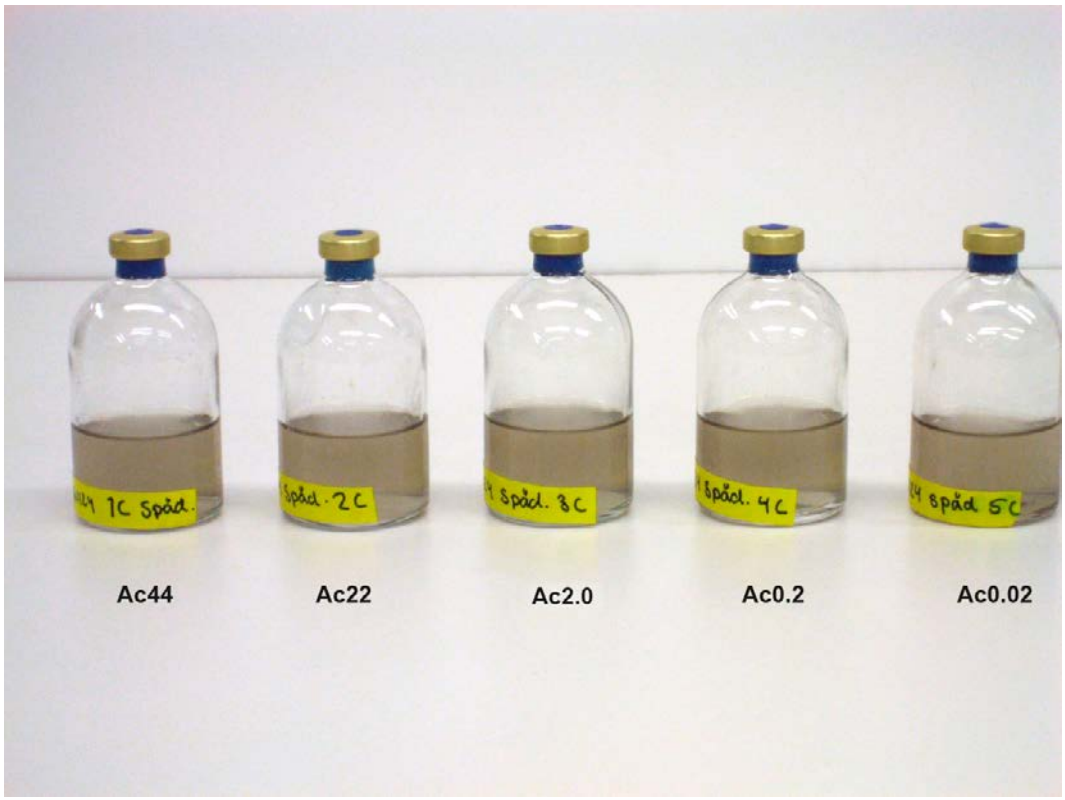

Figure 3-8. *AcRateH2 – Replicate series C at 403 hours, not sampled during the experiment.*

3.2.3 H_2S production

From the growth curve in Figure 3-9 appropriate times for measurement of H_2S from the preserved H_2S samples were chosen. H_2S concentrations for the whole series of experiments are shown in Figure 3-10. The highest production of H_2S was found in experiment Ac2.0 with a maximum concentration of 247 mg L^{-1} (7.7 mM) in replica A and 285 mg L^{-1} (8.9 mM) in replica C.

3.2.4 Stoichiometry of the reactions

The chemical reactions of the growth on acetate and H_2 are coupled to H_2 oxidation with SO_4^{2-} reduction but also to a reaction with electrons from H_2 oxidation that reduce CO_2 in a reaction where CO_2 and acetate-CoA, where CoA is co-enzyme A, produce pyruvate. Equation 3-1 below describes the energy yielding reaction and Equation 3-2 the carbon assimilation reaction.

$$4H_2 + SO_4^{2-} + H^+ \rightarrow HS^- + 4H_2O \qquad \text{Equation 3−1}$$

$$CH_3COO\text{-}CoA + CO_2 + H_2 \rightarrow CH_3COCOO^- + CoA + H_2O \qquad \text{Equation 3−2}$$

The measured stoichiometry of the SO_4^{2-} reduction was 12.24 mM consumed SO_4^{2-} and 8.1 mM produced H_2S. The discrepancy between the SO_4^{2-} and H_2S values can be explain by the same reasons as for the values in LacRate, see discussion of the results in LacRate.

For H_2 oxidation with acetate as carbon source the optimum concentration of acetate was 118 mg L^{-1} (2 mM) with the maximum H_2S concentration of 285 mg L^{-1} (8.9 mM) in Ac2.0:C and 247 mg L^{-1} (7.7 mM) in Ac2.0:A after 403 hours. In experiment Ac2.0 the highest average sulphide production rate was 1.4 mg L^{-1} h^{-1} (43 µM h^{-1}) in the exponential growth phase of the cultures. This lasted between 92 and 236 h. The two experiments with lower concentrations of acetate, Ac0.2 and Ac0.02, as well as the two with higher concentrations than Ac2.0, i.e. experiments Ac44 and Ac22, showed lower sulphide production rates than Ac2.0 up to 236 hours. Between 236 and 403 hours the sulphide production rate in Ac44, was 1.2 mg h^{-1} (40 µM h^{-1}) which is almost as high as the rate in Ac2.0.

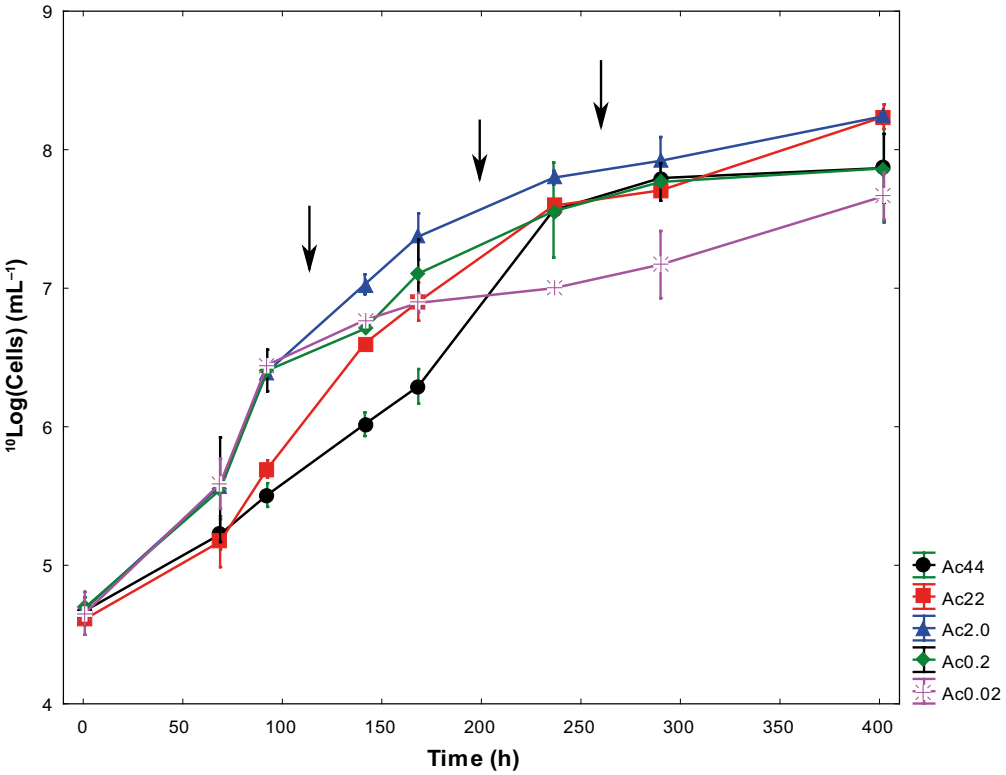

Figure 3-9. *TNC in sulphate reduction experiment AcRateH2, with acetate as carbon source and H_2 as energy source. Values are mean values of replicate series A and B and the whiskers denote the sample standard deviation. Arrows show when H_2 was added into the growth bottles.*

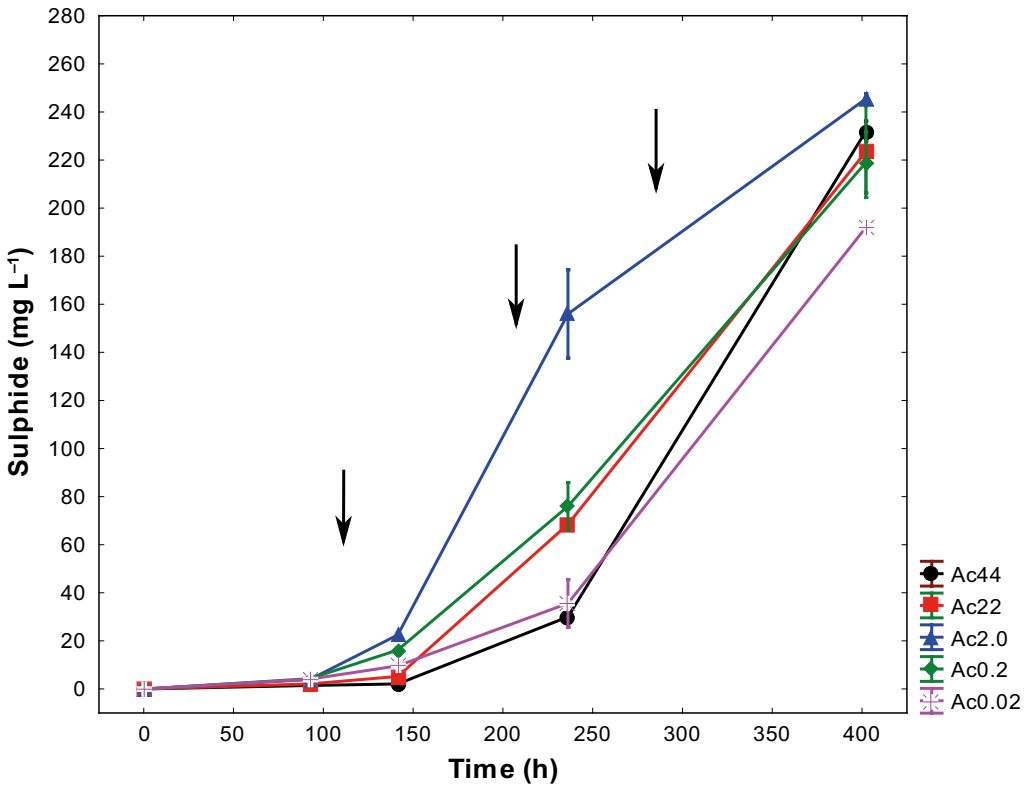

Figure 3-10*. H_2S production in the AcRateH2 experiments with acetate and H_2. Values are mean values of replicates A and B and the whiskers denote the standard deviation. Arrows show when H_2 was added into the growth bottles.*

This increase in sulphide production rate was observed in all experiments, except in Ac2.0. The late increase in the rate resulted in H_2S concentrations over 200 mg L^{-1} (6.25 mM) in the other experiments as well, except for the lowest acetate concentration, Ac0.02, which reached 135 mg L^{-1} (4.2 mM). It has to be noted that also in the experiment with ten times lower concentration, Ac0.2, the concentration of H_2S reached over 200 mg L^{-1} (6.25 mM) at the end of the experiment. Important to note is that the H_2 concentration was high along all experiments and probably not limiting. It is the H_2 that is energy and electron source while the acetate is incorporated into biomass and therefore not directly involved in H_2S production even though some H_2 oxidation is needed for the incorporation of acetate into biomass, see Section 1.1.2.

Figure 3-11 shows the sulphide production rate for the time period 92 to 236 hours, the time period with exponential growth. During this period Ac2.0 had its highest sulphide production rate (Figure 3-10). This curve would look different if another time period was chosen.

The prolonged lag-phase of especially experiments Ac44 and Ac22 but also to some extent Ac0.2 and Ac0.02, is probably a result of adaptation to both high ionic strength as in Ac44 and Ac22 but also due to a lower concentration of the carbon source as in Ac0.2 and Ac0.02.

3.3 H2RateAc – sulphate-reduction with varied H_2 and fixed acetate concentrations

H2RateAc – Growth of *D. aespoeensis* on varied H_2 and fixed acetate concentrations at room temperature. The aim of the laboratory experiments was to measure minimum and optimum initial concentration of H_2 for *D. aespoeensis* when grown on the optimal concentration of acetate of 2.0 mM at room temperature. The optimum acetate concentration of 2.0 mM was established in the growth experiment AcRateH2 (see Section 3.2). Four different H_2-concentrations were used in the experiment, 553, 258, 132 and 33 ‰ of the gas phase in the growth tubes.

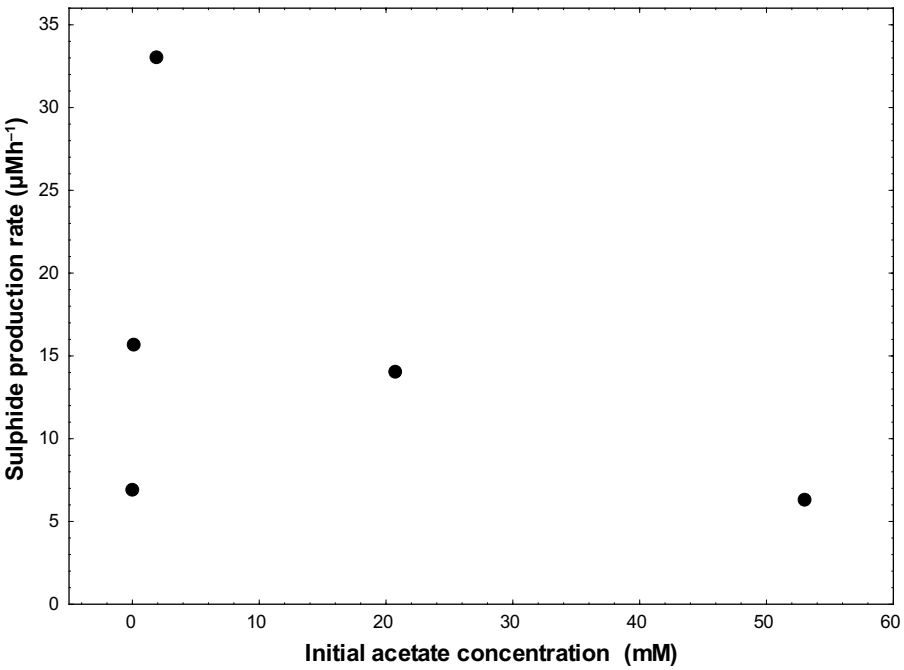

Figure 3-11. *The maximum sulphide production rate in the exponential growth phase versus the initial acetate concentration in AcRateH2 for the time period 92 to 236 hours. Note that these data would look different if another time period was chosen.*

3.3.1 Sampling times for H2rateAc

The growth tubes were sampled at seven occasions, according to the growth curve obtained in AcRateH2 (see Section 3.2). For each hydrogen concentration level three replicate growth tubes were sampled at each sampling occasion (4 × 3 × 8 = 96). For each hydrogen level concentration three sterile tubes (4 × 3 = 12) were prepared and sampled at the end. The experiment included 96 inoculated tubes and 3 sterile tubes for each growth series, 12 sterile tubes (3 for each H_2 concentration), which gave a total of 108 tubes.

The three replicate tubes for each H_2 concentration were sampled at every sampling occasion except the sterile controls that were only sampled at the last occasion at 364 h. The time of samplings are found in Figure 3-12.

3.3.2 TNC

The results from the H2RateAc experiment are presented below. The bacteria grew in all concentrations of hydrogen, see Figure 3-12. Experiments H2*553 and H2*258 had a slightly higher growth rate than the other two, H2*132 and H2*33. The number in the names of the experiments refers to the H_2 concentration in ‰ of the gas phase in the growth tubes.

3.3.3 H_2S

From the growth curve in Figure 3-12 appropriate times for analyses of the preserved H_2S samples were chosen. H_2S production from all replica series is shown in Figure 3-13. At the end of the experiment the highest production of H_2S was found in series H2RateAc with a maximum concentration of 209 mg L^{-1} (6.53 mM) in H2*553:3. There was also high concentration of H_2S in H2*258:3 with 195 mg L^{-1} (6.09 mM).

Figure 3-14 shows the H_2 concentration over time for the four experiments in H2RateAc. It can be seen that in H2*33 the H_2 was totally consumed and in H2*132 it was only a minor part of the H_2 left.

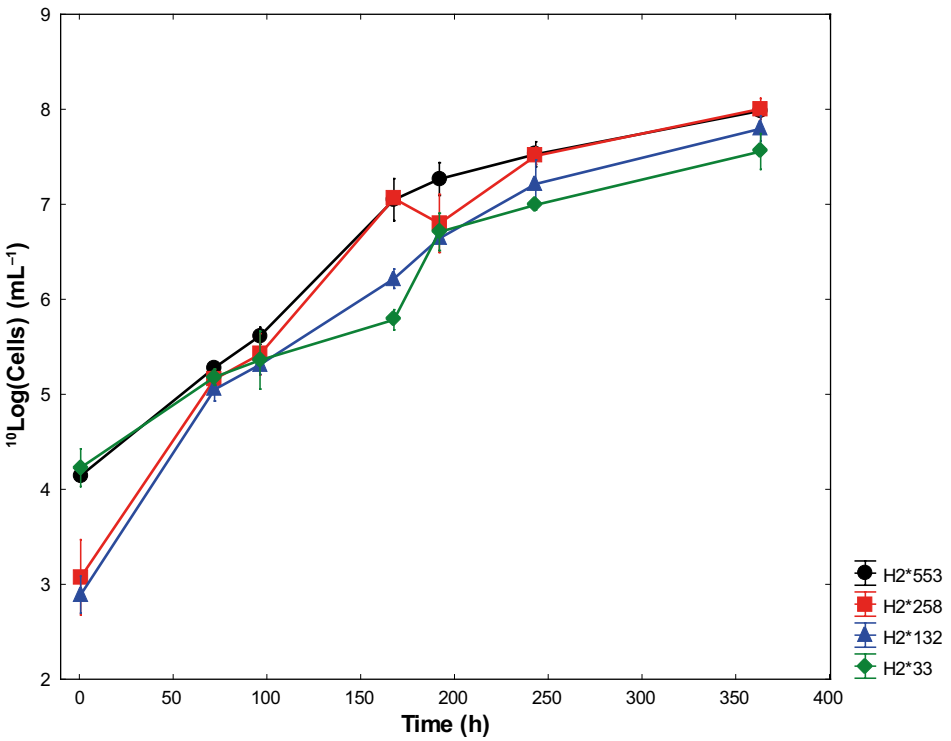

Figure 3-12. *TNC in the sulphate reduction experiment H2RateAc with varied H_2 concentration as energy source and fixed acetate concentration (2mM) as carbon source., Values are mean values of replicate series A and B and the whiskers denote the sample standard deviation.*

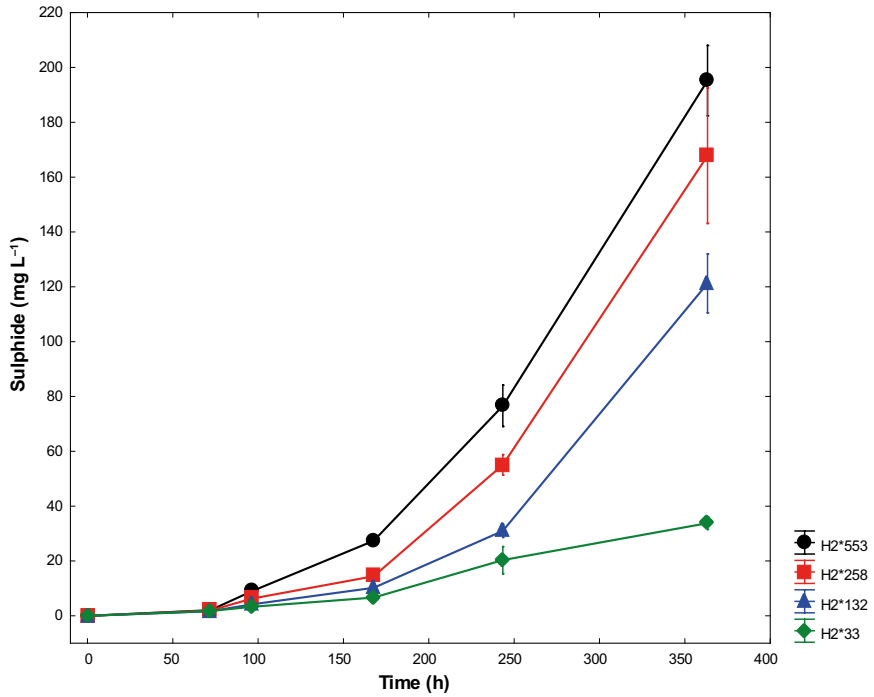

Figure 3-13. *H_2S production in the different experiments in H2RateAc with varied H_2 concentration and fixed acetate concentration (2mM). Values are mean values of replicate series A, B and C and the whiskers denote the sample standard deviation.*

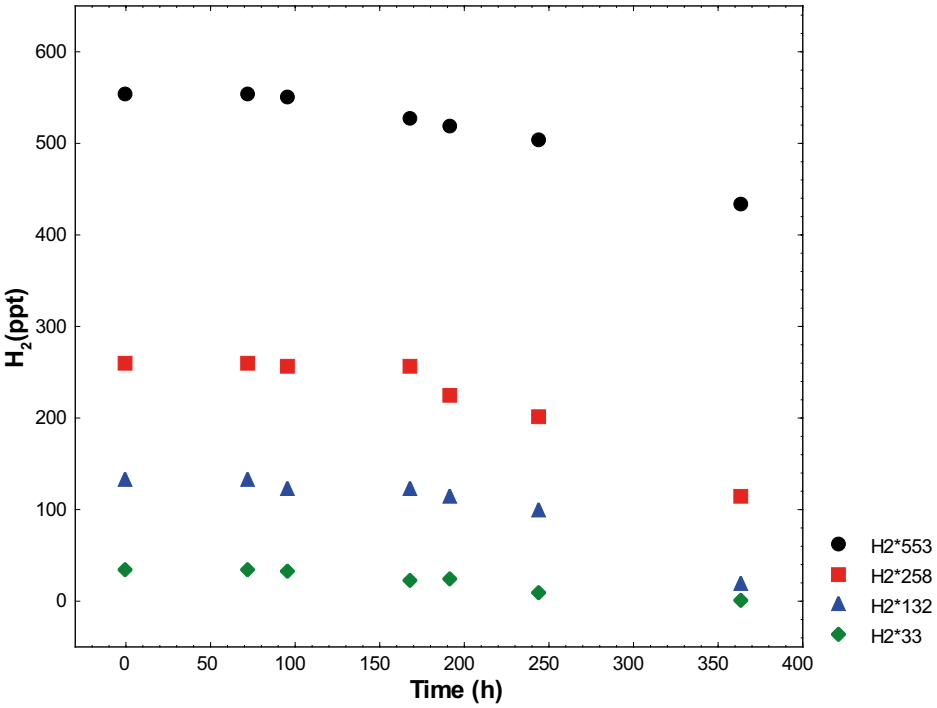

Figure 3-14. *H_2 consumption in the sulphate reduction experiment H2RateAc. Values are mean values of replicates A, B and C.*

The acetate concentrations in the H2RateAc experiments are shown in Figure 3-15. In the three experiments with the highest concentration of H_2 about the same amount of acetate was consumed but less was consumed in H2*33, which correspond to the limited supply of H_2 in this experiment as seen in Figure 3-14.

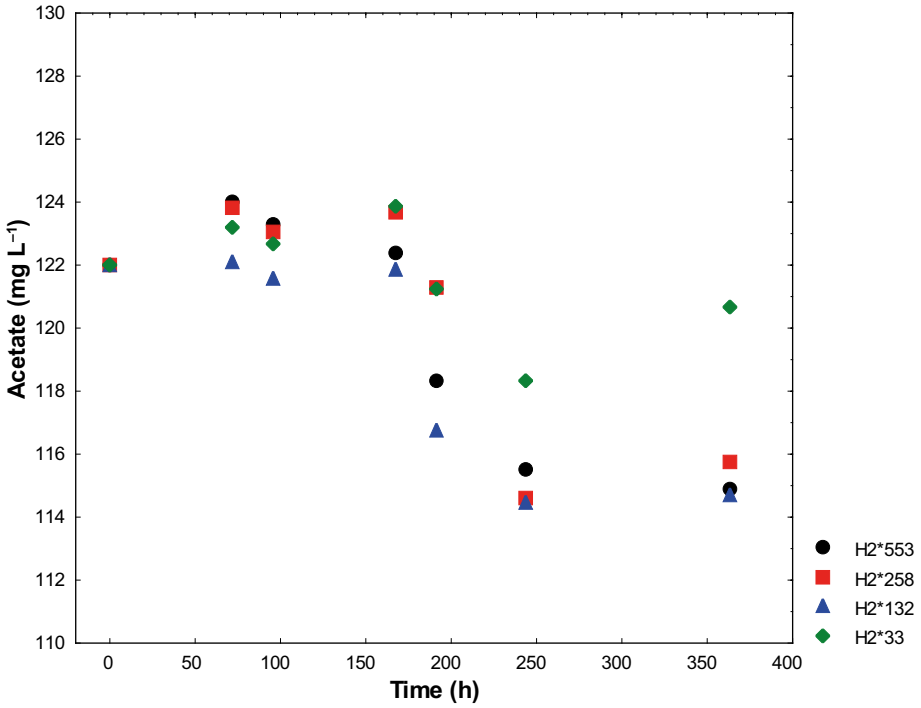

Figure 3-15. *Acetate consumption in the sulphate reduction experiment H2RateAc. Values are mean values of replicates A, B and C.*

3.3.4 Stoichiometry of the reactions

The sulphate reduction reactions using acetate and H_2 are coupled to H_2 oxidation with SO_4^{2-} reduction but also to a reaction with electrons from H_2 oxidation that reduce CO_2 in a reaction where CO_2 and acetate-CoA, produce pyruvate. The energy yielding reaction is:

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

see Equation 2-1, and the carbon assimilation reaction is:

$$CH_3COO\text{-}CoA + CO_2 + H_2 \rightarrow CH_3COCOO^- + CoA + H_2O$$

In the following tables the measured stoichiometry of the reactions for the growth series are shown.

Acetate consumption

The value used as the start concentration is the mean values of the measured acetate concentration from all 12 tubes sampled on t=0 h. This value was 122 mg L^{-1} (standard deviation, SD ± 1.8) which corresponds to 2.0 mM, SD ± 0.03 of acetate. In Table 3-2, the mean values of the consumed amounts of acetate are shown for the four growth series.

Table 3-2. The mean start and end concentrations ± the standard deviation of acetate in the sulphate reduction experiment H2RateAc.

Experiment	Start concentration (mM)	End concentration (mM)	Consumed amount (mM)
H2*553	2.0 ± 0.03	1.92 ± 0.003	0.08 ± < 1.5%
H2*258	2.0 ± 0.03	1.93 ± 0.004	0.07 ± < 1.5%
H2*132	2.0 ± 0.03	1.90 ± 0.0008	0.10 ± < 1.5%
H2*33	2.0 ± 0.03	2.0 ± 0.001	0.03 ± < 1.5%

H_2 consumption

The measured H_2 data are treated as mean values of three replicates from each of the four sulphate reduction experiments (initial concs. of H_2). The data are presented in Table 3-3. The unit measured is parts per thousand, ‰, of the gas phase in the growth tubes. Because of technical problems with the analyses at t=0 h, data from t=72 hours are used as start values. In order to do this, the value for series A was compared to the A series in two sterile bottles and it was concluded that no or only a minor amount of H_2 was consumed during the first 72 hours of the experiment.

Below and in Table 3-4 the conversion of data from (‰) in the gas phase to mmol of gas per tube is described. The volume of the gas phase in the tubes was 16 mL. The pressure in the tubes was 2 bars so the gas volume at one atmosphere pressure was 32 mL. The constant (RT) for converting gas volume to concentration in mole at 22°C is 24.2 $dm^3\ mol^{-1}\ K^{-1}$, as calculated from the law of ideal gas, PV = nRT.

H_2S production

The H_2S data are treated as mean values of the replicates from each experiment (initial amount of H_2). The data are presented in Table 3-5.

SO_4^{2-}- consumption

The SO_4^{2-} data are treated as mean values of the replicates from each experiment (initial amount of H_2). The data are presented in Table 3-6.

The discrepancy between the consumed SO_4^{2-} and produced H_2S is the same as in LacRate and AcRateH2, the accuracy of the methods and the sampling of H_2S which is in both soluble and gaseous phase. To be able to compare the values of the gaseous H_2 with the water dissolved compounds some calculations and comparisons of the different compound were done and compiled in the tables below. In Table 3-7 the amounts of consumed SO_4^{2-} is compared with produced H_2S. Table 3-8 shows the consumed amounts of acetate in the growth tubes.

The aim of H2RateAc experiment was to establish the optimum concentration of H_2 by measuring the sulphide production rate. The acetate concentration was 118 mg L^{-1} (2 mM) as determined from AcRateH2, see Section 3.3. Exponential bacterial growth occurred between 72 and 168 hours and the maximum sulphide production rate was measured during these hours. The rate was 8.4, 4.1, 2.8 and 1.47 µM h^{-1} (269, 131, 90 and 47 µg $L^{-1}\ h^{-1}$) for the experiments H2*553, H2*258, H2*132 and H2*33, respectively. The relation between hydrogen concentration and the sulphide production rate is shown in Figure 3-16. The relation does not fit well at very low levels of hydrogen as revealed by the intercept of 0.98.

It was only in H2*33 that the H_2 concentration was limiting for growth and H_2S production given the condition of 2 mM acetate (Figure 3-12). It has to be considered that this experiment was done with individual growth tubes for each sampling occasion. From that point of view the results must be regarded as highly sufficient for an introductory experiment. For future experiments our suggestion is to develop continuous culture equipment where pressure and gas concentrations can be controlled during the experiments.

Table 3-3. The mean H_2 proportion of the gas phase ± SD at start and end in the sulphate reduction experiment H2RateAc. SD = sample standard deviation.

Experiment	Start concentration (‰)	End concentration (‰)	Consumed amount (‰)
H2*553	553 ± 6	432 ± 12	121 ± < 5%
H2*258	258 ± 14	114 ± 9	144 ± < 5%
H2*132	132 ± 2	19 ± 5	113 ± < 5%
H2*33	33 ± 2	b.d.*	33 ± < 5%

*b.d. = below detection

Table 3-4. Conversion of gas data from ‰ in the gas phase to mmol of gas per growth tube in the sulphate reduction experiment H2RateAc.

Experiment	Consumed amount of H_2 (ppt)	Consumed amount of H_2 per tube (mmol)
H2*553	121 ± < 5%	0.160 ± < 5%
H2*258	144 ± < 5%	0.189 ± < 5%
H2*132	113 ± < 5%	0.149 ± < 5%
H2*33	33 < 5%	0.044 ± < 5%

Table 3-5. The mean start and end concentrations ± SD of H_2S in replicates A–D in the sulphate reduction experiment H2RateAc. SD = standard deviation.

Experiment	Start concentration (mg L^{-1})	Produced amount (mM)
H2*553	0	6.1 ± 0.4
H2*258	0	5.2 ± 0.8
H2*132	0	3.8 ± 0.3
H2*33	0	1.1 ± 0.06

Table 3-6. The mean start and end concentrations ± SD of SO_4^{2-} in the sulphate reduction experiment H2RateAc. SD = standard deviation.

Experiment	Start concentration (mM)	End concentration (mM)	Consumed amount (mM)
H2*553	14.1 ± 1.7	7.5 ± 0.5	6.6 ± < 12%
H2*258	14.1 ± 1.7	7.5 ± 0.9	6.6 ± < 12%
H2*132	14.1 ± 1.7	7.8 ± 0.6	6.3 ± < 12%
H2*33	14.1 ± 1.7	10.5 ± 0.3	3.6 ± < 12%

Table 3-7. The consumed SO_4^{2-} and the produced H_2S and their quota in the experiment H2RateAc. The highest standard deviation in these figures is < 12%.

	Reactants		Products		
Experiment	SO_4^{2-} (mM)	SO_4^{2-} (µmol $tube^{-1}$)	H_2S (mM)	H_2S (µmol $tube^{-1}$)	S^{2-}/SO_4^{2-}
H2*553	6.6	66	6.1	61	0.92
H2*258	6.6	66	5.2	52	0.79
H2*132	6.2	62	3.8	38	0.61
H2*33	3.5	35	1.1	11	0.31

Table 3-8. The amount of acetate consumed in H2RateAc.

Experiment	Acetate (mM)	Acetate (µmol $tube^{-1}$)
H2*553	0.08 ± < 1.5%	0.8 ± < 1.5%
H2*258	0.07 ± < 1.5%	0.7 ± < 1.5%
H2*132	0.10 ± < 1.5%	1.0 ± < 1.5%
H2*33	0.03 ± < 1.5%	0.3 ± < 1.5%

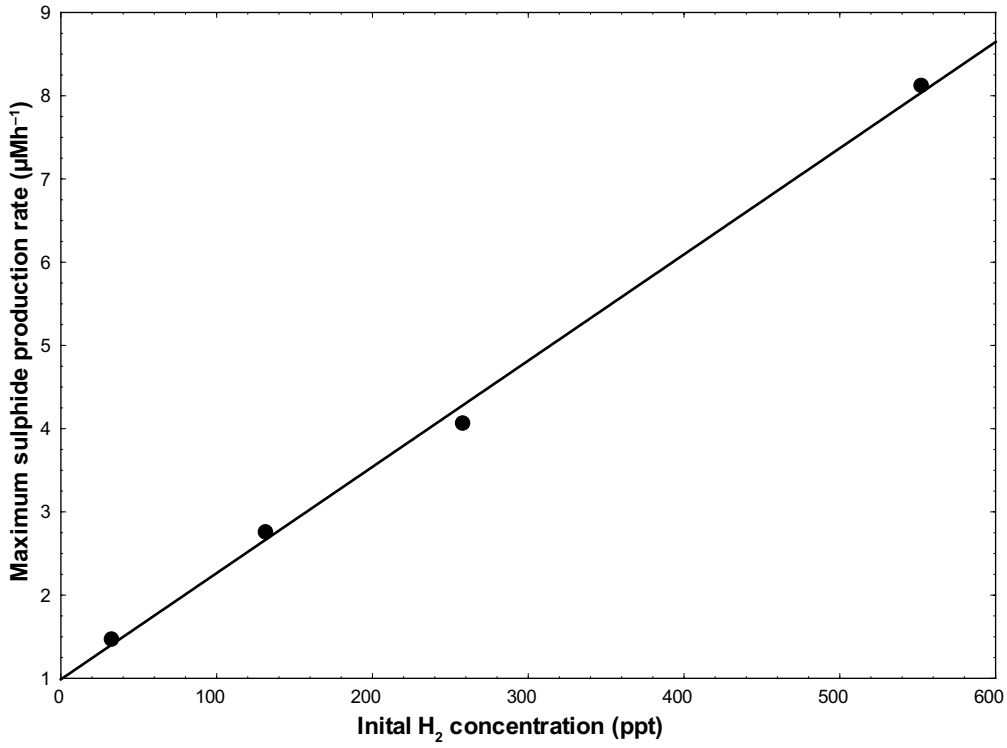

Figure 3-16. *The maximum sulphide production rate versus the initial H_2 concentration in the gas phase in the H2RateAc experiments. The rate was measured between 72 and 168 hours. The relation is described by the equation y=0.98 + 0.013x with r=0.9987 and significant at p=0.0013.*

E_h and pH measured in sterile controls and growth tubes from the experiment H2rateAc

E_h

Redox measurements in the H2rateAc experiment were done in sterile controls and growth tubes that were sampled 2007-05-15. The tubes with growth had been standing after sampling with a volume of about 6 mL in 20°C until E_h measurements but not opened. It is important to notice that the ratios of liquid/gas volumes were different compared to the circumstances during bacterial growth and sulphate reduction.

The data are found in Table 3-9 and Table 3-10.

pH

After Eh was measured pH was measured in the same tubes see Table 3-9 and Table 3-10. The values from the sterile tubes are averages with sample standard deviation of three tubes. The tubes from the replicas A-D had to be merged because of the small volumes left after sampling for the other parameters. No standard deviation value is therefore available.

It has to be noted that the tubes were opened on 2007-05-15 for the E_h measurement and pH was measured the next day. The carbon dioxide/carbonate system was disturbed and may have affected pH compared to the system in closed tubes. pH was also measured in growth tubes sampled 2007-05-08. These tubes were not opened during growth. The data are shown in Table 3-11.

Table 3-9. E_h calculated from ORP and pH measured in sterile control tubes from H2RateAc, at time t=364h. Values show the average of measuring three replicate samples.

Sample	E_h (mV)	pH
H2*553 sterile	−395	7.45 ± 0.006
H2*258 sterile	−348	7.47 ± 0.1
H2*132 sterile	−340	7.33 ± 0.08
H2*33 sterile	−254	7.45 ± 0.2

Table 3-10. E_h calculated from ORP and pH measured in growth tubes from H2RateAc, at t=364h. Single measurements of the mixture of replicas A-D.

Sample	E_h (mV)	pH
H2*553	−288	7.78
H2*258	−243	7.72
H2*132	−215	7.57
H2*33	−185	7.41

Table 3-11. pH measured in growth tubes from H2RateAc, at t=192h. Values show the average of measuring three replicate samples.

Sample	pH
H2*553	7.31 ± 0.03
H2*258	7.32 ± 0.03
H2*132	7.16 ± 0.01
H2*33	7.06 ± 0.006

Important aspects on the measured E_h and pH values

The following points have to be noted:

- The growth medium was redox buffered with cysteine which has to be taken in consideration when interpreting differences and changes during the experiments.
- Possibly H_2 can react with the redox electrodes.
- The medium is pH buffered with HCO_3^-. Since this buffer is in equilibrium with the gaseous CO_2, changes in gas and liquid volumes and gas pressure will affect pH.
- The produced H_2S will affect pH since it is a base that will participate in the acid-base reactions during growth.
- E_h measurements made in the site investigations in Sweden are considered to have an error of ± 50 mV. This great error could not be seen in the measurements of the redox references in the laboratory. However this should be kept in mind when interpreting the measured E_h data in this experiment.

4 Discussion and conclusions of the laboratory experiments

Recent work in Äspö HRL has shown that acetate and H_2 are efficient energy and carbon sources for SO_4^{2-} reduction in deep groundwater (Hallbeck and Pedersen 2008a, Pedersen 2012a, b). In the experiments presented in this report the growth- and sulphide production rates for different concentrations of lactate, acetate and H_2 for SRB were determined. These parameters were used in modelling of the H_2S production. In the experiments it was shown that growth on acetate and H_2 gave the highest amounts of H_2S.

4.1 Comparison of cell growth and H_2S production between different energy sources

The production of H_2S, both the sulphide production rate and the total amount of H_2S that can be produced, is dependent on the type and concentration of energy source. This is illustrated by some correlation plots. For sample standard deviation values of the data used in the figures see Figure 3-5, Figure 3-6, Figure 3-10, Figure 3-11, Figure 3-13 and Figure 3-14.

The first correlation studied is that between the TNC and the H_2S concentration. Figure 4-1a and b show the correlations for LacRate, experiments Lac11.3 and Lac1.13. There is in both experiments an initial growth of cells with minor increase in H_2S. After this phase an increase in both cells and H_2S followed. In Lac1.13, the maximum cell number is the same as in Lac11.3 but the lactate as energy source is limiting and the H_2S concentration is therefore lower than in Lac11.3. Lactate serves both as energy source but also as carbon source for the biomass production. In comparison with AcRateH2, the maximum H_2S value of Lac11.3, 170 mg S^{2-} L^{-1} (5.3 mM), is lower than most of the experiments in AcRateH2. This may be due to the fact that AcRateH2 continued for 403 hours but LacRate for 334 hours only. The H_2S versus time curves for both experiments, Figure 3-6 and Figure 3-10, on the other hand show different shapes in this time interval and it is not expected that even if the LacRate experiment was prolonged it would have produced H_2S concentrations as high as those in AcRateH2. In this experiment the energy source H_2 is repeatedly added to the growth cultures keeping the concentration high during the whole experiment.

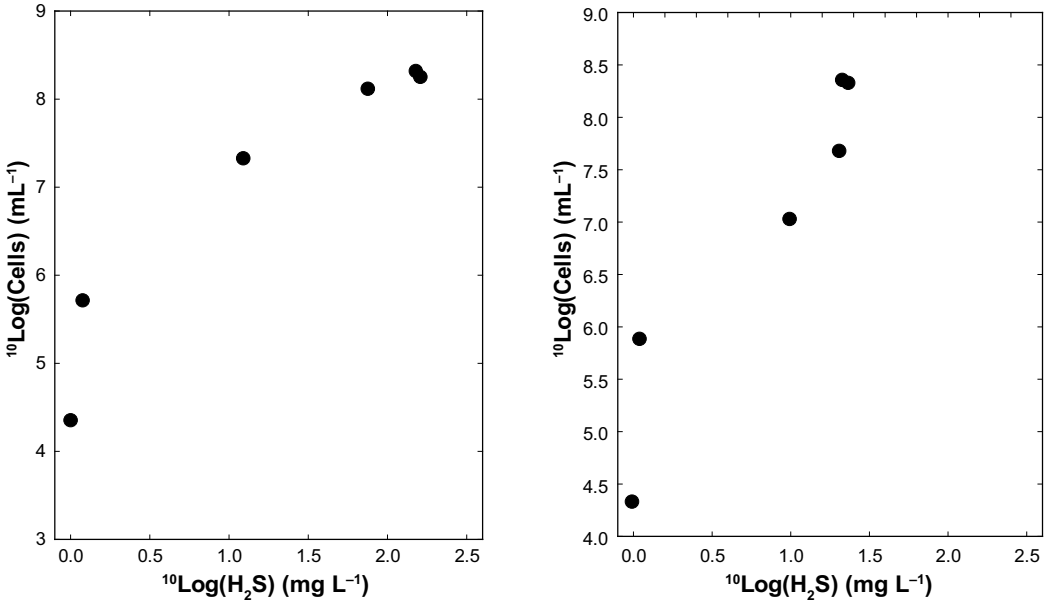

Figure 4-1a and 4-1b. *Correlation between Log_{10} (Cells) and Log_{10} (H_2S) concentration in the LacRate experiment Lac11.3, left and Lac1.13, right.*

With lactate as energy and carbon source, the lactate concentration influenced the sulphide production rate and the maximum H_2S concentration but not the maximum cell number.

The experiments AcRateH2 and H2RateAc both had H_2 as the energy source and acetate and CO_2 as the carbon sources. In Figure 4-2 and Figure 4-3 the Log_{10} of the total number of cells and Log_{10} of H_2S are plotted for AcRateH2 and H2RateAc. The figures show that the greatest difference between the different concentrations of acetate is how large the sulphide production rate becomes. The final sulphide concentration did not differ very much.

The same pattern was found in the H2RateAc experiment. It is very clear that the highest H_2 concentration had the highest sulphide production rate but the maximum H_2S concentrations is close to each other for H2*553, H2*258, and H2*132. The experiment H2*33 on the other hand was energy limited which is demonstrated by the low maximum H_2S concentration and lower sulphide production rate.

In Table 4-1, the maximum H_2S values are compiled for the three sulphate reduction experiments. In AcRateH2, the H_2 concentration was the same for all series and H_2 was added three times during the experiment: in this way the amount of H_2 available was most probably close to unlimited as shown by the high maximum H_2S values for the three experiments Ac44, Ac22 and Ac2.0. The differences in growth in the AcRateH2 experiments depended on the different acetate values as discussed in Section 3.2. The maximum H_2S values in H2RateAc were directly correlated to the available energy, i.e., the H_2 concentration. Figure 3-16 clearly shows that the concentration is linearly connected to the H_2S production rate for the concentrations used here.

The difference in the maximum H_2S value between the AcRateH2 experimentAc2.0 and the H2RateAc experiment H2*553, can depend on that the cultures in AcRateH2, grew longer, 403 h compared to 364 hours for the cultures in H2RateAc. The additions of H_2 during growth in AcRateH2 gave also a higher total amount of H_2 available during growth than the highest initial H_2 concentration in H2RateAc.

From the comparison of the parameters measured in AcRateH2 and H2RateAc it can be concluded that the H_2S production rate is clearly coupled to the H_2 concentration. It is the hydrogenase activity and the following electron transport to SO_4^{2-} that regulate the sulphide production rate (Figure 1-2).

The parameter "H_2S per cell" should be used with caution because it will differ depending on where in the growth curve it is measured. In the stationary phase of a growth curve, the cells often make one cell division without any increase in biomass, a so called reduction division, which increase the cell number but not the biomass. This division will change the H_2S per cell value with a factor 0.5.

Table 4-1. Compilation of growth parameters measured at the end of the three experiments.

Experiment	Experiment	Time (h)	Energy source and concentration (mM or ‰)	Carbon source and concentration (mM)	Maximum cell number (mL^{-1})	Maximum H_2S concentration ($mg\ L^{-1}$)	H_2S per cell ($mg\ cell^{-1}$)
LacRate	Lac11.3	334	Lactate 11.3	Lactate, 11.3	1.7×10^8	162	9.3×10^{-7}
	Lac1.13	334	Lactate 1.13	Lactate 1.13	2.1×10^8	23	1.1×10^{-7}
AcRateH2	Ac44	403	Hydrogen n.m*.	Acetate 44	9.0×10^7	232	2.6×10^{-6}
	Ac22	403	Hydrogen n.m.	Acetate 22	1.8×10^8	224	1.3×10^{-6}
	Ac2.0	403	Hydrogen n.m.	Acetate 2	1.8×10^8	246	1.4×10^{-6}
	Ac0.2	403	Hydrogen n.m.	Acetate 0.2	8.0×10^7	219	2.8×10^{-6}
	Ac0.02	403	Hydrogen n.m	Acetate 0.02	4.8×10^7	136	2.8×10^{-6}
H2RateAc	H2*553	364	Hydrogen 553	Acetate 2	1.0×10^8	197	2.0×10^{-6}
	H2*258	364	Hydrogen 258	Acetate 2	1.2×10^8	178	1.5×10^{-6}
	H2*132	364	Hydrogen 132	Acetate 2	7.3×10^7	121	1.6×10^{-6}
	H2*33	364	Hydrogen 33	Acetate2	2.9×10^7	34	1.2×10^{-6}

*n.m. = not measured

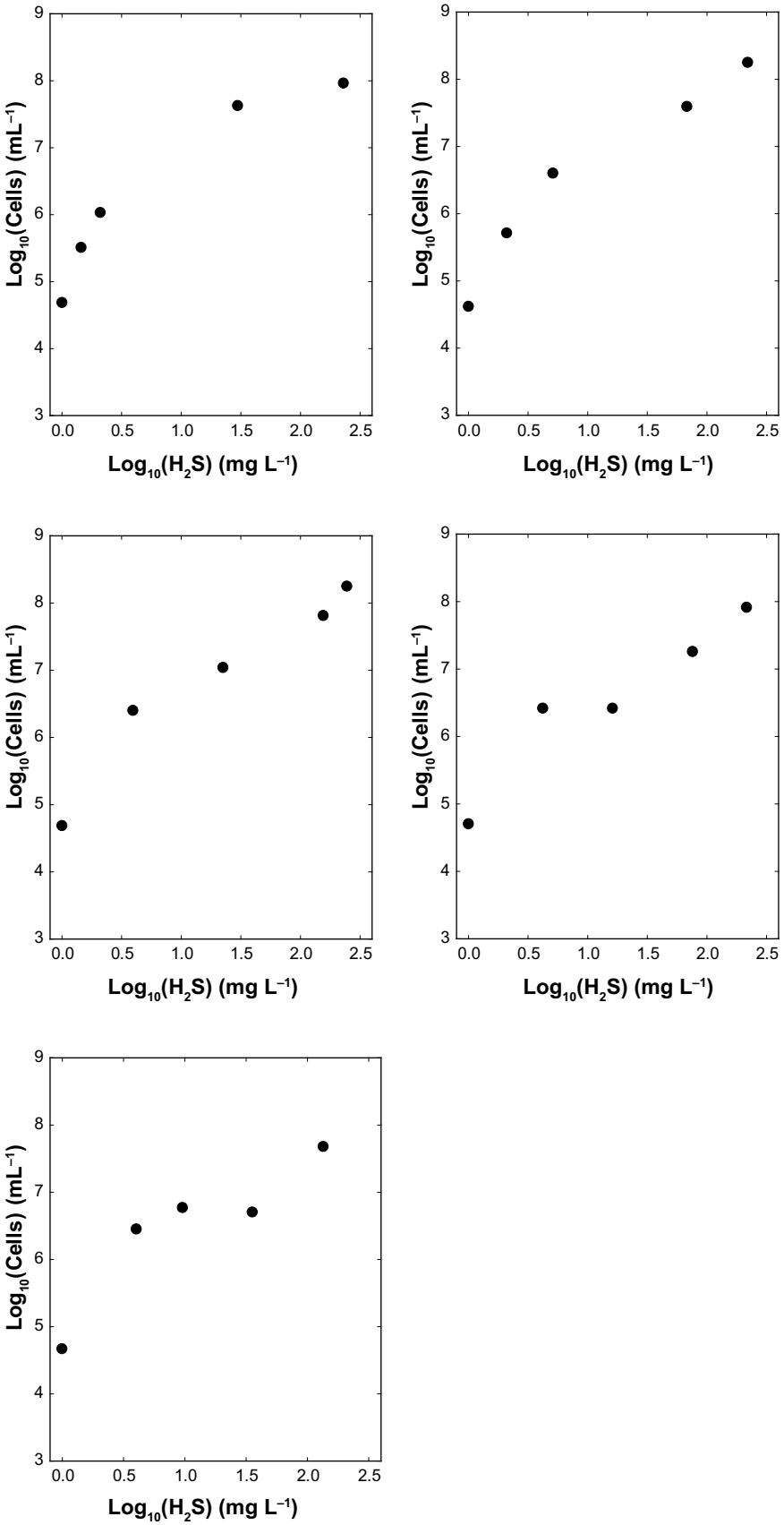

Figure 4-2. *Correlation between Log_{10} TNC and Log_{10} H_2S concentration in AcRateH2 for experiments Ac44 (top left), Ac22 (top right), Ac2.0 (middle left), Ac0.2 (middle right) and Ac0.02 (bottom).*

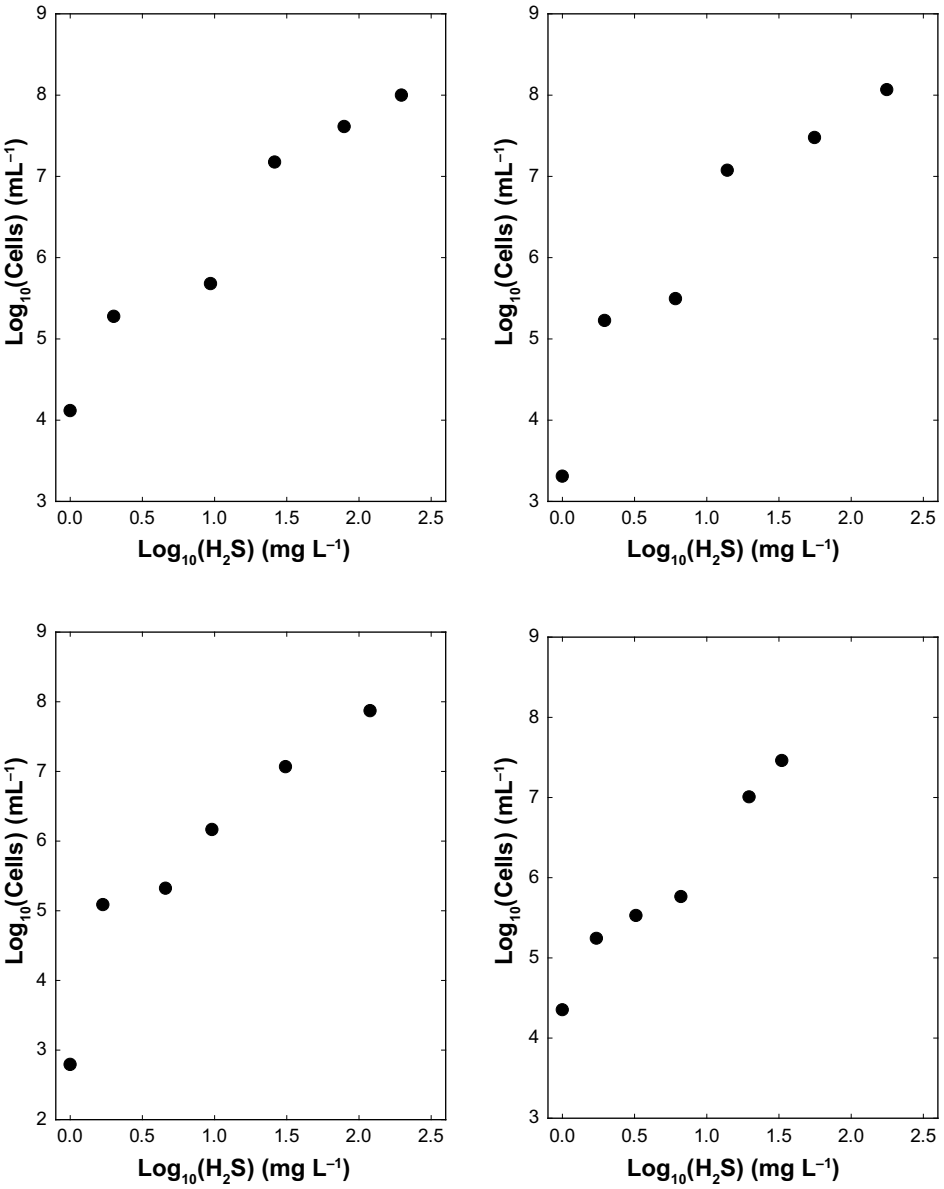

Figure 4-3. *Correlation between Log_{10} (TNC) and Log_{10} (H_2S) concentration in H2RateAc experiments H2*553 (top left), H2*258 (top right), H2*132 (bottom left) and H2*33 (bottom right).*

In Figure 4-4, the amounts of H_2S produced with different energy sources are illustrated. Here the maximum H_2S concentrations produced in LacRate and H2RateAc are plotted versus the H_2S produced per cell at the end of the experiments. H_2 utilizing cultures produced both more H_2S in total and more H_2S per cell than cultures grown on lactate. The times for the last measurements were for LacRate 334 h and for H2RateAc, 364 hours. Note that this figure does not show rates, it shows only the values at the end of the experiments.

4.2 Comparison of the experimental results with literature data

SRB studies have mostly been conducted in the context of degradation of different types of organic contaminants (Reichenbecher and Schink 1997, Muyzer and Stams 2008). These are often high molecular weight hydrocarbons, and they can be both aliphatic and aromatic compounds. Another aspect on SRB activity that has been studied is their presence in methane bioreactors where they compete with methanogens for the substrate, which often is H_2 (Kalyuzhnyi and Fedorovich 1998,

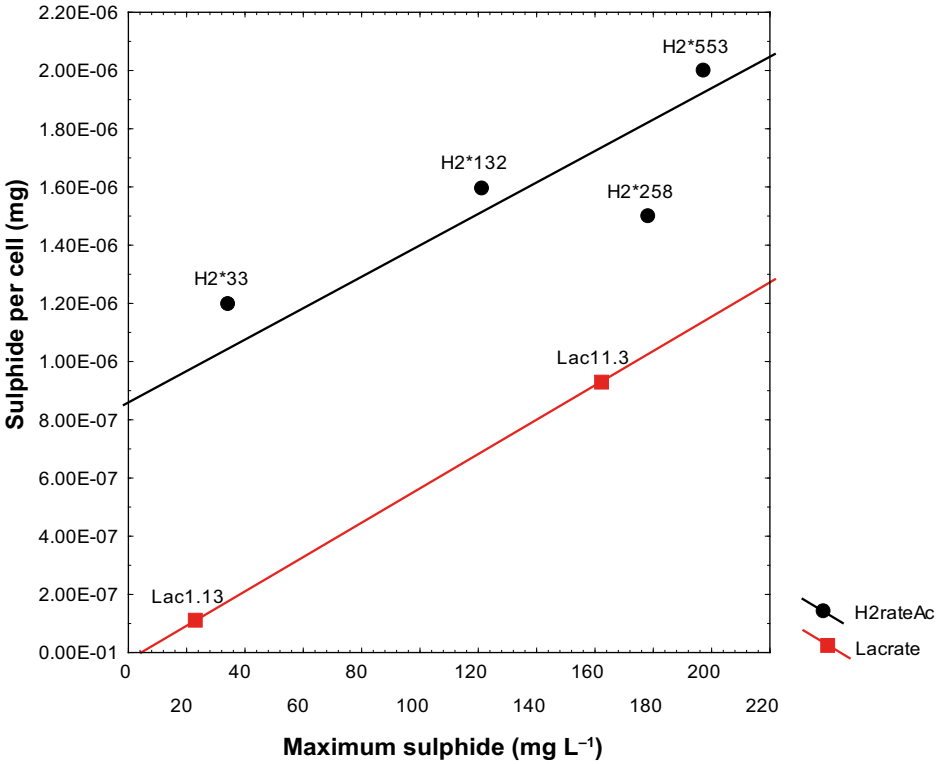

Figure 4-4. *The maximum H_2S concentration versus H_2S produced per cell at the end of the growth experiments LacRate and H2RateAc.*

Moosa et al. 2002, Muyzer and Stams 2008). Presence of SRB in such bioreactors decreases the production of methane and is thus unwanted. These systems are quite different from an uncontaminated groundwater system. In contaminated systems and in bioreactors there is no limitation in the energy sources which are the contaminant or the substrate to be fermented and further degraded. Also the energy source is different, since it is high molecular organic molecules in contaminated sites and bioreactors, which is not the case in a groundwater system. The energy sources in deeper groundwater are not yet conclusively elucidated. To a certain depth it is of course organic compounds from degradation of organic material produced by photosynthesis at the ground surface. The hypothetical source of energy at deeper depth is H_2 produced in the mantel and/or crust. At certain places the energy can also be CH_4 produced at depth and transported upwards.

Sulphate reduction in connection with acid mine drainage and removal of heavy metal contamination by precipitation of metal-sulphides has also been studied (Moosa et al. 2002, Muyzer and Stams 2008). In such systems different types of energy and carbon sources are present and there are often mixed microbial populations. A set up of a continuous culture system to study sulphate reduction with acetate as energy and carbon source was used by Moosa et al. (2002). In this system SO_4^{2-} was limiting, because the SO_4^{2-} reduction increased when the SO_4^{2-} concentration was increased. The values in Moosa et al. (2002) correspond to a sulphide production rate of 0.06 – 0.07 and 0.3 µM h^{-1}. The sulphide production rate in H2RateAc was in the range 1 – 10 µM h^{-1}, hence about 10 to 100 times faster than in the continuous culture study. This difference in rates emphasizes the importance of using equivalent studies in comparisons of growth variables. Sulphate reduction in wetlands has been studied and stimulated by laboratory studies using addition of sucrose (Lloyd et al. 2004). In such environment and with sucrose as carbon and energy source the initial step is the fermentation of sucrose by fermenting organisms like *Clostridium* and *Bacteriodes*-species. A similar study but with fresh-water sediment showed sulphate consumption rates, which should be almost the same as sulphide production rates, between 1 and 61 µM L^{-1} depending on treatment of the sample (Scholten et al. 2002). The lowest rate was for unsupplemented control sediment and the highest for a sample supplied with acetate and SO_4^{2-}. These values were in the same order as the rates measured in the present project.

One paper describes lab culture experiments with a strain of *Desulfovibrio vulgaris* (strain Marburg) in batch cultures but with continuous gassing with hydrogen and carbon dioxide and at a temperature of 35°C (Badziong and Thauer 1978, Badziong et al. 1978, 1979). The sulphide production rate for this strain under the circumstances described above gave a sulphide production rate of approximately 0.8 mM h^{-1}. This is around 20 times higher than the maximum rate, 0.033 mM h^{-1} in AcRateH2, experiment Ac2.2, in the present report. The higher sulphide production rate can be explained by the more than ten degrees higher temperature and continuous supply of H_2. The cultures were also on continuous shaking which also can increase the growth in batch cultures. In spite of these differences our results are in good agreement with the literature.

In conclusion:

- The source of energy, and by that the type of metabolism, used by the SRB (sulphate reducing bacteria) influence both the production rate of H_2S together with the total amount of H_2S that can be produced in batch cultures. The maximum sulphide production rate measured in this project was 1.5 mg L^{-1} h^{-1} or 46 µM h^{-1}. The highest H_2S concentration measured was 285 mg L^{-1} or 8.9 mM .
- The production of H_2S is linearly correlated to the concentration of energy source, both the sulphide production rate and the maximum H_2S concentration produced in batch cultures.
- The cell number produced is not directly correlated to the concentration of the energy source.

5 Acknowledgements

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Appendix

A1 Data LacRate

Table A-1. Total number of cells (TNC) for the LAcRate experiments.

TNC (cells/mL) Sample/Time (h)	0	45	70	93	168	219	262	334	364
Lac1.13A	45,000	163,000	550,000	1,000,000	42,000,000	2.6E+08	4.14E+08	347,000,000	
Lac1.13B	30,000	77,000	277,000	450,000	6,300,000	85,000,000	4.9E+08	456,000,000	
Lac1.13C	14,000								1.08E+08
Lac1.13A	20,000	86,000	220,000	790,000	11,000,000	48,000,000	1.26E+08	86,600,000	
Lac1.13B	22,000	70,000	153,000	700,000	10,000,000	47,000,000	3.22E+08	330,000,000	
Lac1.13C	15,300								15,200,000
Lac0.11A	18,000	66,000	148,000	690,000	2,600,000	2,900,000	3,800,000	4,700,000	
Lac0.11B	15,000	52,500	151,000	370,000	1,960,000	2,000,000	4,180,000	2,500,000	
Lac0.11C	13,000								2,930,000
Lac0.01A	9,300	90,000	188,000	500,000	1,500,000	970,000	1,480,000	1,330,000	
Lac0.01B	d m*	61,000	156,000	480,000	1,200,000	1,800,000	1,730,000	1,910,000	
Lac0.01C	d m								890,000
Lac0.001A	13,300	90,000	190,000	690,000	1,220,000	720,000	1,580,000	1,320,000	
Lac0.001B	4,200	61,000	156,000	340,000	1,040,000	1,330,000	2,100,000	1,140,000	
Lac0.001C	7,600								548,000

*d m = data missing

Table A-2. Lactate concentration at start and at the end of LacRate.

Lactate (g/L) Sample/Time (h)	0	334	364
Lac11.3	1.34	0.002	
Lac11.3B	1.25	0.0014	
Lac11.3C	1.03		0.0018
Lac1.13A	0.163	0.0006	
Lac1.13B	0.096	0.0013	
Lac1.13C	0.045		0.0014
Lac0.11A	0.05	0.0005	
Lac0.11B	0.04	0.001	
Lac0.11C	0.06		0.0016

Table A-3. Acetate concentration at start and at the end of LacRate.

Acetate(g/L) Sample/Time (h)	0	334	364
Lac11.3A	0	0.804	
Lac11.3B	0	0.751	
Lac11.3C	0		0.574
Lac1.13A	0	0.099	
Lac1.13B	0	0.112	
Lac1.13C	0		0.069
Lac0.11A	0	0.009	
Lac0.11B	0	0.009	
Lac0.11C	0		0.006

Table A-4. Sulphide concentration at start and at the end of LacRate.

Sulphide (mg/L)						
Sample/Time (h)	**0**	**93**	**168**	**219**	**262**	**334**
Lac11.3A	0	1.4	15.2	74.8	154	170
Lac11.3B	0	1	9.8	76.8	150	153.6
Lac11.3C	0					121
Lac1.13A	0	1.1	11.2	21.2	20.8	25.2
Lac1.13B	0	1.1	8.6	20	22.2	21.6
Lac1.13C	0					13.6
Lac0.11C	0					0.008
Lac0.01C	0					0.001
Lac0.001C	0					0.001

Table A-5. Sulphate concentration at start and at the end of LacRate.

Sulphate (mg/L)		
Sample/Time (h)	**0***	**334**
Lac11.3A	1,300	570
Lac11.3B	1,300	640
Lac11.3C	1,300	808
Lac1.13A	1,300	1,120
Lac1.13B	1,300	1,020
Lac1.13C	1,300	1,280
Lac0.11C	1,300	1,420
Lac0.01C	1,300	1,420
Lac0.001C	1,300	1,460

*Start value is the mean value from samples and controls

A2 Data AcRateH2

Table A-6. Total number of cells data in experiment AcRateH2.

TNC cells/mL								
Sample/Time (h)	**0**	**68**	**92**	**141**	**169**	**236**	**290**	**403**
Ac44A	4.0E+04	1.4E+05	3.70E+05	9.1E+05	2.4E+06	2.1E+07	6.0E+07	3.9E+07
Ac44B	5.5E+04	2.0E+05	2.80E+05	1.2E+06	1.6E+06	6.4E+07	6.5E+07	1.4E+08
Ac22A	4.4E+04	1.1E+05	4.50E+05	4.0E+06	6.4E+06	3.7E+07	4.8E+07	1.5E+08
Ac22B	3.8E+04	2.0E+05	5.50E+05	4.0E+06	1.0E+07	4.2E+07	5.4E+07	2.0E+08
Ac2.0A	5.2E+04	3.7E+05	2.50E+06	9.5E+06	3.1E+07	5.8E+07	6.3E+07	1.7E+08
Ac2.0B	4.5E+04	3.8E+05	2.50E+06	1.2E+07	1.8E+07	6.8E+07	1.1E+08	1.8E+08
Ac0.2A	4.7E+04	1.9E+05	2.00E+06	5.1E+06	8.6E+06	d.m.*	4.7E+07	4.9E+07
Ac0.2B	5.2E+04	6.5E+05	3.25E+06	d.m.	1.9E+07	3.6E+07	7.3E+07	1.1E+08
Ac0.02A	3.5E+04	5.2E+05	2.70E+06	6.3E+06	7.0E+06	d.m.	2.2E+07	3.5E+07
Ac0.02B	5.8E+04	2.9E+05	2.90E+06	5.5E+06	8.8E+06	1.0E+07	1.0E+07	6.1E+07

*d.m. data missing

Table A-7. Sulphide data in experiment AcRateH2.

Sulphide mg/L Sample / Time (h)	0	92	141	236	403
Ac44A	0	1.6	2.1	27	229
Ac44B	0	1.3	2.1	33	235
Ac22A	0	1.9	4.2	67	210
Ac22B	0	2.3	6.2	70	237
Ac2.0A	0	3.8	23	169	247
Ac2.0B	0	4.1	22	143	244
Ac0.2A	0	4.85	16	69	228
Ac0.2B	0	3.7	16.4	83	210
Ac0.02A	0	3.6	9.6	28.6	79
Ac0.02B	0	4.55	d.m.	42.6	192

Table A-8. Sulphide data in experiment AcRateH2.

Sulphate mg/L Sample/Time (h)	0	403
Ac2.2A	1300	25
Ac2.2B	1300	25

Table A-9. Acetate data in experiment AcRateH2.

Acetate mg/L Sample/Time (h)	0	403
Ac44A	3,358	2,846
Ac44B	2,913	2,739
Ac44C	2,278	2,105
Ac22A	1,227	973
Ac22B	1,229	1,137
Ac22C	1,096	1,087
Ac2.0A	115	71
Ac2.0B	124	62
Ac2.0C	118	62
Ac0.2A	10.7	1.7
Ac0.2B	9.6	3.5
Ac02C	10.7	2.9
Ac0.02A	4.2	1.7
Ac0.02B	4.8	0.3
Ac0.02C	3.6	1.5

A3 Data H2RateAc

Table A-10. Total number of cells data in experiment H2RateAc.

TNC cells/mL							
Sample/Time (h)	**0**	**72**	**96**	**168**	**192**	**244**	**364**
H2*553A	1.20E+04	1.65E+05	4.86E+05	1.98E+07	2.88E+07	3.70E+07	1.09E+08
H2*553B	1.60E+04	2.00E+05	3.28E+05	7.48E+06	1.34E+07	2.40E+07	9.16E+07
H2*553C	1.40E+04	2.10E+05	4.40E+05	9.47E+06	1.63E+07	4.30E+07	9.03E+07
H2*258A	3.40E+03	1.70E+05	4.46E+05	1.23E+07	2.80E+06	2.66E+07	1.31E+08
H2*258B	7.00E+02	1.10E+05	2.50E+05	1.13E+07	8.90E+06	4.00E+07	7.89E+07
H2*258C	7.00E+02	1.60E+05	1.65E+05	1.10E+07	9.97E+06	3.18E+07	1.00E+08
H2*132A	6.00E+02	8.60E+04	2.50E+05	1.57E+06	4.83E+06	1.22E+07	6.04E+07
H2*132B	6.00E+02	1.50E+05	1.58E+05	1.34E+06	4.32E+06	1.13E+07	8.59E+07
H2*132C	1.30E+03	1.10E+05	2.18E+05	2.12E+06	4.00E+06	3.20E+07	4.70E+07
H2*33A	2.30E+04	1.72E+05	1.68E+05	4.60E+05	3.56E+06	8.78E+06	2.48E+07
H2*33B	1.00E+04	1.21E+05	1.40E+05	7.20E+05	4.45E+06	9.80E+06	5.79E+07
H2*33C	2.10E+04	1.68E+05	5.10E+05	6.80E+05	8.50E+06	1.12E+07	3.24E+07

Table A-11. Sulphide data in experiment H2RateAc.

Sulphide mg/L						
Sample/Time (h)	**0**	**72**	**96**	**168**	**244**	**364**
H2*553A	0.00	1.68	7.85	22.21	72.37	178.05
H2*553B	0.00	1.81	6.64	24.70	60.31	162.73
H2*553C	0.00	1.78	8.39	22.88	62.60	156.99
H2*258A	0.00	1.70	5.09	10.82	45.76	137.84
H2*258B	0.00	1.60	5.55	12.97	44.22	124.44
H2*258C	0.00	1.67	5.40	13.07	50.35	165.60
H2*132A	0.00	1.44	3.84	8.09	28.43	112.00
H2*132B	0.00	1.42	2.79	9.48	26.13	103.38
H2*132C	0.00	1.45	4.08	8.52	24.41	93.81
H2*33A	0.00	1.52	2.43	5.22	12.83	30.63
H2*33B	0.00	1.44	2.81	5.50	17.80	28.72
H2*33C	0.00	1.46	3.15	6.22	21.16	26.80

Table A-12. Sulphate data in experiment H2RateAc.

Sulphate mg/L		
Sample/Time (h)	**0**	**364**
H2*553A	1,050	670
H2*553B	1,300	730
H2*553C	1,600	760
H2*258A	1,600	800
H2*258B	1,600	730
H2*258C	1,300	620
H2*132A	1,300	710
H2*132B	1,300	730
H2*132C	1,300	820
H2*33A	1,300	1,040
H2*33B	1,300	1,022
H2*33C	1,300	980

Table A-13. Acetate data in experiment H2RateAc.

Acetate mg/L Sample/Time (h)	0	72	96	168	244	364
H2*553A	119	125	123	117	116	113
H2*553B	122	123	123	118	114	116
H2*553C	120	124	123	120	116	116
H2*258A	124	124	122	121	114	116
H2*258B	123	125	124	122	115	119
H2*258C	125	123	124	121	115	113
H2*132A	122	121	121	118	116	114
H2*132B	120	122	122	115	113	115
H2*132C	122	123	121	117	115	115
H2*33A	122	123	123	121	119	121
H2*33B	121	124	123	121	116	121
H2*33C	120	123	122	121	120	120

Table A-14. H_2 data in experiment H2RateAc.

H_2 (‰) Sample / Time (h)	0	72	96	168	244	364
H2*553A	481	586	577	531	557	447
H2*553B	527	587	584	534	576	474
H2*553C	530	575	577	521	561	465
H2*258A	252	275	281	257	241	132
H2*258B	251	285	261	249	215	122
H2*258C	249	256	242	214	209	112
H2*132A	131	141	118	101	105	dm*
H2*132B	127	140	115	102	110	16
H2*132C	128	126	117	96	110	24
H2*33A	21	31	27	15	0	0
H2*33B	27	32	33	17	7	0
H2*33C	33	35	30	22	12	0

*dm = data missing