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Contribution of sulfate-reducing bacteria in soil to degradation and retention of COD and sulfate

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Contribution of sulfate-reducing bacteria in soil to degradation and retention of COD and sulfate

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S. Koydon

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1. Introduction

Sewer systems are expected to drain the wastewater into a treatment station. The pollutants and the toxic substrates of sewage must be eliminated before discharging it into a natural watercourse. Defects of pipes are caused by many reasons, such as the age of sewers, the type of materials, the age of connecting seals and the corrosion of pipes. Leakage is one of the major problems in a sewer system that affects the quality of groundwater. Health problems may arise especially in countries that use groundwater as a source of drinking water. In Germany, it was reported that 10 - 26 % of total wastewater was trickling through leaks directly into the unsaturated underground and from there to the groundwater. If calculated from the total length of sewer pipes (8 x 10^5 km) and it would cost at least 200 million Euro to repair all of them (Winkler, 2001). In another report (Berger et al., 2002) the total sewer system of Germany was estimated at 4.45 x 10^5 km of which 75 % were inspected. The costs for rehabilitation were calculated at 1.64 billion Euro (year 2000). In other countries, for example in the UK 23 % (7.3 x 10^4 km) of the total sewer system (3.2 x 10^5 km) is in critical condition (Ellis and Revitt, 2002). 150 million Pounds/annum were invested on infrastructure rehabilitation. In Berlin (Germany) more than 50 % of the sewer pipes must be repaired (Milojevic et al., 1999). Mueller (2000) reported that about 1.06 million Euro were required to repair only 5 km of leaky pipes from a total of 600 km. As a result of the high costs for repairing, renovation of sewers was postponed. This might affect the quality of groundwater or increase the risk of outbreak of epidermies, if uncleaned groundwater was the source of drinking water. In previous research, Dohmann (1999) mentioned that wastewater, trickling from the surface through soil into a depth of 1 - 4 meter from the surface, significantly contaminated groundwater, due to the low degradation of pollutants during trickling. The trickling time of sewer water through the unsaturated zone of soil was not long enough to degrade all of its toxic substrates.

There are a lot of ideas on using principal markers as an indicator or tracer to follow or predict the process of sewage leaking through sediments or soils. The ionic ratio or organics or bacteria like faecal bacteria or *E. coli* or even stable isotopes like N^{15}, 0^{18}, S^{34} or H^2 were suggested as tracers (Ellis and Revitt, 2002).

The degradation of substances in wastewater is carried out by both, aerobic or anaerobic bacteria or only by anaerobic bacteria in the unsaturated underground zone. Microorganisms, which will dominate, depend on parental parameters of soil and

wastewater, such as pH, temperature, redox potential, organic matter, toxic substrates etc. Studies reported that the primary organisms should be fungi, followed by bacteria such as *Pseudomonas* sp., *Arthrobacter* sp., clostridia, cellulose degrading bacteria, sulfate reducing bacteria and methane bacteria, thereby changing the growth conditions from aerobic to anaerobic. The DFG-research group at the University of Karlsruhe (Germany) intended to investigate the degradation processes or activities of microorganisms under leaky sewers, the possibility to predict diffusion processes from leaks by mathematical modelling and to estimate the danger of leaks for the quality of groundwater and for human health. The sulfate reducing bacteria (SRB) are one group of microorganisms that are essential or might be some of the dominant microorganisms. They produce hydrogen sulfide as the end product of sulfate reduction. The hydrogen sulfide reacts with heavy metals and precipitates the heavy metal ions as metal sulfides such as FeS, NiS, CoS and by this causes elimination of the toxic heavy metals in wastewater before the water enters into the groundwater.

The respiration of sulfate is possibly the dominant activity in shallow marine or even freshwater sediments (Cooling et al., 1996). It depends on the sulfate concentration. Although sulfate is not highly concentrated in wastewater and soil, its concentration is high enough for using sulfate isotope distribution during respiration of sulfate by sulfate reducing bacteria as parameter to predict or decide the level of quality of water. Since sulfur isotopes are stable in the environment, S-isotope discrimination can be used as a tracer to determine the ratio of chemical and biological reactions in the underground. For analysis of the kinetics of this process, batch cultures of sulfate reducers and enrichment cultures from soil as well as sulfate reducers from biofilms in soil columns were cultured to predict sulfate degradation rates. The sampled soils were investigated for the potential of sulfate reducing activity at different temperatures.

The Objectives of this work were:

: to investigate the degradation rates by sulfate reducing bacteria with different carbon sources, under normal and under stress conditions (e.g. in the presence of molybdate).

: to determine the transformation of sulfur compounds in sewage in soil column studies as models for studying processes occurring under sewer leaking areas.

: to enumerate the bacterial community during anoxic and anaerobic trickling conditions: either in suspension or as biofilms on the sand.

: to characterize the bacterial community at different leaking sites in soil samples.

2. Literature review

2.1 The cycle of sulfur

Sulfur is one of the essential elements in the global world. It contributes 1 % of the total dry weight of organisms. In microorganisms, the sulfur is utilized in protein synthesis e.g. for cystine, cysteine and methionine or as a component of coenzymes, hormones or vitamines. In nature we can find 4 forms of sulfur: sulfate (SO_4^{2-}, +6), sulfide (H_2S, HS^-, S^{2-}, -2), elemental sulfur (S^o, 0) and organic sulfurhydryl compound (R-SH). The type of sulfide depends on the pH. If the pH is acidic the sulfide will be volatile as H_2S. HS^- is the predominant form at neutral pH and S^{2-} is the predominant form at alkaline pH.

The main inert source of sulfur on earth is sulfur in sediments, fossils, fuels and in rocks such as gypsum ($CaSO_4$) and pyrite (FeS_2). Seawater contains sulfur in form of sulfate and dimethylsulfide (CH_3SCH_3), an excretion product of algae. Dimethylsulfide is the end product of degradation of dimethylsulfonium propionate, which is used for osmoregulation by algae. In air we can find sulfurdioxide (SO_2), sulfate (on dust particles), hydrogensulfide and dimethylsulfide. All sulfur compounds may come back to earth by precipitation. Sulfur compounds effect the quality of surface water or soil in terms of acid rain, and these compounds may be biotransformed, precipitated in soil or washed out into the groundwater.

The dissimilatory sulfate reduction is the main source of sulfide. Vice versa the assimilatory sulfate reduction is the main biological source of organic sulfur, R-SH. Microorganisms play the dominant role in oxidation, reduction and degradation of sulfur compounds within the sulfur cycle (Fig. 2.1).

2.2 Biotransformation of sulfur and sulfate (Fig. 2.1)

2.2.1 Biological sulfur transformation process

2.2.1.1 Sulfur oxidation:

Sulfur oxidation occurs under three conditions: 1) Abiotic: hydrogen sulfide reacts with oxygen and releases elemental sulfur. 2) Biotic phototrophic sulfur conversion (e.g. S^o- deposition in single cells) and 3) Biotic chemoautotrophic sulfur metabolism.

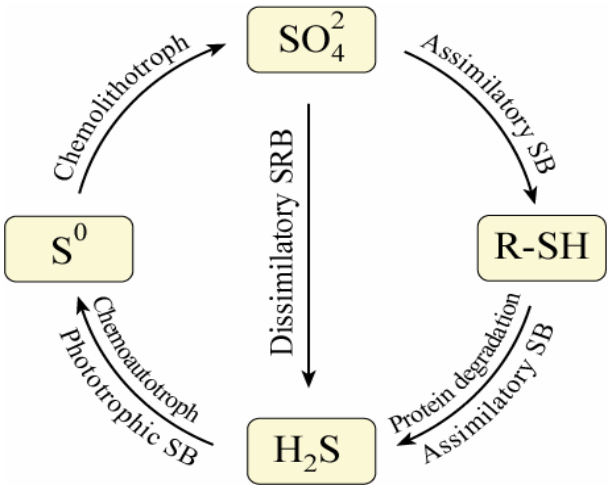


Figure 2.1 Biotic activity involved in the cycle of sulfur.

Phototrophic bacteria are bacteria that utilize hydrogen sulfide as an electron donor source, CO_2 as a carbon source and light as energy source under anaerobic conditions, e.g. Chromatiaceae or Chlorobiaceae. The bacteria use sulfide as an electron donor and fix carbon dioxide (1). For comparison in oxygenic photosynthesis water is the electron donor and oxygen is released (2).

$$6\ CO_2 + 12\ H_2S \rightarrow 6\ (CH_2O) + 12\ S + 6\ H_2O \quad (1)$$

$$6\ CO_2 + 6\ H_2O \rightarrow 6\ (CH_2O) + 6\ O_2 \quad (2)$$

Chemoautotrophic bacteria are found in anoxic areas beneath the oxic zone, such as *Beggiatoa* sp. and *Thiothrix* sp.. These are microaerophilic filamentous bacteria that oxidize H_2S to elemental sulfur (3).

$$2\ H_2S + O_2 \rightarrow 2\ S^o + 2\ H_2O \quad (3)$$

The elemental sulfur (S^o) is chemically quite stable under most condition. Sulfur oxidizing bacteria, such as chemolithotrophic *Thiobacillus* sp., oxidize sulfide or sulfur with oxygen to sulfate (4). This process could effect the dissolvation and mobilization of phosphorus, since it is accompanied by a drastic drop of the pH.

$$S^o + 1.5\ O_2 + H_2O \rightarrow H_2SO_4 \quad (4)$$

2.2.1.2 Sulfate reduction

Sulfate reducing bacteria are anaerobic bacteria that utilized sulfate as an electron acceptor and different organic substrates or hydrogen as electron donors. They produce sulfide as an end product. Sulfate reducers can be found in typical watercourse, wastewater or under different anaerobic conditions, a wide range of pH, salinities or temperatures and they are capable to eliminate various substrates.

The sulfate reacts with ATP, forming APS and pyrophosphate. APS is cleaved to AMP and sulfite (SO_3^{2-}). After that sulfite forms hydrogen sulfide. A major electron carrier is cytochrome C_3. It transfers electrons to a membrane associated with a protein complex, called Hmc that carries them across the cytoplasmic membrane (5).

$$4\ H_2 + SO_4^{2-} \rightarrow H_2S + 2\ H_2O + 2\ OH^- \quad (5)$$

According to Fritsche (1998) sulfate reducing bacteria can be divided into three groups:

- *Completely oxidizing sulfate reducers*: SRB will utilize lactate, propionate, ethanol or any substrate and oxidize it to carbon dioxide (CO_2) and hydrogen (H_2). Examples are *Desulfobacter* sp., *Desulfobacterium* sp., *Desulfococcus* sp., *Desulfonema* sp. or *Desulfotomaculum acetoxidans*.
- *Incompletely oxidizing sulfate reducers*: SRB degrade the carbon sources to acetate and CO_2. Examples are *Desulfovibrio* sp., *Desulfomicrobium* sp., *Desulfotomaculum* sp., *Desulfobulbus* sp. (Fig. 2.2).
- *H_2 oxidizing sulfate reducers*: *Desulfovibrio desulfuricans* can use H_2 as an electron donor, but in some reports it is mentioned that it requires the presence of acetate. A small number of SRB that are chemolithotrophic, use H_2 as an energy source.

Assimilatory sulfate reduction: Sulfate bacteria utilized sulfate and produced organic sulfur like the SH-group of amino acids such as cystine, cysteine and methionine. Sulfate reacts with ATP to APS and with another ATP to form PAPS (Fig. 2.2). A sulfate molecule will be reduced to sulfite and further to sulfide reacts with amino acids to form cystine, cysteine or methionine.

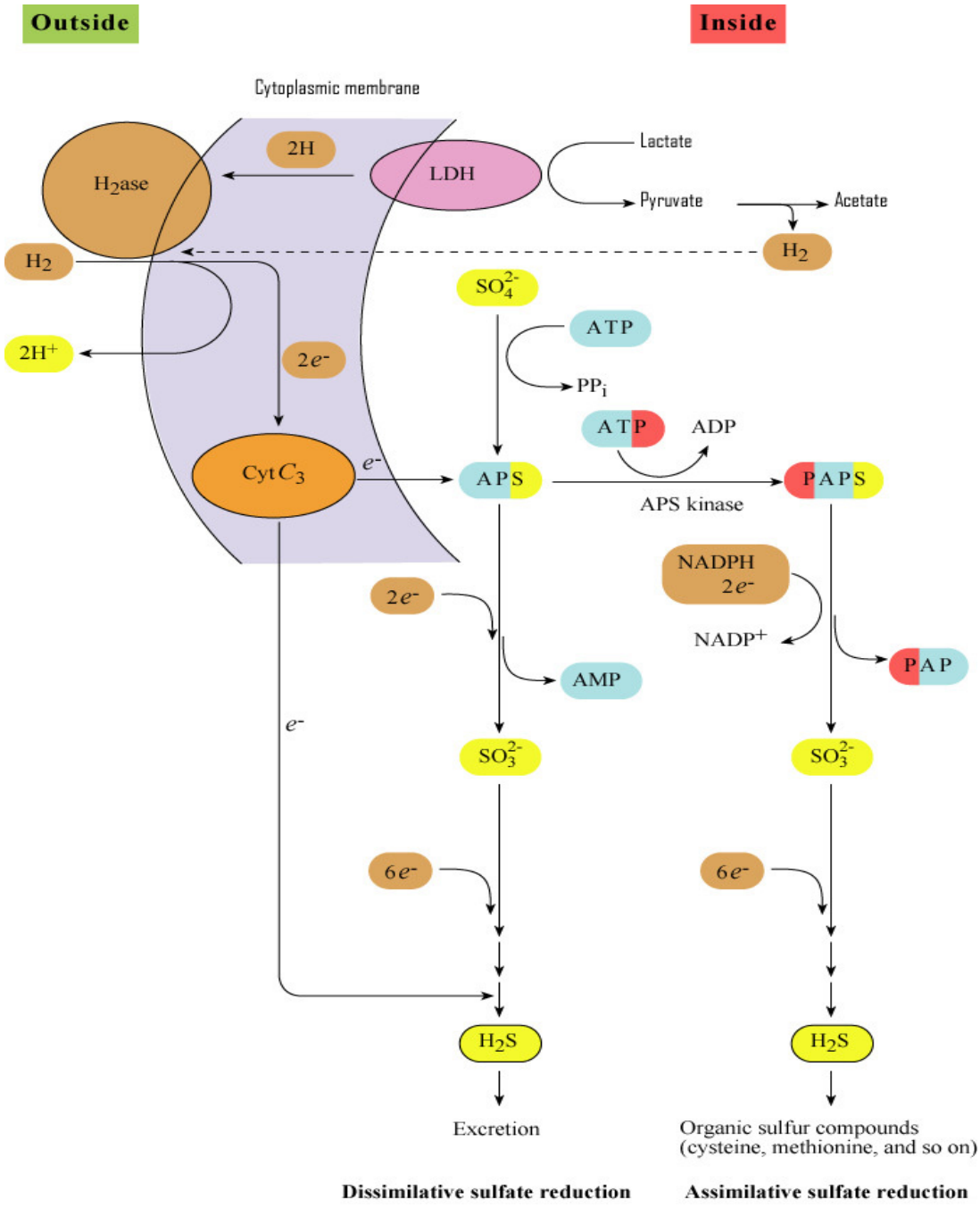


Figure 2.2 The process of incomplete sulfate reduction with lactate as a carbon source (Madigan et al., 2000).

2.2.2 Chemical or physical degradation

Sulfate and elemental sulfur are stable sulfur forms. They are difficult to degrade under normal conditions. Sulfide can be oxidized from H_2S to S^o and thiosulfate in an abiotic process.

2.3 Factors influencing growth or the sulfate reduction rates

Carbon: In many marine sediments, it was well known that limiting supply of carbon is the main restricting effect on the SRB activities. Under low concentration or in the presence of unsuitable carbon sources the bacterial growth is decreased.

Sulfide: Sulfide is the end product of sulfate reduction and may be inhibitory for the SRB themselves at high concentration. It reacts for instance with iron of cytochrome and other essential iron containing-compounds in cells (Madigan et al, 2000).

Oxyanion molybdate: Mo is one of the chemical elements of group V. The oxyanion molybdate inhibits growth of sulfate reducing bacteria. It has a stereochemical similarity with sulfate and competes with sulfate in biological reactions, such as transport or ester formation. Normally adenosine 5´- triphosphate (ATP) reacts with sulfate under catalysis of the enzyme adenosine 5´- triphosphate sulfurylase quantitatively to APS and PP. During sulfate respiration in the presence of molybdate rapid formation of unstable molybdo-phospho anhydrides takes place, which results in depletion of ATP levels. Molybdate corrupted the reaction of intracellular ATP with sulfate like other elements of group V. Selenate inhibited SRB by blocking the transportation of sulfate into cells (Chen et al., 1998). Taylor and Oremland (1979) reported that chromate is the most effective inhibitor and selenate is the least: $CrO_4^{2-} > MoO_4^{2-} = WO_4^{2-} > SeO_4^{2-}$. Molybdate, as a component of bacterial media, is recommended as an inhibitor for SRB, although molybdate is a component of several coenzymes and cofactors in SRB.

Cooling et al. (1996) reported that a group of structure related derivatives of 9,10-anthracenedion; (common name: anthraquinones) results in a severe inhibition of SRB at micromolar concentration. It caused the uncoupling of electron transfer from ATP synthesis. It is possible in future to use anthraquinones as inhibitors for SRB, due to the extremely effective inhibition of growth of SRB. Even though, derivatives of these compounds cannot inhibit all of the SRB.

2.4 Source of sulfate in groundwater (Fig. 2.3)

In groundwater the main source of sulfate came from untreated acid air pollutants (69.5 %), 25.5 % from fertilizers, 4.7 % from industry wastewater and 0.3 % from sewage wastewater (Brettschneider, 1990). The main sources of sulfate and sulfur in wastewater came from components of natural drinking water, from industrial wastes, detergents, as well as from bio-wastes.

The drinking water contains 100 - 300 mg/L SO_4^{2-}, average 200 mg/L (Haerig, 1991). In industrial wastewater, it is possible to enclose high sulfate upto 100 g/L. Grimm-Strehle et al. (1999) reported an additional sulfate concentration at more than 100 mg/L in groundwater, caused by leaking of sewers.

In sewage from Neureut's treatment plant sulfate concentrations varied from 60- 120 mg/L (data part 3.1)

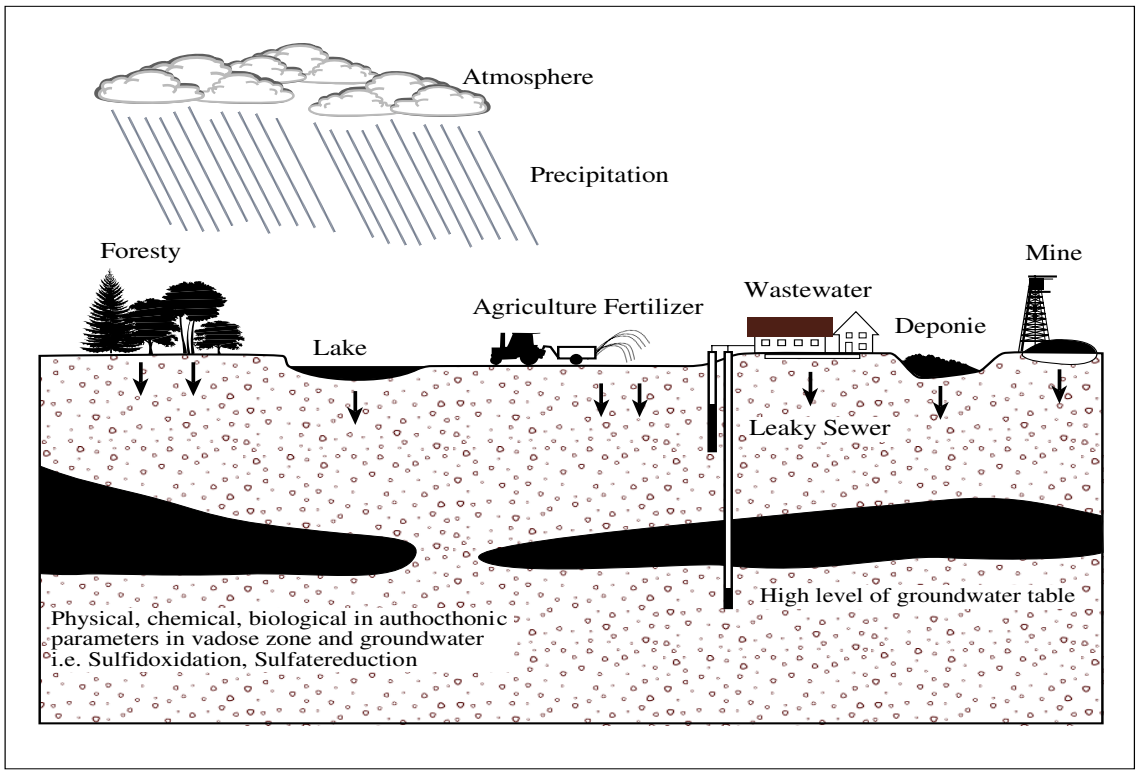


Figure 2.3 Sources of sulfate in groundwater (Haupt, 2000).

2.5 How dangerous are leaks in sewers for the quality of groundwater and is there an advantage of having SRB in sewage and soil?

The main contamination of the unsaturated zone that effects the quality of groundwater are leaky sewers, sanitary, landfills, precipitations, septic tanks and land application of wastewater.

If the groundwater is used as drinking water, quality criteria such as the total number of bacteria, pathogens or toxic substrates that effect human health were defined by the WHO as standard quality criteria. To analyse the effect of untreated wastewater on groundwater quality, a sand column reactor was used and sewage was continuously trickled through the sand. It was found that most of the COD or DOC was quite rapidly degraded (70 – 90 % degradation; Hua, unpublished, University Karlsruhe). In addition, the number of bacteria after trickling through sand was reduced 90 – 99 %, (data in results section). The numbers of bacteria, especially those of *E. coli* that is not allowed to appear in drinking water were still too high in the effluent of the sand columns. The level of heavy metals in untreated wastewater and after trickling through soil was also of concern. According to the pioneer knowledge that metals such as Co, Cu, Ni, Pb and Zn, are extremely reactive with sulfide this leads to precipitation of metallic sulfide. Sulfide from sulfate reduction decreases the concentration of heavy metal ions in groundwater to very low concentrations. Due to the inhibition of growth of bacteria by metal ions at high concentration, these must be removed to less than 50 mg/L. The biological sulfate reduction process is more rapid and more stable than the hydroxide heavy metal precipitation in a chemical treatment process (Jalali and Baldwin, 2000). The advantage of SRB for treatment of contaminated groundwater that contains sulfate and heavy metals was extensively discussed (Barnes et al., 1991, 1992; Rowley et al., 1997; Jalali and Baldwin, 2000). So in the research, activity of SRB that produced sulfide as end product was studied to find out the potential of SRB for heavy metal precipitation and to predict elimination of heavy metal ions from groundwater, including the fractionation of sulfur isotopes (Stoegbauer, PhD-thesis, University Karlsruhe). The ratio of S^{32}/S^{34} is normally constant in the environment. However, microorganisms prefer S^{34} for sulfate reduction, leading to a more “heavy” sulfide, whereas a chemical reaction does not discriminate between S^{34} and S^{32}. Thus, sulfur isotopes may serve as useful tracers for analysis of chemical and biological sulfur conversion processes under leaking areas of sewers and

may help to answer the question of the danger of untreated wastewater for the quality of groundwater.

2.6 Which marker species is specific to trace the processes under leaking sewer areas.

To reduce field studies and save costs, the analytical parameters of sulfur compounds were used. The parameters signalled that the process is very complex.

The specific monitoring of leaks is separated in two types of analytical parameters. The one group consists of synthetic or inorganic substrates that act as a marker. Their concentrations indicated the contamination. The second are substrates, which normally occur in nature, like sulfate, nitrogen, nitrate or heavy metals. When these substrates change their concentrations or isotope compositions this may be used to follow their fates in the environment.

The idea of using specific parameters as markers to detect wastewater leaking areas of sewers lead to several suggestions (Table 2.1). In pilot studies hydro-chemical parameters were used, that cost time but only a few were successful. Some specific chemicals were applied for the identification of leaks (Lerner, 1986; Rivers et al., 1996; Barrett et al., 1999). The parameters that should be used according to the literature are listed in Table 2.1.

Addition of chloride and sulfate could reasonably be used as a conservative marker substrate. If the concentration changes, it shows a leak. Nitrate is the end product of degradation of ammonia in sewer wastewater, but might be denitrified if a carbon source is available at anoxic condition. Boron and phosphorus are the main component of detergents but they are not really specific. Phosphorus is not unique in sewer wastewater and its solubility depends on the pH. Chlorination by-products such as bromodichloromethane or chloroform or trihalogenated methane could also be used as markers. Faecal organics, like coprostanol were reported by Hatch and McGillivary (1979) and Kelly (1995) as a selective marker for detection of sewage-contaminated coastal wastewater. Barrett et al. (1999) commented that coprostanol was not suitable for groundwater studies because it is highly hydrophobic and tends to accumulate in wastewater sludge. Aminopropanone, a urine substrate from humans was a useful marker in the studies of Fitzsimmons et al. (1995). Some organic substrates from detergents are present due to recommendation, like sulfonic acid, diaminostibene

disulfonic acid or distyrylbiphenyl. EDTA accumulation in sediment was found in groundwater in Dresden, Germany.

Table 2.1 Potential marker species for sewer leaks (Barrett et al., 1999)

Group	Group	Species
Inorganic	Major cations	Ca^{2+}, Mg^{2+}, K^{+}, Na^{+}
	Major anions	HCO_3^{-}, SO_4^{2-}, CL^{-1}
	Nitrogen species	NO_3^{-}, NH_4^{+}, organic nitrogen
	Other minor ions	B, PO_4^{3-}, Sr, F, Br, CN
	Metals	Fe, Mn, trace metal
Organic	Detergent related	Optical brightness, EDTA, limonene
Microorganism	Faecal bacteria	
	E. coli	

By publications of the past it is obvious that water is a carrier of gastrointestinal human pathogens. The WHO (world health organisation) mentioned that the behaviour of pathogens in water is important. Most countries have laws that rule out the presence of *Escherichia coli* in drinking water. The faecal group of bacteria was taken as an indicator of leaking sewers, despite the knowledge that coliforms can be detected in areas without contamination. Because of this reason now *E. coli* is used as a specific marker of sewage contaminated areas. Viruses or enteroviruses will also be transferred into groundwater, which was contaminated by faecal bacteria. Stable isotopes are popular as markers, such as N^{15}, S^{34}, O^{18} Barrett et al. (1999) suggested that S^{34} and O^{18} might be useful, but in their own work there was no significant advantage.

2.7 Bacterial activity in soil and groundwater

We can divide the microorganisms in two groups. First, the allochthonic microorganisms, which enter into the soil through pollutants, for example sewage, solid wastes etc. The other group are autochthonic microorganisms that are native in soil. The diversity of bacteria that inhabit the soil is related to parameters such as the redox potential, pH, conductivity and concentration of substrates. The redox potential in soil mainly influences the distribution of bacteria with depth. It is the basis of

biochemical reactions, involving oxidation processes or anaerobic reactions of compounds or electrons. The sequence of electron transferring reaction with decreasing redox potential is shown in Fig. 2.4.

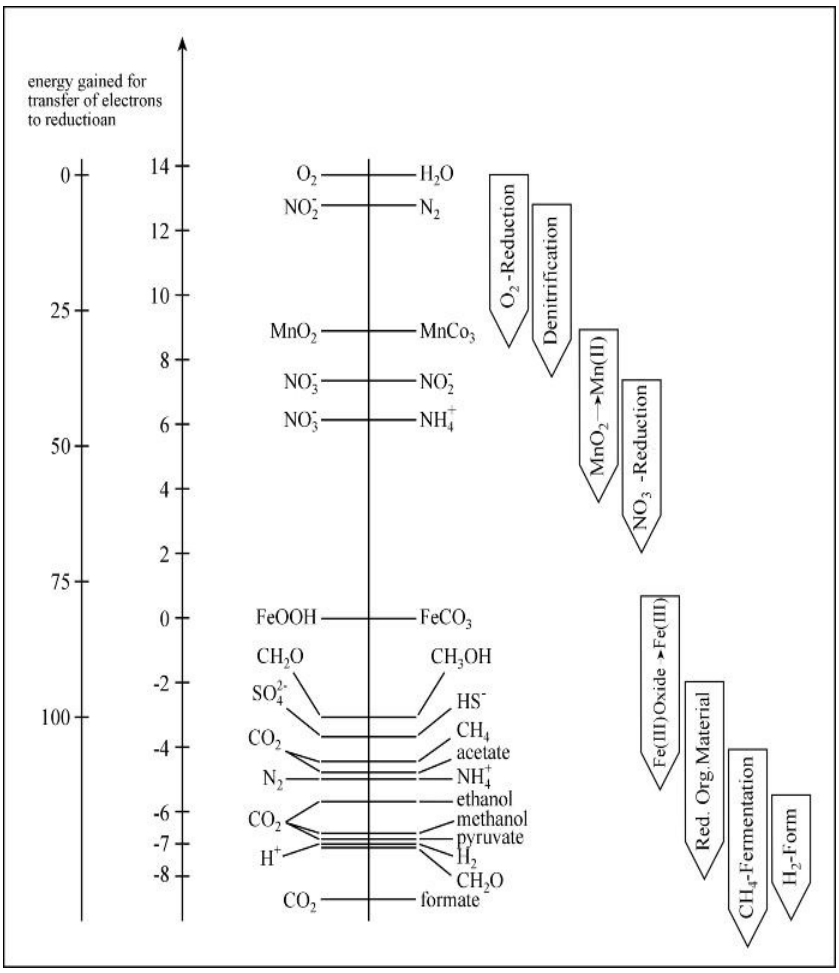


Figure 2.4 The pattern of redox potential in closed aqueous systems (Stumm and Morgan, 1996).

2.7.1 Oxidation-reduction with molecular oxygen

Carbohydrate is degraded to CO_2 and reducing equivalents by glycolysis, pyruvate clevage and the tricarboxylic acid cycle. Then the (H) equivalents react with oxygen to form water in the respiration chain, conserving 66 % of the energy in the phospho anhydride bonds of ATP for growth of bacteria.

$$CH_2O + O_2 \rightarrow CO_2 + H_2 \quad (6)$$

2.7.2 Nitrate reduction or denitrification

The resistant form of nitrogen in the environment is nitrogen in air and ammonia. Nitrate is denitrified in the process of denitrification under the lack of oxygen. The nitrate is reduced to nitrite in the periplasma membrane. After that the nitrite will be reduced to nitric- and nitrous oxide. The final product dinitrogen gas is easily released to the atmosphere (7a, 7b).

$$\underset{\text{nitrate}}{NO_3^-} \xrightarrow{(1)} \underset{\text{nitrite}}{NO_2^-} \xrightarrow{(2)} \underset{\text{nitric oxide}}{NO} \xrightarrow{(3)} \underset{\text{nitrous oxide}}{N_2O} \xrightarrow{(4)} \underset{\text{dinitrogen}}{N_2} \qquad (7a)$$

$$CH_2O + 4/5\ NO_3^- + 4/5\ H^+ \rightarrow 2/5\ N_2 + CO_2 + 3\ H_2O \qquad (7b)$$

1 = nitrate reductase
2 = nitrite reductase
3 = nitric oxide reductase
4 = nitrous oxide reductase

2.7.3 Manganese reduction and Iron (III) reduction

The bacteria of the manganese reduction group develop under anaerobic conditions after the elimination of oxygen and the reduction of nitrate is finished (8). The anaerobic heterotrophic Fe^{3+}-reducing bacteria produce increasing amounts of Fe (9). A Fe (II) utilizing reaction is the reaction with hydrogen sulfide produced by sulfate reducing bacteria, generating iron-sulfide (FeS), which precipitates due to low solubility.

$$CH_2O + 2\ MnO_2 + 4\ H^+ \rightarrow 2\ Mn^{2+} + CO_2 + 3\ H_2O \qquad (8)$$

$$CH_2O + 4\ Fe(OH)_3 + 8\ H^+ \rightarrow 4\ Fe^{+2} + CO_2 + 11\ H_2O \qquad (9)$$

2.7.4 Sulfate reduction

Sulfate reducing bacteria grow under strictly anaerobic conditions with sulfate (10) or sulfur dioxide (the polluting gas from vehicles). Winter (1999) report that HgS, As_2S_3, CdS, CuS or PbS precipitated at acid pH, but ZnS, FeS, NiS or MnS are precipitated at neutral or alkaline pH. Al_2S_3 or Cr_2S_3 is difficult to precipitate due to a much higher solubility.

$$CH_2O + 0.5\ SO_4^{2-} + 0.5\ H^+ \rightarrow 0.5\ HS^- + CO_2 + H_2O \qquad (10)$$

In the microbial metabolism process, microorganisms fractionate the sulfur isotope. The hydrogen sulfide formed by sulfate reducing bacteria has a higher proportion of S^{32} than the original sulfate. The alteration of the relative abundances of S32 and S34 signs for the differentiation of biologically generated sulfide and nonbiogenic sulfide.

2.7.5 Methane fermentation by methanogenesis

Methane bacteria require strictly anaerobic conditions for growth. They are capable of using hydrogen as an electron acceptor and carbon dioxide as a carbon source and require a low redox potential (11). However methanogens utilize also other substrates like formate, carbon monoxide, acetate (12) as well as alcohols.

$$4\ H_2 + CO_2 \rightarrow CH_4 + 2\ H_2O \quad (11)$$

$$CH_3\text{-}COOH \rightarrow CO_2 + CH_4 \quad (12)$$

3. Materials and methods

3.1 Analytical procedures

Total solids (TS) were determined by evaporation of well-mixed samples in an oven at 105 °C (Heraeus GmbH Type T6060, Hanau, Germany), for about 24 hours until constant weight. **The volatile total solids (OTS)** were obtained by subtracting the ash content (550 °C for 3 hours; Heraeus GmbH Type Labor Muffelofen-M110, Hanau, Germany) from the total solid (APHA, AWWA and APCF, 1995).

Biogas components were analysed with a PACKARD model CP 9001. The gas chromatograph was equipped with a Maestro's concentration calculation program, had a TCD-detector and was run with a Teflon column (1.5 mm x 1.8 m), that was packed with Poropack N (80 - 100 mesh). The temperature was set at 40 °C for the column and at 100 °C for the injector and detector. Nitrogen was used as carrier gas with a flow rate of 10 mL/min. A 100 µL-sample was taken from test bottles or reactors with a syringe (Pressure Lock, Precision sampling corp., Baton Rouge, Louisiana) and injected on the column of the gas chromatograph. Standard gases such as methane, carbon dioxide, hydrogen and oxygen with desired content were prepared. Standards were injected first as the reference concentrations under the same condition as the sample. Alternatively the following set-up was used:

Injection temperature 250 °C
Detector temperature 250 °C
Oven temperature 110 °C
Detector Thermal conductivity detector (Micro-WLD) with filament temperature 280 °C
Carrier gas N_2 (25 mL/min)
Column for separation: Carboplot 007, length 2.75 m, diameter 0.53 mm.
Pressure 0 kPa for analysis cell, 150 kPa for reference cell
Split ratio 4 : 1

Fatty acids were determined with a PACKARD model 437 A gas chromatograph. The fatty acids were separated in a Teflon-column, packed with Chromosorb, and with nitrogen as a carrier gas. The samples were centrifuged and the clear supernatant was acidified 1 : 1 with 4 % phosphoric acid (H_3PO_4). The acidified sample was injected

with a micro syringe (Hamilton) on-column. The mixed standard fatty acids (acetate, propionate, butyrate and valerate) were injected as reference before and after analysis of test samples. The concentration of fatty acids in samples should be less than 5 mM. If it was higher, an appropriate dilution was required. The peak areas were calculated from the daily peaks of a standard reference concentration. Set-up of the gas chromatograph:

Injection temperature 210 °C

Detector temperature 210 °C

Oven temperature 180 °C

Detector Flame ionisation detector (FID) with H_2 (30 mL/min) and synthetic gas (300 mL/min) as burning gas

Carrier gas N_2 (30 mL/min)

Column for separation: Teflon column, length 2 m, diameter 1.5 mm, packed with Chromosorb C101 (Sigma)

The pH was measured with a pH-meter using an Ingold electrode model Inlab Nr. 412 (Mettler Toledo, Germany) and a pH-meter (Inolab, Germany).

***Centrifuge*:** To remove particles, samples were centrifuged, with a centrifuge, model 543 (Eppendrof, Germany) and Elmer.

The optical density of bacteria (OD) was determined with a Spectrophotometer (LKB, Biochrom Pharmacia Biotech, model Ultrospec II 4050) at the wave length of 578 nm with a cuvette 12.5 x 12.5 x 45 mm, total volume 1.5 mL (Neolab, Germany). To samples from pure cultures that contained resazurine as a redox indicator, sodium dithionite ($Na_2S_2O_4$) should be added before measurement, to get rid of the indicator's colour, that would interfere with the turbidity measurement of the sample.

Sulfate (Cypionka and Pfennig, 1984) was determined by precipitation of barium ions with sulfate ions as bariumsulfate ($BaSO_4$). The analysis method is as following

Reagent 1: Citrate-glycerine solution, made from 5 % (w/v) citric acid in 60 % (v/v) glycerine

Reagent 2: Barium solution, prepared from 1 % (w/v) $BaCl_2$ x 2 H_2O in 10 % (w/v) citric acid

Analysis: Add subsequently into a test tube

- 2 mL centrifuged sample, only supernatant.
- 2 mL citrate-glycerine solution

• 0.5 mL barium solution and mix well with vortex

• Wait for 30 minutes until the precipitation of bariumsulfate is completed.

The sample in the test tube was mixed again and the extinction was measured at λ 436 nm (Ultrospec II).

Sulfate in pure cultures was determined by suppressing anion chromatography with conductivity detection (DIONEX DX 100 Ion Chromatography). For separation a 4 mm column (DIONEX IonPac AS4A-SC column) and as an eluent $Na_2CO_3/NaHCO_3$ with a flow rate 2 mL/min were used. The sulfate concentration in sample should be in a range of 0 - 40 mg/L.

Lactate (modified according to Horost, 1966) was determined with an enzyme test. The L-lactate reacted with the enzyme L-lactate dehydrogenase and NAD^+ and produced $NADH_2$ as end product.

Reagent:

♦ Glycine hydrazine puffer, pH 9.0, was prepared from glycine (22.8 g) plus 50 mL of 25 % hydrazine-hydrate in 500 mL distillated water. The solution could be kept at 4 °C for 6 months.

♦ NAD^+ solution was made from 100 mg NAD^+ in 10 mL distillated water.

♦ Lactate dehydrogenase (LDH) was L-LDH from rabbit muscle (Boehringer Mannheim) and contained 5 mg protein/mL.

Procedure:

• Add 3 mL glycine hydrazine puffer pH 9 to a test tube.

• Pipet 0.2 mL NAD^+-solution

• Add 0.2 mL centrifuged sample or standard solution.

• Mix well, measure absorption at λ 366 nm as zero point.

• 20 µL of enzyme Lactate dehydrogenase (LDH) was given into the test tube, mixed and incubated at 37 °C for one hour. Then the absorption was measured again at λ 366 nm.

The blind sample (distilled water) was ran similarly as the sample. The lactate concentration in samples should not exceed **100 µg/mL.** The sample should be diluted, if the difference of the sample and the control was not more than $\Delta E_{corr} = 0.22$. The calculation was as follows.

$$\mu\text{mol Lactate/mL} = \frac{\text{Test volume} \times \Delta E \times \text{dilution factor} \times 2}{\in_{366} \times d \times \text{sample volume}}$$

Test volume = total volume in standard test, 3.42 mL

ΔE = the difference of extinctions between sample and control

($\text{extinction}_{\text{sample}} - \text{extinction}_{\text{blind cuvette}}$)

$\in_{366}$ = 3.30 $cm^2/\mu mol$ (Standard test)

d = cuvette's diameter (1 cm)

Test volume of sample = 0.2 mL

Dilution = dilution factor

Chemical oxygen demand (COD; Wolf and Nordmann, 1977): It was analyzed by the reaction of organic matter of the sample with a strong oxidant such as potassium dichromate. Potassium dichromate with addition of silver sulfate as catalyst was recommended as an effective oxidizer. It could oxidize almost all of the organic matter or inorganic compounds (13). In the presence of silver sulfate the nuisance of chloride ion oxidation in test sample was eliminated.

$$Cr_2O_7^{2-} + 6\,e^- + 14\,H^+ \rightarrow 2\,Cr^{3+} + 7\,H_2O \qquad (13)$$

COD-Reagent: $K_2Cr_2O_7$ 15 g

Ag_2SO_4 10 g

H_2SO_4 (98 %) 750 mL

H_3PO_4 (85 %) 250 mL

Analysis:

- 1 mL of sample was pipetted into the test tube
- Add 1.5 mL of COD-reagent, close with test tube cap and mix well with vortex.
- Incubate in thermo block at a temperature of 150 °C for 2 hours.
- After cooling, determine the extinction of the solution with a spectrophotometer at λ_{615} nm.
- Calculate the concentration of COD from a standard curve. Glucose was used as a reference substrate for making the standard curve (1 g = 1000 mg/L COD).

Total COD in soil: Soil was suspended in Milli-Q water and stirred for 30 minutes. The suspension was used as a sample for COD determination. It was analyzed with the same method as fluid samples (Wolf and Nordmann, 1977).

***Total organic carbon and dissolved organic carbon (TOC, DOC); DOC*:** Samples were filtrated through cellulose acetate membrane filters with 0.45 µm pore size (Schleicher & Schuell, Germany). The filtrated samples were acidified with HCl to pH 2 and were purged with air for 5 minutes to strip of inorganic carbon like CO_2 gas. The organic carbon in the samples was oxidized with air at 800 ^oC to CO_2 that was detected with an infrared detector and converted to mg-C/L (Maihak Typ TOCOR2, Germany). A 0.1 N of acetic acid (Type Titrisol solution, Merck, Germany) was referenced for 2.4 g-C/L. The standard stock solution of acetic acid was diluted to 240 mg/L and used as a reference concentration for adjusting the flow rate of samples into the equipment. The range of analysis was 0 - 240 mg/L DOC.

TOC in soil: Soil samples were suspended in Milli-Q water and stirred for 30 minutes. After sedimentation of the solution the supernatant was trickled through folded filter paper Nr. 595 (Schleicher & Schuell, Germany). The filtrated sample was analysed like the DOC measurement of fluid samples. The recommendation for DOC analysis of filtrated samples through 0.45 µm filter was case adapted for TOC analysis of soil suspensions.

Total carbon in soil: The carbon and sulfur in soil were analyzed with an ELTRA CS500 Carbon Sulfur Determinator (ELTRA GmbH, Neuss, Germany). The sample was burned in an oven at a temperature of 1300 ^oC with oxygen as a carrier gas. The gas flow was constantly 180 L/hr. Carbon or sulfur in samples were oxidized to CO_2 and SO_2. They were detected with an infrared analyzer, related to the sample weight and finally integrated to % C and % S. $BaSO_4$ (13.7 % S) and $CaCO_3$ (12 % C) were used as references. The range of analysis were 0 - 12 % carbon and 0 - 13.7 % sulfur .

3.2 Chemicals

All the chemicals were analytical grade products that can be purchased from Merck, Fluka and Sigma Company, using Millipore water (Milli-Q, Germany) for preparation of solutions.

3.3 Gases

All gases that were used in dispensed oxygen and gas chromatography were obtained from Linde (Hoellkriegelsreut**,** Germany).

3.4 Media

3.4.1 Desulfovibrio desulfuricans was cultured in Medium No. 63 of DSM-catalogue of strains (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, 1993).

The media were prepared from solution A, B and C in Schott bottles (Fischer, Germany). Solution A was dispended in an anaerobic chamber into serum bottles (120 mL total volume) in approximate portion. The serum bottles were closed with a rubber stopper, which was fixed with an aluminium cap. The gas phase of the anaerobic chamber was replaced by N_2 : CO_2 (4 : 1) at a gas station. The serum bottles were autoclaved. Solution C was always freshly made. After autoclaving, sterile solution B and filtrated solution C (through 0.2 µm filter) were injected into serum bottles with sterile syringes.

Formular	Chemicals	Weight
Solution A		0.5 g
NH_4Cl	Ammonium chloride	1.0 g
Na_2SO_4	Sodium sulfate	1.0 g
$CaCl_2$ x 2 H_2O	Calcium chloride dihydrate	0.1 g
$MgSO_4$ x 7 H_2O	Magnesiusulfate heptahydrate	2.0 g
L-CH_3COH_2COONa	Sodium lactate	2.0 g
$C_{12}H_7NO_4$	Resazurine	1.0 mg
H_2O	Distilled water	980 mL
Solution B		
$FeSO_4$ x 7 H_2O	Ironsulfateheptahydrate	0.5 g
H_2O	Distilled water	10 mL

Solution C		
$NaH_3SC_2O_2$	Sodiumthiogycolate	0.1 g
$C_6H_8O_6$	Ascorbic acid	0.1 g
H_2O	Distilled water	10 mL

3.4.2 Medium Freshwater II (Widdel, 1988) This medium was used for enrichment cultures.

All solutions were prepared separately. After preparation of solution A, the pH was adjusted by $NaHCO_3$ solution to 7.3. The solution was flushed with nitrogen gas for 10 minutes to obtain anaerobic conditions, before autoclaving at 121 °C for 15 minutes. After cooling filtrated Na_2S solution and mineral solution, prepared anaerobically, were added to the sample bottle before use.

Formular	Chemicals	Weight
Solution A		
NaCl	Sodium chloride	1.0 g
$MgCl_2$ x 6 H_2O	Magnesium chloride hexahydrate	0.4 g
$CaCl_2$ x 2 H_2O	Calcium chloride dihydrate	0.15 g
KCl	Potassium chloride	0.5 g
NH_4Cl	Ammonium chloride	0.25 g
KH_2PO_4	Potassium dihydrophosphate	0.2 g
H_2O	Distilled water	1000 mL
Solution B		
1. Na_2S solution		2 mL/L
Na_2S x 9 H_2O	Disodium sulfide heptahydrate	120 g
H_2O	Distilled water	1000 mL
2. $NaHCO_3$ solution		15 - 30 mL/L
$NaHCO_3$	Sodium bicarbonate	84 g
H_2O	Distilled water	1000 mL
3. Mineral solution	SL 10	2 mL/L

3.4.3 Medium R2A for heterotrophic bacteria

The R2A medium (Reasoner and Geldreich, 1985) had the following composition:

Formular	Chemicals	Weight
	Yeast extract	0.5 g
	Peptone	0.5 g
	Casamino acids	0.5 g
$C_6H_{12}O_6$ x H_2O	Glucose	0.5 g
$(C_6H_{10}O_5)n$	Starch	0.5 g
$C_3H_3O_3Na$	Sodium pyruvate	0.3 g
K_2HPO_4	Di-potassium hydrophosphate	0.3 g
$MgSO_4$ x 7 H_2O	Magnesiumsulfate heptahydrate	0.05 g
	Agar-agar	15.0 g
H_2O	Distilled water	1000 mL

After autoclaving, the pH was adjusted with filtrated $NaHCO_3$ or HCl to 7.2

3.4.4 Medium for sulfate reducing bacteria (Drews, 1968; modified)

Sulfate reducers were cultured in the following medium:

Formular	Chemicals	Weight
KH_2PO_4	Potassium dihydrogen phosphate	0.5 g
NH_4Cl	Ammonium chloride	1.0 g
$MgSO_4$ x 7 H_2O	Magnesiumsulfate heptahydrate	2.0 g
$NaSO_4$	Sodium sulfate	1.0 g
L-$NaCH_3CHOHCOO$	L- sodium lactate	3.5 g
	Ascorbic acid	0.1 g
$C_2H_3NaO_2S$	Sodium thioglycolate	0.1 g
	Yeast extract	0.5 g
	Bromoethane sulfonic acid	2.11 g
	Agar	15 g
H_2O	Distilled water	1000 mL

Ascorbic acid and sodium thioglycolate were filtrated via 0.2 µm filters and added after autoclaving.

3.4.5 Medium for CO_2 reducing bacteria: methane bacteria and acetogenic bacteria (modified after Braun et al., 1979)

Formular	Chemicals	Weight
NH_4Cl	Ammonium chloride	1.0 g
KH_2PO_4	Potassium dihydrogen phosphate	0.68 g
K_2HPO_4	Dipotassium hydrogen phosphate	0.87 g
$MgSO_4$ x 7 H_2O	Magnesium sulfate heptahydrate	0.04 g
	Vitamine solution	20 mL
	Mineral solution	20 mL
	Yeast extract	2.0 g
$NaHCO_3$	Sodium hydrogen carbonate	10.0 g
	Sodium molybdate	2.42 g
	Agar	15.0 g
	Distilled water	950 mL

Vitamine solution was filtrated with 0.2 µm filters and added after autoclaving. The pH of media should be 7.8.

3.4.6 Cellulose medium for cellulose bacteria

Solution A:

Formular	Chemicals	Weight
$(NH_4)_2SO_4$	Ammonium sulfate	1.0 g
NaCl	Sodium chloride	2.0 g
K_2HPO_4	Dipotassium hydrophosphate	13.0 g
H_2O	Distilled water	1000 mL

The pH of solution A was supposed to be 7.0.

Solution B:

Formular	Chemicals	Weight
$MgSO_4$ x H_2O	Magnesium sulfate heptahydrate	0.05 g
$CaCl_2$	Calcium chloride	0.05 g
H_2O	Distilled water	50 mL

Cellulose solution was prepared from 2 % (W/V) cellulose

Medium preparation:

Formular	Chemicals	Weight
	Solution A	500 mL
	Cellulose suspension	200 mL
	Bromoethane sulfonic acid	2.11 g
Na_2MoO_4	Sodium molybdate	2.42 g
	Yeast extract	1.0 g
	Agar	15.0 g
H_2O	Distilled water	250 mL

After autoclaving, 50 mL of sterile-filtered solution B was added to the media. For pouring plates with this medium, it had to be mixed well immediately before pouring to avoid cellulose sedimentation.

3.5 Sources of bacteria

3.5.1 Enrichment cultures were prepared from wastewater and sludge from the wastewater treatment plant of Karlsruhe in Neureut (Germany), from soil below leaky sewer areas in Mannheim and Rastatt (Germany) and from soil of laboratory sand columns (filled with sand, ϕ 0.4 - 2 mm, Wissemburger, Karlsruhe, Germany). The sand columns were continuously supplied with sewage from Neureut's wastewater station for a different time span before unpacking.

3.5.2 Desulfovibrio desulfuricans was used as a reference strain. It was obtained from DSMZ (Braunschweig), DSM-Catalogue (1993), strain-number 642^T =

NCIB 8307, VKM B-1799 (G. Gottschalk, NCIB, M.E. Adams and K.R. Butlin, strain Essex 6. Soil near gas main; England (732, 733). Type strain (1300). (734, 735). (Medium 63, 37 °C, anaerobic). Teaching strain. For all experiments with these bacteria DSM media number 63 were used.

3.5.3 Wastewater samples to determine the numbers of bacteria by plate counting were freshly withdrawn periodically from the reactors at different sampling positions with a sterile syringe through butyl rubber stoppers. Seven different soil columns (Table 3.1) were investigated.

3.6 Conditions of reactors before sampling

Sand Columns for trickling of sewage

Data of the studied columns (Part 4.3) are compiled in Table 3.1, Fig. 3.1 and Fig. 3.2.

NRT and SAW-columns: ***The 20 cm diameter columns*** were constructed from 30 cm pieces of PE columns (total length 1.2 m, ∅ 20 cm) connected with screws and filled with quartz sand (~80 % quartz, 10 - 20 % feldspart and < 5 % clay minerals). The total carbonate content was 1.2 %, determined by using the Scheibler-method. The saturated hydraulic conductivity as calculated with the Hazen-Formula was 6 x 10^{-3} m/s. The columns contained an inner and an outer outflow (Fig. 3.1) 0.5 L of gravel was filled to serve as supporting layer on the bottom of the columns to prevent excessive drag-out of the wet sand. The sand was filled into the columns in portions of 2 liters and compressed by four hits of a falling weight (2.46 kg, 30 cm height of fall). In each depths of 5, 10, 20, 40, and 90 cm (below upper soil surface), five suction roots (Saugkerzen) were built into the columns to observe vertical distribution profiles. During the experiments, the columns were kept in the dark and thermostated at 17 °C. Deionized water was pumped into the four different columns at a flow rate of approximately 3 L/day. After a week, wastewater was pumped into three of the columns with a flow rate of 2 L/day. The fourth column served as a reference and was run with tap water (DFG project, 2002).

Wastewater was taken routinely by every three weeks from the influent of a sewage treatment plant (Neureut, Germany) after it had passed the silting tank. The samples were then frozen and stored at –20 °C. Every half week the frozen wastewater

was thawed, filled into a storage tank that stayed in a refrigerator (4 °C), and served to run the experiments. This procedure was chosen to maintain a single batch composition of wastewater during a three-week period. To analyse biological and chemical processes taking place in the refrigerator, influent samples were taken both at the beginning (NRT in_{new}) as well as at the end (NRT in_{old}) of the half-week-experimental period. Effluents from the columns were taken as mixed samples over a period of 24 hr (NRT 1, NRT 2, NRT 3). Samples from the reference column are named blank (DFG project, 2002).

IBA-columns: ***The 10 cm diameter columns*** (IBA1 and IBA2) were obtained from Technische Glasblaeserei H+K Paris, Karlsruhe. The total length was 1.50 m, the sand bed was 120 cm at a diameter of 10 cm. The thickness of the glass walls was 2 mm. Columns were operated in a room at constantly 20 ^{o}C temperature. Gas columns were wrapped with aluminum foils to protect the reactor from light. The columns had an inner funnel for collection of trickling sewage from the center and an outer ring-like area for collection of sewage that trickled along the glass walls and in the peripherical zone (Fig. 3.1). Gravel and coarse sand were filled into the bottom of the column as supporting layer (height 8 - 10 cm) followed by wet sand of 0.4 – 2 mm grain size and filled to give 120 cm sand height. During filling the sand was compressed every 10 cm. On the top of columns there were 10 cm water column (sludge + wastewater + sediment). Each column had 6 small outlet points for sampling of the gas at different depths (0, 20, 50, 80, 110 cm from upper surface). Before operation with sewage tap water was trickled for a week and then the sewage was pumped with constant speed to the top of the column (Gilson pump model Minipuls, Abimed Analysen Technik GmbH, Germany, Fig. 3.1). The overflow of influent was recirculated back to the influent bottle. Initially a high flow rate was expected which should decrease with time due to a sludge layer sediment on top of the sand and due to biofilm formation on the sand. The sewage was homogenized with a stirrer at 177 rpm speed (Janke & Kunkel GmbH, IKA Labortechnik, Germany).

Table 3.1 Experimental details of the 7 investigated soils columns

Column designation	NRT1	NRT2	SAW1	SAW2	Blank	IBA1	IBA2	Remark
Temperature	17 °C	17 °C	17 °C	17 °C	17 °C	20 °C	20 °C	
Flow rate	2 L/day	2 L/day	2 L/day	2 L/day	2 L/day	0.04 - 3.3 L/day average = 0.75 L/d	0.063 - 9.6 L/day average = 1.1 L/d	
Operation time	10 months	21.5 months	21.5 months	8.2 months	21.5 months	4 months	18 months	
Column: diameter	20 cm	20 cm	20 cm	20 cm	20 cm	10 cm	10cm	
: Length	120 cm	120 cm	120 cm	120 cm	120 cm	120 cm	120 cm	
: Material	PE	PE	PE	PE	PE	glass*	glass*	* = Glass column from Technishe Glasblaeserei H + K Paris, Karlsruhe.
Filling material	sand (0.4 - 2 mm)	sand (0.4 - 2 mm)	sand (0.4 - 2 mm)	sand (0.4 - 2 mm)	sand (0.4 - 2 mm)	sand (0.4 - 2 mm)	sand (0.4 - 2 mm)	
Influent	wastewater	wastewater	wastewater	wastewater	wastewater	wastewater	wastewater	Neureut – wastewater treatment plant, Karlsruhe, Germany

Wastewater was taken routinely every three weeks from the influent of a sewage treatment plant (Neureut, Germany) after it had passed the silting tank. The samples were then frozen and stored at –20 °C. Every half week the frozen wastewater was thawed, filled into a storage tank that stayed in a refrigerator (4 °C), and served to run the experiments. This procedure was chosen to maintain a single batch composition of wastewater during a three-week period. To analyse biological and chemical processes taking place in the refrigerator, influent samples were taken both at the beginning (NRT in_{new}) as well as at the end (NRT in_{old}) of the half-week-experimental period. Effluents from the columns were taken as mixed samples over a period of 24 hr (NRT 1, NRT 2, NRT 3). Samples from the reference column are named blank (DFG project, 2002).

IBA-columns: ***The 10 cm diameter columns*** (IBA1 and IBA2) were obtained from Technische Glasblaeserei H+K Paris, Karlsruhe. The total length was 1.50 m, the sand bed was 120 cm at a diameter of 10 cm. The thickness of the glass walls was 2 mm. Columns were operated in a room at constantly 20 ^{o}C temperature. Gas columns were wrapped with aluminum foils to protect the reactor from light. The columns had an inner funnel for collection of trickling sewage from the center and an outer ring-like area for collection of sewage that trickled along the glass walls and in the peripherical zone (Fig. 3.1). Gravel and coarse sand were filled into the bottom of the column as supporting layer (height 8 - 10 cm) followed by wet sand of 0.4 – 2 mm grain size and filled to give 120 cm sand height. During filling the sand was compressed every 10 cm. On the top of columns there were 10 cm water column (sludge + wastewater + sediment). Each column had 6 small outlet points for sampling of the gas at different depths (0, 20, 50, 80, 110 cm from upper surface). Before operation with sewage tap water was trickled for a week and then the sewage was pumped with constant speed to the top of the column (Gilson pump model Minipuls, Abimed Analysen Technik GmbH, Germany, Fig. 3.1). The overflow of influent was recirculated back to the influent bottle. Initially a high flow rate was expected which should decrease with time due to a sludge layer sediment on top of the sand and due to biofilm formation on the sand. The sewage was homogenized with a stirrer at 177 rpm speed (Janke & Kunkel GmbH, IKA Labortechnik, Germany).

Sewage from the sewage treatment plant of Neureut was sieved through a cooking sieve and stored at 4 ^{o}C maximally for 2 weeks before use. The daily required

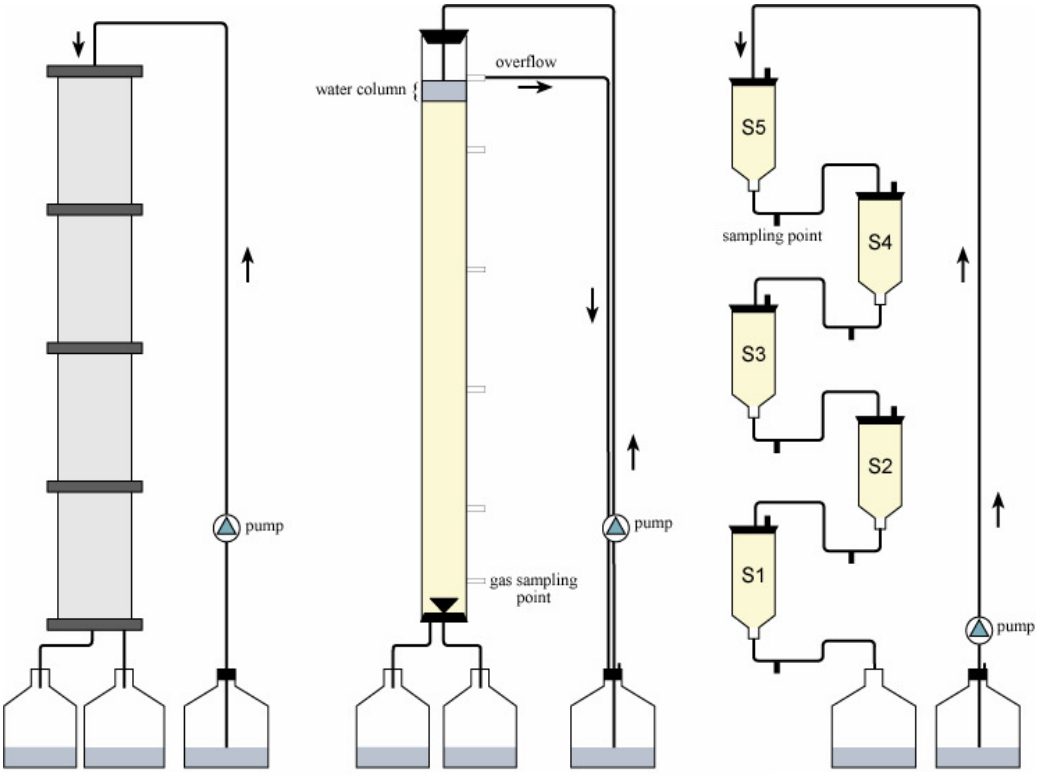
amount of sewage was pre-stored in a glass carboy at 20 °C and was continuously purged with nitrogen gas to prevent aerobic degradation. Less than 5 % of the COD was lost by anaerobic degradation. Samples for chemical analyses were 24 hour composite samples from effluent, $influent_{new}$ and $influent_{old}$. After 6 months of running time, the top of the column was sealed with a butyl rubber stopper, thus eliminating oxygen and maintaining a constant flow rate, equal to the flow rate of the pump.

The segmented column (total length 125 cm, 10 cm in diameter): It was represented by 5 glass columns in sequence (Technische Glasblaeserei H+K Paris, Karlsruhe; length of each 25 cm, sand bed 20 cm, diameter 10 cm, thickness of glass wall 2 mm) that were connected with silicone or tygon tubings (Fig. 3.1). The columns were run in a thermo-stated room at 20 °C. Each single column was filled with gravel and coarse sand at the bottom (5cm) followed by a 20 cm wet sand layer. The top of each column was sealed with a butyl rubber stopper. The columns were wrapped with aluminum foil to prevent light (Fig. 3.2). Each connection between 2 sub-columns had a siphon shape for withdrawal of samples for analysis of chemical parameters representative for each depth. Before operation, the column was run with tap water for a week and then the wastewater was pumped with a constant flow rate (Gilson pump model Minipuls, Abimed Analysen Technik GmbH, Germany) to the top of column No. S5. The sewage flow followed gravity. In the first month, the combined columns were run with about 1 L/day flow rate, which was increased to 2 L/day in the second month and then to about 3 L/day in the third month. The maximal flow rate was 4.5 L/day.

3.7 Experimental operation

Part 1 Batch culture*: Medium "Freshwater II"* were prepared in Schott bottles (0.5 or 1 L) or serum bottles (total volume 125 mL) under strictly anaerobic conditions. The gas phase was flushed with carbon dioxide : nitrogen (1 : 4) or 100 % nitrogen gas. Media were then autoclaved. Then 10 % of a supernatant from suspended soil, or soil, or sludge or another inoculums were injected into the test bottles. If soil was added directly into the bottles, after addition of the soil sample, the bottle was purged again with nitrogen gas.

Samples were periodically withdrawn from the bottle with a syringe for chemical analyses. Before sampling, a sterile syringe was flushed several times with CO_2/N_2 gas. Gas samples were withdrawn with a gas tight pressure lock syringe for gas measurement.



a. ϕ 20 cm column **b**. ϕ 10 cm column **c**. ϕ 10 cm seg. column

Figure 3.1 Scheme of the different sand columns

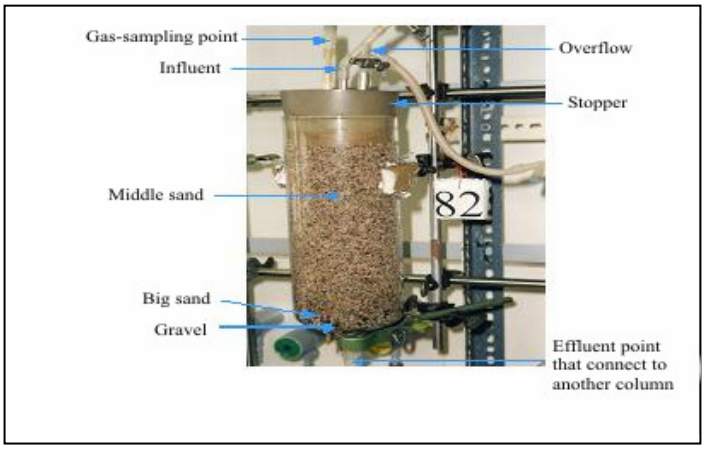


Figure 3.2 Detail of one of the five sub-columns of the segmented column. Five segments were connected top to bottom.

***Part 2**: The operation of fermenters for continuous cultures*

Infors-Fermenters model 4103 with 2.5 L volume of the culture vessel (Infors AG 4103, Bottmingen, Switzerland) were used for this research. Medium Nr. 63 (DSMZ, Braunschweig) was prepared and dispensed with nitrogen gas for 10 minutes without autoclaving. The 10 % bacterial inocula were injected into the reactors as initial biomass. During the experiment, the pH was automatically adjusted to a range of 7 - 7.2 with 3 mM sodium hydroxide or 10 % (v/v) hydrochloric acid. The fermenters was run at room temperature.

***Part 3**: Bacterial culture and preparation of samples*

Medium: The total count of aerobic and anaerobic bacteria were determined by culturing of diluted samples on the surface of R2A agar media at pH 7.2, according to Reasoner et al. (1985). Denitrifying bacteria were determined in complex media using nitrate as an N-source (Alef, 1991). Sulfate reducing bacteria were examined with sulfate reducing-lactate medium (Drews, 1968). Coliform bacteria were grown in Endo-agar (DIN 38411).

Sampling: The samples were collected from soil of an unpacked column (such as NRT I-column after several months of trickling sewage through it) at different depths. The samples were kept in sterile Schott-bottles and closed with butyl rubber stopper. Bottles were flushed with nitrogen at normal pressure and stored at 4 ^{o}C before analysis of the soils. All samples were examined within 2 hours after collection. 10 g of soil from each depth were suspended with 50 - 100 mL of 0.85 % sodium chloride solution (w/v) and stirred with a magnetic stirrer (Janke & Kunkel GmbH & Co Type IKA Combimag Reo, Breisgau, Germany) at 300 rpm speed for 30 minutes. These suspensions were used as an initial inoculum. The supernatant was sampled for preparation of serial dilutions into 0.85 % of sodium chloride solution.

The fluid samples to determine the population of suspended bacteria were periodically withdrawn with sterile syringes and serial dilutions were made with 0.85 % of sodium chloride to approximate concentrations.

Spread plate enumeration and counting: 100 µL of sample from an appropriate dilution with approximately 1000 CFU/mL was pipetted onto agar plates and spreaded for the plate count procedure (n = 3). For culturing anaerobic bacteria, an anaerobic

chamber was used (Coy Laboratories, Ann Arbor, Mi, USA). For aerobes the working procedures were performed in a sterile cabinet.

Plates for culturing of anaerobic bacteria were incubated in anaerobic jars under 1.5 bar nitrogen gas atmosphere at 20 ^{o}C for 4 weeks to allow colonies to grow (Balch et al., 1979). Plates for culturing of aerobic bacteria were placed in plastic bags and incubated at 20 ^{o}C for 7 days. The colony forming units (CFU) on each plate should not exceed 20 - 300 CFU/plate to allow counting. The number of bacteria was calculated as mentioned below.

C*alculation of the population density of bacteria:*

The bacterial cell number was calculated with the following formular:

$$\textbf{Total bacteria/mL} = \frac{Y \times (\sum K_x + \sum K_{x+1}) \times 10^{x}}{N_x + 0.1N_{x+1}}$$

Y = volume of sample (mL) that was spread on plates

$\sum K_x$= the total bacterial colonies at dilution factor 10^{-x}

$\sum K_{x+1}$ = the total bacterial colonies at dilution factor 10^{-x+1}

N_x = the number of replications of spread plates at dilution factor 10^{-x}

N_{x+1} = the number of replications of spread plates at dilution factor 10^{-x+1}

Total bacterial cell: total aerobic + total anaerobic bacteria

% Elimination = $[X_{initial}-X_t)/X_{initial}] \times 100$

$Bacteria_{initial}$ = number of cells in first profile.

3.8 Calculation of the stoichiometric ratio of substrate to electron acceptor for sulfate reduction:

The theoretical ratio of carbon source per mole of sulfate during incomplete carbon source degradation can be seen from the equations 14 - 18 below (Kleikemper et al., 2002).

Butyrate as a carbon source for sulfate reduction:

$$2/3\ CH_3(CH_2)_2COO^- + SO_4^{2-} \rightarrow 2/3\ CH_3COO^- + 2/3\ HCO_3^- + HS^- + 1/3\ H^+ \qquad (14)$$

Propionate as a carbon source for sulfate reduction:

$$4/3\ CH_3CH_2COO^- + SO_4^{2-} \rightarrow 4/3\ CH_3COO^- + 4/3\ HCO_3^- + HS^- + 1/3\ H^+ \quad (15)$$

Lactate as a carbon source for sulfate reduction: ($\Delta G = -38.2$ kcal·reaction^{-1})

$$2\ CH_3CHOHCOO^- + SO_4^{2-} \rightarrow 2\ CH_3COO^- + 2\ HCO_3^- + HS^- + H^+ \quad (16)$$

Acetate as a carbon source for sulfate reduction:

$$CH_3COO^- + SO_4^{2-} \rightarrow 2\ HCO_3^- + HS^- \quad (17)$$

Hydrogen as a carbon source for sulfate reduction:

$$4\ H_2 + SO_4^{2-} \rightarrow 4\ H_2O + S^{2-} \quad (18)$$

Hydrogen could also be used for carbon dioxide reduction to methane in competition to sulfate reduction (19):

$$4\ H_2 + 2\ HCO_3^- + H^+ \rightarrow CH_4 + 3\ H_2O \quad (19)$$

In addition, lactate could syntrophically be converted to acetate and methane by sulfate reducers and methanogens, if sulfate was not available (20, 21):

$$2\ CH_3CHOHCOO^- + 4\ H_2O \rightarrow 2\ CH_3COO^- + 2\ HCO_3^- + 2\ H^+ + 4\ H_2 \quad (20)$$

$$2\ CH_3CHOHCOO^- + H_2O \rightarrow 2\ CH_3COO^- + HCO_3^- + H^+ + CH_4 \quad (21)$$

The calculation of DOC and COD of fermentation products:

$$DOC = c\ (C_xH_yO_z) \times M \times 12 \times 1000 \quad mg/L$$

$$COD = C_xH_yO_z + n\ O_2 \rightarrow x\ CO_2 + y\ H_2O$$

Formular (1mM)	DOC (mg/L)	COD(mg/L)
Acetate: CH_3COOH	24	64
Propionate: CH_3CH_2COOH	36	112
Butyrate: $CH_3(CH_2)_2COOH$	48	160
Lactate: $CH_3CHOHCOOH$	36	96
Glucose: $C_6H_{12}O_6$	72	192

4 Results

4.1 Batch culture of sulfate reducing enrichments from different sources

Reaction conditions for the experiments that were undertaken with soil and sludge samples to enrich sulfate reducing bacteria and to investigate sulfate reduction of *D. desulfuricans* are summarized in Table 4.1.1.

Table 4.1.1 Conditions for batch culture experiments with sewage sludge, contaminated soils and pure cultures of *D. desulfuricans*

Test	Strain of bacteria	Temperature	Medium	Electron source	Remark
4.1.1	Neureut's sludge	4 , 15 and 37 ^{o}C	Freshwater II	SO_4^{2-}: Acetate and Butyrate	with BESA
4.1.2	Mannheim's soil	15 ^{o}C and 37 ^{o}C	Freshwater II	SO_4^{2-}: Acetate Butyrate Lactate	with or without BESA
4.1.3	*D. desulfuricans*	15 ^{o}C and 37 ^{o}C	DSM Nr. 63	SO_4^{2-}: Lactate	

4.1.1 Batch culture experiments with sewage sludge from the sewage treatment plant of Neureut.

Sludge from Neureut's domestic sewage treatment station was sampled and tested in batch assays at different temperatures for its capability to reduce sulfate with acetate and butyrate as carbon sources. The assays were incubated at conditions as mentioned in Table 4.1.1.

At a temperature of 4 ^{o}C, sulfate reduction proceeded over a 30 days incubation period. Finally 9.6 mM sulfate were still present (39.4 % reduction) in the test unit. The SRR was 8.6 µM/hr (0.8 mg/L·hr). The SRB were incompletely oxidizing sulfate reducers, since they did not degrade acetate. Butyrate was converted to acetate and acetate accumulated in the test-unit (Fig. 4.1.1a).

At a temperature of 15 ^{o}C, sulfate reduction proceeded for 44 days. The 16 mM of initial sulfate were eliminated to finally 5.6 mM of sulfate (64.8 % reduction). The SRR was 11.7 µM/hr (1.1 mg/L·hr). The enriched sulfate reducing bacteria belonged to the incompletely oxidizing SRB group. Therefore they could not utilize acetate. The bacteria could degrade butyrate to acetate and acetate accumulated in water (Fig. 4.1.1b).

In samples that were incubated at 37 °C, 100 % of the initial sulfate was degraded within 30 days. The SRR of bacteria was 22.1 µM/hr (2.1 mg/L·hr). The enriched SRB in this experiment belonged also to the incompletely oxidizing group of SRB. The enrichments could only use butyrate as a carbon source (Fig. 4.1.1c).

The temperature had a pronounced effect on the activity of the sulfate reducing bacteria. It turned out that a temperature of 37 °C was the optimal growth temperature for SRB (Fig. 4.1.1d). The sulfate reduction rate declined with decreasing temperature. Most of the known SRB were mesophilic bacteria.

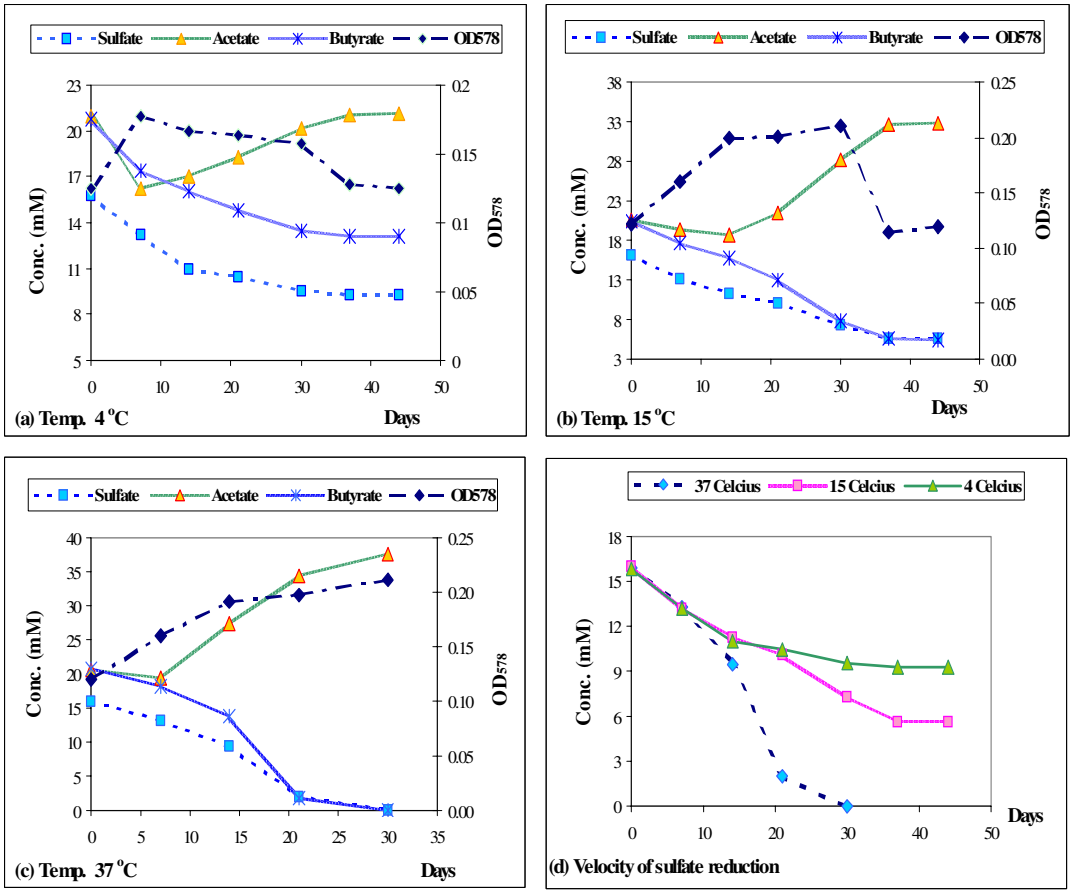


Figure 4.1.1 Concentration of sulfate, butyrate, acetate, and growth (OD_{578}) of sulfate reducing enrichment cultures from sewage sludge of Neureut. The sludge was incubated at different temperatures: (a) = 4 °C, (b) = 15 °C, (c) = 37 °C. Comparison of the velocity of sulfate reduction as effected by temperature (d).

4.1.2 Enrichment of sulfate reducing bacteria in batch culture with soil taken from below a leakage of a sewer in Mannheim

Soil below a leaking sewer from Mannheim was investigated for its sulfate reduction activity with a variety of carbon sources, such as acetate, butyrate and lactate. Parallel assays with and without the addition of BESA (bromoethane sulfonic acid) were incubated at 15 ^{o}C and 37 ^{o}C. BESA acted as an inhibitor for methane bacteria, the competing group of anaerobes with the SRB under an anaerobic atmosphere.

***Incubation temperature 15 ^{o}C*:**

Soil samples were incubated with lactate as a carbon source in the presence or absence of BESA. The sulfate reduction began after a lag phase of one week. 76 % and 53.5 % of the initial sulfate were reduced in samples with and without BESA after a month of incubation (Fig. 4.1.2), respectively. The SRR was 24.4 µM/hr (2.3 mg/L^{-}hr) and 13.9 µM/hr (1.3 mg/L^{-}hr) in the presence and absence of BESA, respectively. Enrichments contained incompletely oxidizing SRB, thus lactate was converted to propionate and acetate. Methane gas was not found in both treatments.

During incubation with acetate as an electron donor, 34.3 % and 32.3 % of the initial sulfate concentration were degraded by completely oxidizing SRB in both assays in the presence or absence of BESA (Fig. 4.1.3). The SRR of samples with and without BESA were 10.7 µM/hr (1 mg/L^{-}hr) and 11.0 µM/hr (1.1 mg/L^{-}hr). The sulfate reduction activity was not restricted to bacteria of the incompletely oxidizing SRB, but methane gas was not found. It could be assumed that only SRB grew in these assays.

If butyrate was the carbon source for bacteria 42.4 % of the initial sulfate concentration was degraded in the presence of BESA and 44.4 % in samples without BESA (Fig. 4.1.4). The SRR of samples with and without BESA were 8.7 µM/hr (0.8 mg/L^{-}hr) and 8.1 µM/hr (0.8 mg/L^{-}hr). Methane gas was not found until the third week in the samples without BESA, but totally little methane was generated (0.1 %). Butyrate was converted to acetate. No propionate was produced.

The best carbon source for SRB was lactate, followed by butyrate and acetate (Fig. 4.1.5). The addition of BESA had almost no effect on the competition between SRB and methane bacteria or other bacteria for acetate and butyrate as a carbon source. The presence of 10 mM BESA had affected the growth of sulfate reducing bacteria that used lactate as a carbon source.

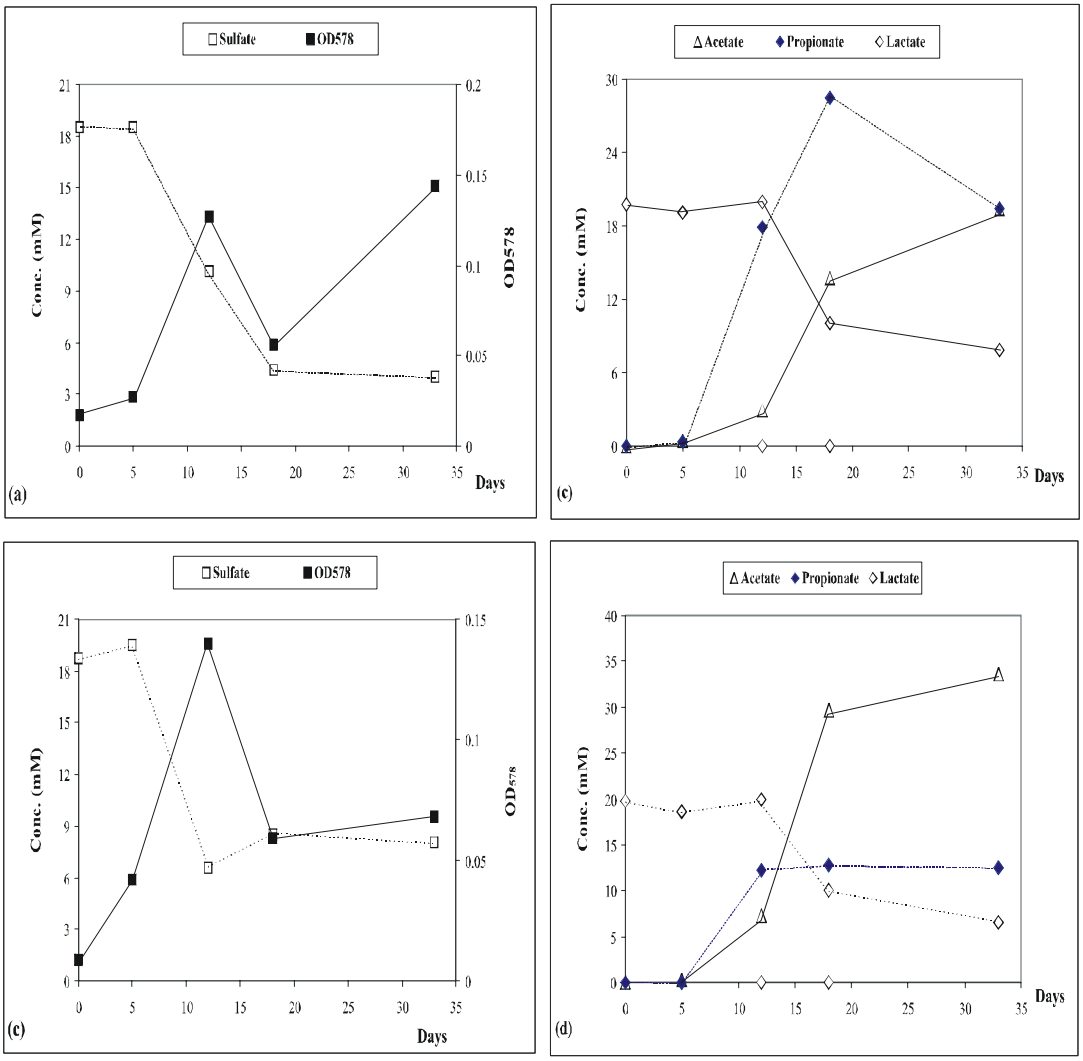


Figure 4.1.2 Concentration of sulfate, lactate and fatty acids and growth of biomass (OD_{578}) during incubation of soil from below leaking sewers of Mannheim, with BESA (a - b) and without BESA addition (c - d). The soil was used as an inoculum for Freshwater II media and lactate was the C-source at 15 ^{o}C.

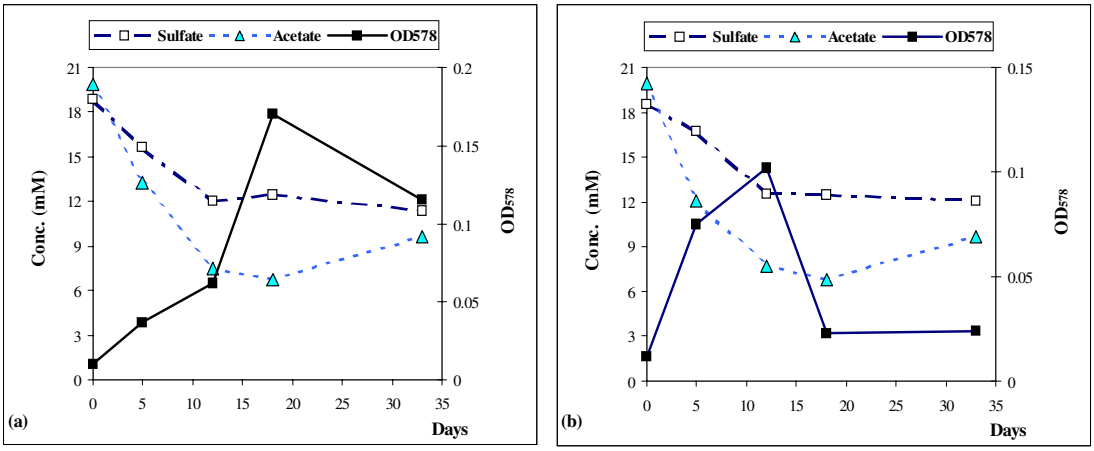


Figure 4.1.3 Concentration of sulfate and acetate, growth of biomass (OD_{578}) during incubation of soil from below leaking sewers of Mannheim, (a) with and (b) without BESA addition. Soil was incubated in Freshwater II media. Acetate was the C-source at 15 ^{o}C.

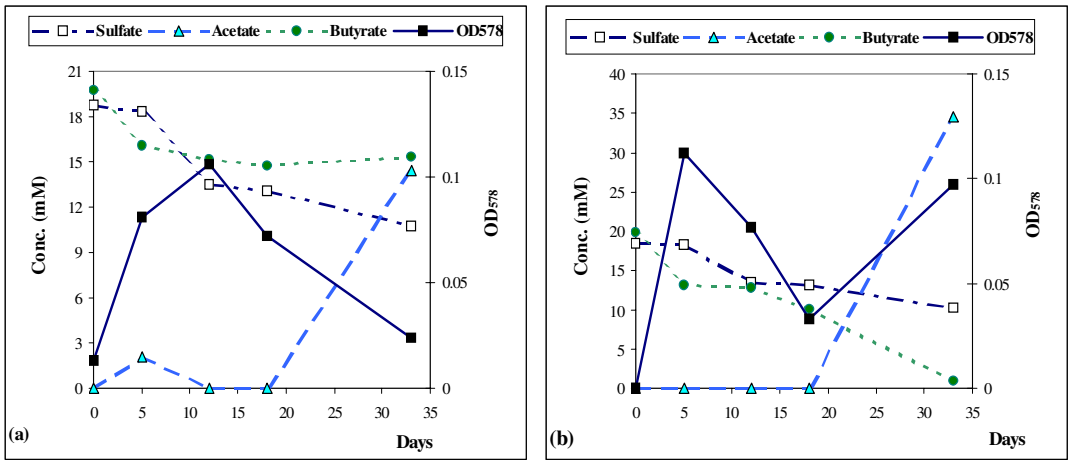


Figure 4.1.4 Concentration of sulfate, butyrate, acetate and biomass (OD_{578}) that grew in soil from below leaking sewers of Mannheim. Enrichments were incubated in Freshwater II media (a) with and (b) without BESA. Butyrate was the C-source at 15 ^{o}C.

It might be that the carbon sources in the test were better suitable for SRB than for MB. However test conditions were more appropriate for growth of SRB, such as high concentrations of sulfate (nearly 20 mM) or supply of carbon source.

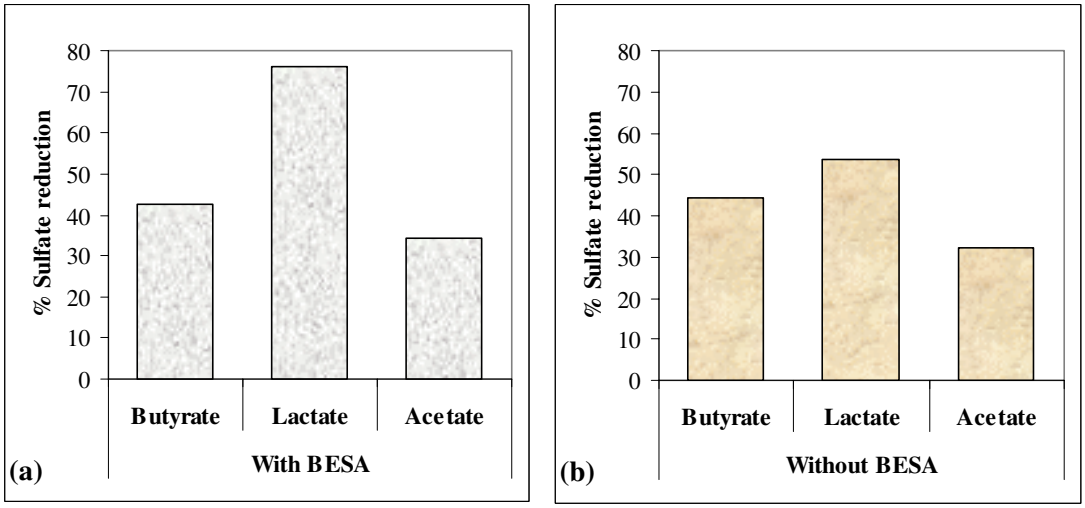


Figure 4.1.5 The ability of SRB to degrade different carbon sources as effected by the presence of BESA (a) or in the absence of BESA (b). Incubation in Freshwater II media at 15 ^{o}C.

Incubation temperature 37 ^{o}C:

Sulfate reduction with lactate (Fig. 4.1.6) as a carbon source was investigated with and without addition of BESA. 98.3 % and 95.8 % of the initial sulfate concentrations were reduced after 2 weeks with a SRR of 53.8 µM/hr (5.2 mg/L·hr) and 34.7 µM/hr (3.3 mg/L·hr) in the samples ± BESA, respectively. After that 10 mM of sulfate was injected into the assay bottle (with BESA). The SRR after re-feeding of sulfate during day 21 - 41 was 12.4 µM/hr (1.2 mg/L·hr). The assays without BESA were also recharged with 7 mM sulfate after 21 days. However, the SRB were no longer active. It might be that the conditions in the bottle were unsuitable for growth of bacteria, due to a high content of sulfide or acidic pH. Incompletely oxidizing SRB were enriched in the assays, and lactate was converted to propionate and acetate. Lactate was injected twice in the sample bottles to offer a non-limiting amount of C-source. However the SRR was decreased after the 2nd lactate addition if compared with the initial SRR, due to unfavourable conditions during the test. Methane gas was found in treatments without BESA after a week of incubation. The content of methane

gas was in the range of 0 - 3 %. In the samples with 10 mM BESA methane production was prevented for the whole time of the experiment.

In a control treatment with addition of sulfate but no addition of a carbon source no activity of sulfate reducing bacteria was observed. No changing of sulfate values was observed in both samples in the absence or presence of BESA. No changing of gas evolution was noticed. Fatty acids could not be detected in the whole incubation.

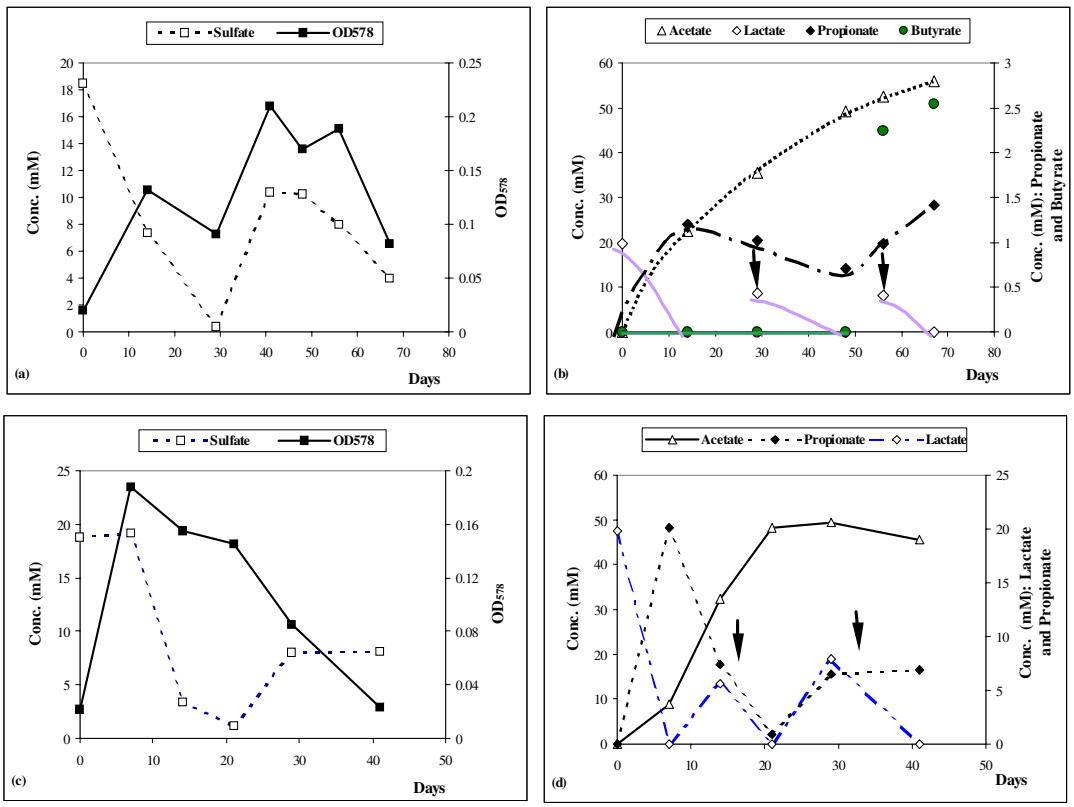


Figure 4.1.6 Concentration of sulfate, lactate, fatty acids, and growth of biomass in enrichment cultures from soil under leaking sewers of Mannheim (a - b) with and (c - d) without BESA. The soil was incubated in Freshwater II medium, lactate was supplied as a C-source at 37 °C. *Arrows* shows the time when the C- or S-source in the test unit was replenished.

During incubation of soil with acetate as electron donor 66.1 % and 62.3 % of the initial sulfate were degraded by completely oxidizing SRB in samples ± BESA during 41 days. The SRR of samples with and without BESA were 12.6 µM/hr (1.2 mg/L·hr) and 11.6 µM/hr (1.1 mg/L·hr), respectively, as shown in Fig. 4.1.7. 0 - 2.3 % methane gas was produced in samples without BESA.

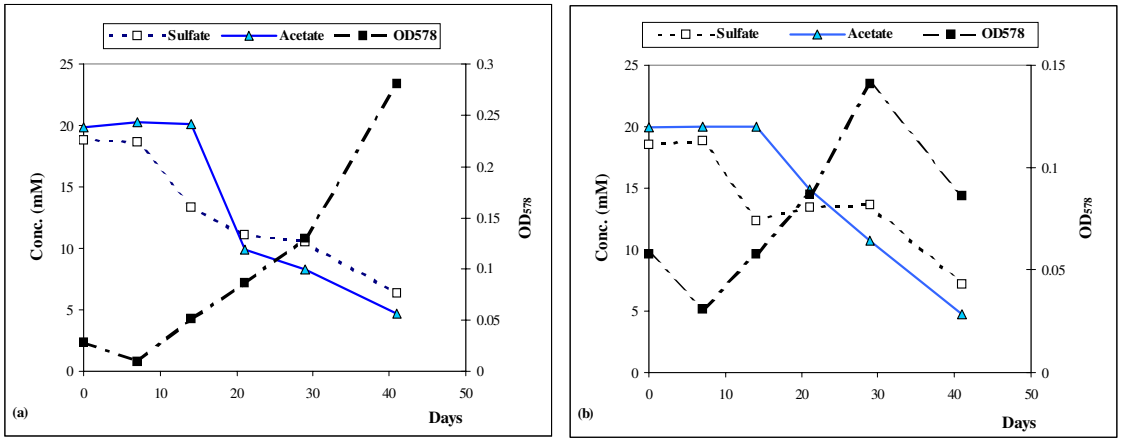


Figure 4.1.7 Concentrations of sulfate, acetate, and growth of biomass (OD_{578}) in enrichment culture from soil below leaking sewers in Mannheim (a) with and (b) without BESA. Soil was incubated in Freshwater II medium with acetate as C-source at 37 °C

Incubation of soil samples with butyrate as carbon source for the bacteria resulted in 70.6 % of the initial sulfate concentration being reduced in the sample with BESA and 55.3 % in the sample without BESA. The SRR of samples with and without BESA was 23.3 µM/hr (2.2 mg/L·hr) and 20.2 µM/hr (1.9 mg/L·hr) (Fig. 4.1.8), respectively. Methane gas was found after a month, ranging from 0 - 1.2 % in the sample without BESA. The butyrate was converted to acetate. No propionate was found in the cultures.

Lactate seemed to be the favourite carbon source for SRB in the test, followed by butyrate and acetate (Fig. 4.1.9 - 10). The addition of BESA had no effect on the competition between SRB and MB in all media, except in the assays with lactate as a carbon source. The conditions were suitable for SRB, such as high concentrations of sulfate (nearly 20 mM) or carbon source with a high affinity to SRB or sulfide concentrations that inhibited the MB. The addition of BESA seemed to be not

necessary. The SRB belonged to both groups, completely and uncompletely oxidizing sulfate reducers. The temperature had an influence on the SRR. At high temperature of 37 °C the SRB activity required a shorter time than at 15 °C and resulted in higher SRR.

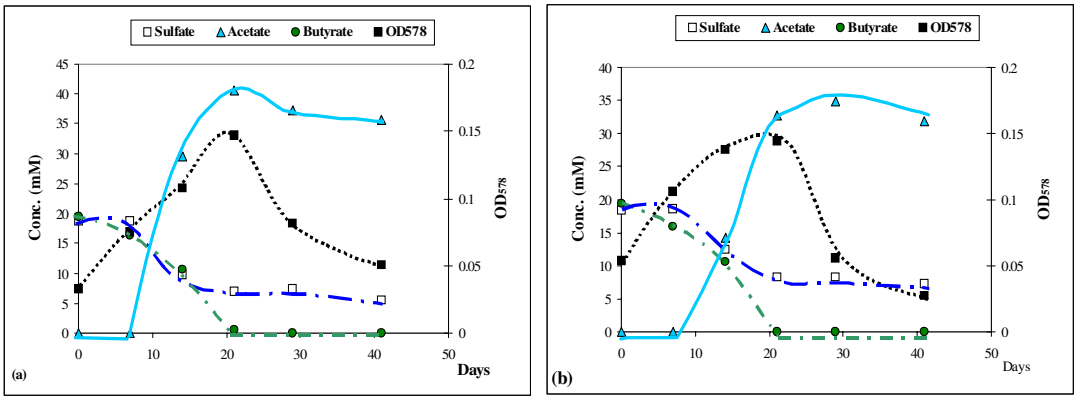


Figure 4.1.8 Concentration of sulfate, butyrate, acetate, and biomass in enrichment cultures from soil below leaking sewers in Mannheim (a) with and (b) without BESA. Soils were incubated in Freshwater II medium with butyrate as C-source at 37 °C.

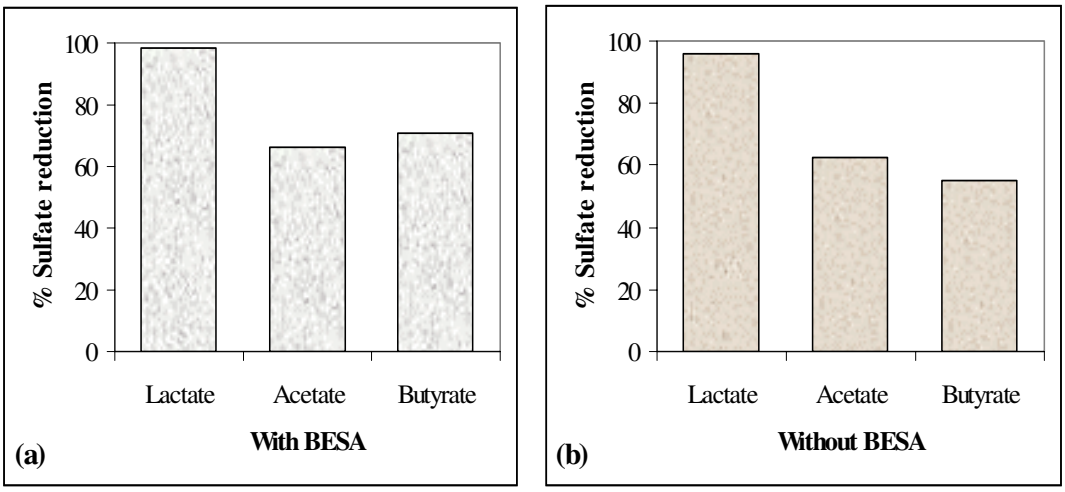


Figure 4.1.9 The ability of SRB to degrade different carbon sources and the effect of BESA (an inhibitor of methane bacteria) on the activity of SRB that were incubated in Freshwater II medium at 37 °C (a). In (b) the sulfate reduction in the absence of BESA is shown.

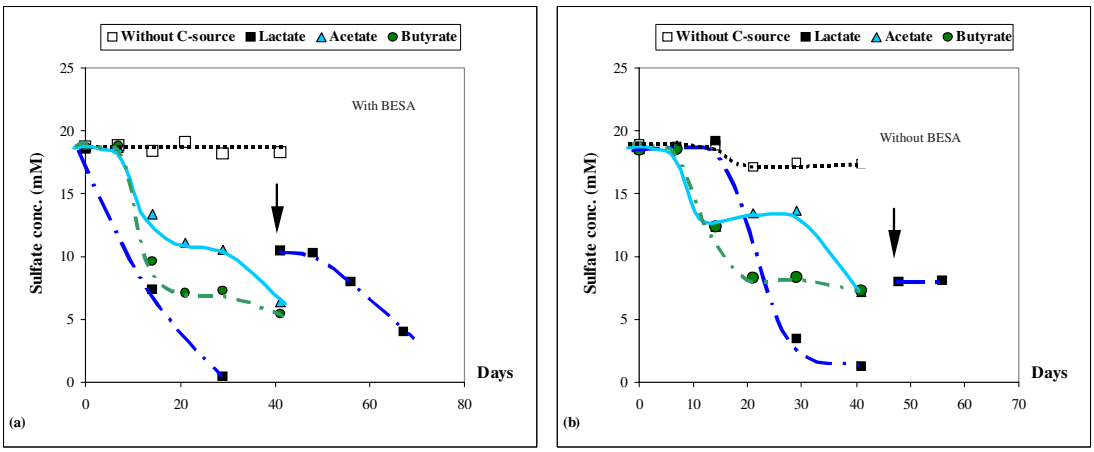


Figure 4.1.10 Comparison of sulfate reduction in enrichment cultures from soil below leaking sewers from Mannheim, with different carbon sources at 37 °C (a) in the presence and (b) absence of BESA. *Arrow* shows the time when the C-source in the test unit was replenished.

4.1.3 Pure culture studies of Desulfovibrio desulfuricans

A pure culture of *Desulfovibrio desulfuricans* was cultured in DSM media Nr. 63 at 15 and 37 °C (Fig. 4.1.11).

Incubation at 15 °C: The sulfate reduction started its log phase after 5 days. The initial sulfate concentration (17 mM) was degraded to finally 5.8 mM (67 % reduction) at day 9 and then growth continued in the stationary phase. The SRR was 51.9 µM/hr or 5 mg/L·hr, respectively. The cell density increased from 2.2 x 10^6 cells/mL at the beginning to 3.2 x 10^8 cells/mL at day 9. Lactate decreased from 30.1 mM to 6.2 mM and acetate accumulated.

Incubation at 37 °C: The growth of bacteria began without a lag phase (only a few hours or a day). The log phase lasted from day 0 to day 5. The SRR was 251.9 µM/hr or 24.2 mg/L·hr during day 0 to day 2 and 68.5 % of the supplied sulfate were reduced to sulfide. The cell numbers in these tests increased from 7.4 x 10^6 cells/mL to 2.8 x 10^8 cells/mL.

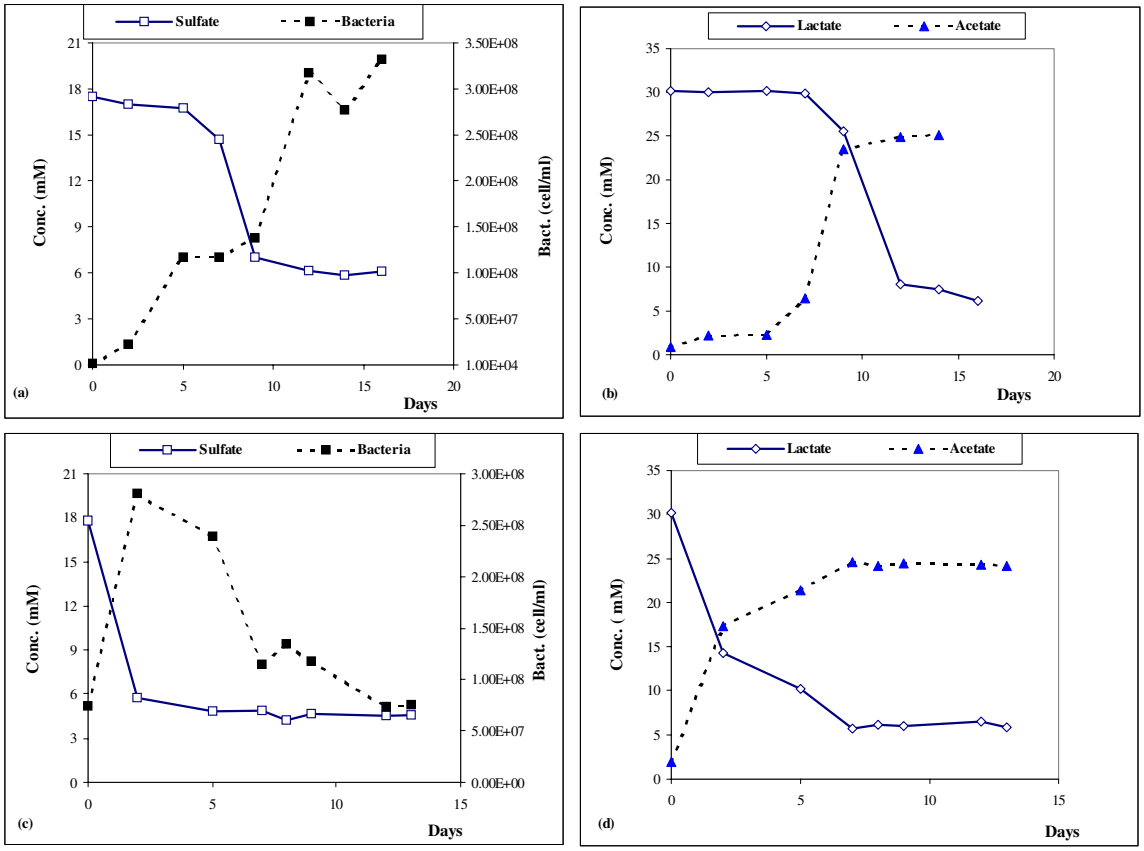


Figure 4.1.11 Change of concentrations of sulfate, lactate and acetate, and cell numbers of *D. desulfuricans* during incubation in DSM medium Nr. 63 at 15 (a - b) and 37 ^{o}C (c - d).

4.1.4 Growth of sulfate reducing bacteria under stress conditions: Molybdate as a stress factor or inhibitor

To investigate the effect of molybdate, a specific inhibitor of sulfate reducers, as a stress factor for sulfate reducing bacteria, experiments with pure cultures and with enrichment cultures from different contaminated soil sources, that were taken below leaking sewers, were conducted. A summary of the test assays is given in Table 4.1.2. The experiments were undertaken in cooperation with the group of Geochemistry (Stüben/Berner/Stögbauer), who investigated the influence of molybdate on sulfur isotope discrimination during sulfate reduction by sulfate reducers in the soil samples.

Table 4.1.2 Overview over experiments of Part 4.1.4

Test	Strain /source of bacteria	Temperature (°C)	Media	SO_4^{2-} : Lactate	Molybdate conc.
1	*D. desulfuricans*	37	DSM Nr. 63	10 : 20	0 - 5 mM
2	*D. desulfuricans*	37	DSM Nr. 63	10 : 20	0 - 0.75 mM
3	*D. desulfuricans*	15 and 37	DSM Nr. 63	10 : 20	0.1 mM
4	Soil from Rastatt	37	Freshwater II	10 : 30	0.1 mM

Test 1-2: ***Inhibiting concentrations of molybdate applied during growth and sulfate reduction of Desulfovibrio desulfuricans:***

To find out the concentrations of molybdate, that acted as stress factors on growth of *D. desulfuricans* the inhibition of growth of *Desulfovibrio desulfuricans* in pure culture at 37 °C by addition of 0 - 5 mM molybdate was investigated.

A concentration of ≥ 1 mM molybdate caused an effective inhibition of bacterial growth, if compared to non-inhibited control samples. Most of the sulfate (94 % reduction) and lactate (98 % reduction) was utilized by the sulfate reducers in the absence of molybdate. In the cultures that were inhibited with molybdate, lactate and sulfate were not completely utilized and only low concentrations of acetate as an end product accumulated. Statistic analysis revealed no effect of different inhibitor concentrations (analysis of variance). Samples with and without Mo had a significant difference of variance. The optical density as a parameter of biomass concentration, measured at λ_{578} nm with a spectrophotometer, increased from 0.237 initially to 0.563 in samples that were not inhibited by molybdate. In the samples that contained molybdate there were no or only little increase of growth.

The percentage of sulfate reduction in cultures with molybdate concentrations of 0, 1, 2, 3, 4 and 5 mM, was 93.7, 14.5, 15.6, 14.5, 14.8 and 14.2 %, respectively. The sulfate reduction started slowly in all assays during day 1 to day 4, but then was completely suppressed in the samples that contained molybdate. No sulfate reduction activity could be observed at molybdate concentrations of > 1 mM, which also completely inhibited bacterial growth. The inhibiting effect was fully manifested after 4 days of incubation. This might be due to a restricted diffusion of the molybdate into the cells. However, since the SRB in samples without molybdate also started with log-phase growth only on day 2 to day 8, this might simply be due to the varying lag-phases of the different cultures.

In further experiments the effect of molybdate at lower concentrations than 1 mM on growth of sulfate reducers was tested. The purpose of these experiments was to test out those molybdate concentrations that would not lead to a complete inhibition of growth. Analysis of sulfate reduction during non-inhibited and partially inhibited growth of sulfate reducers by molybdate would give information about the fractionation of different sulfur isotopes (analysis of sulfur isotope fractionation by IPG, Institute for Petrography and Geology, University of Karlsruhe). For this purpose growth tests were carried out with molybdate concentrations of 0, 0.1, 0.2, 0.3, 0.5, 0.6 and 0.75 mM. It turned out that a molybdate concentration of ≥ 0.2 mM had a significant inhibiting effect on growth of sulfate reducers. No statistic differences at the concentration of Mo ≥ 0.2 mM on bacterial growth were observed. In the assay with 0.1 mM molybdate sulfate reduction was slightly inhibited, reaching 66.7 % of sulfate that was reduced to sulfide after the same incubation time at which in the non-inhibited assays (controls) 88.4 % sulfate had been reduced.

The percentages of sulfate reduction in samples that contained increasing molybdate concentrations from 0.2 - 0.75 mM was 14.8, 13.6, 16.8, 18.9 and 15.0 %, respectively, representing total inhibition. In the assays without and with 0.1 mM of molybdate, 88.4 % and 66.7 % of sulfate were degraded during day 2 to day 8 of incubation (Fig. 4.1.12). The presence of ≥ 0.2 mM molybdate in culture assays showed a slight decrease of sulfate only during day 1 to day 4 and no further sulfate reduction lateron (Fig. 4.1.12 and 4.1.13). The cultures were completely inhibited by molybdate. The OD_{578} increased significantly from 0.231 to 0.486 in samples without molybdate and from 0.252 to 0.465 in samples containing 0.1 mM molybdate. The final OD values were high compared to the assays that contained ≥ 0.2 mM of molybdate. The presence of 0.1 mM of molybdate was suitable to study the effect of molybdate on growth and SRR of pure cultures and enrichment cultures from natural sources, such as sewage-contaminated soil below leaking sewers.

In the assays that contained molybdate, the orange coloration of Mo and sulfate was seen. This could be clearly observed and quantified even with the eyes. The colour did not influence the OD measurement at λ_{578} nm. Samples had an odour of hydrogen sulfide and showed black precipitates of iron sulfide.

A concentration of 0.1 mM molybdate is the recommended concentration for further studies of the effect of molybdate as a stress substrate for SRB and the effect of stress conditions on sulfur isotope discrimination.

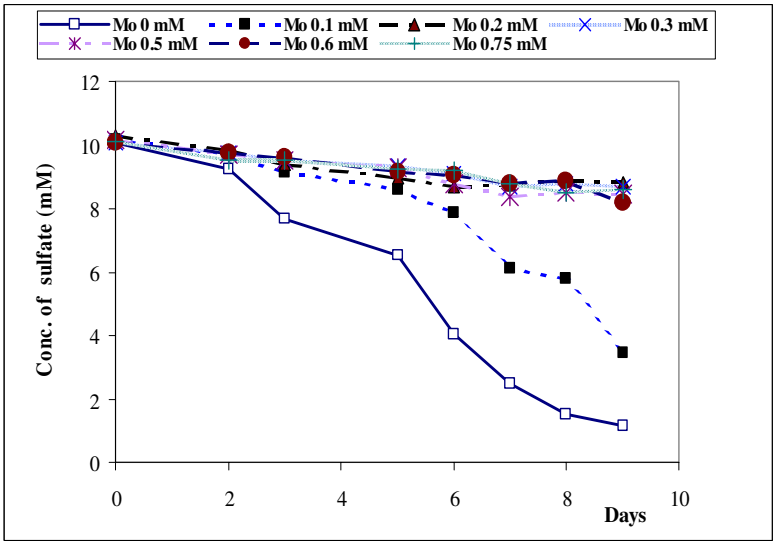


Figure 4.1.12 Comparison of sulfate reduction by *D. desulfuricans* at 37 °C in the presence and absence of molybdate (0 - 0.75 mM).

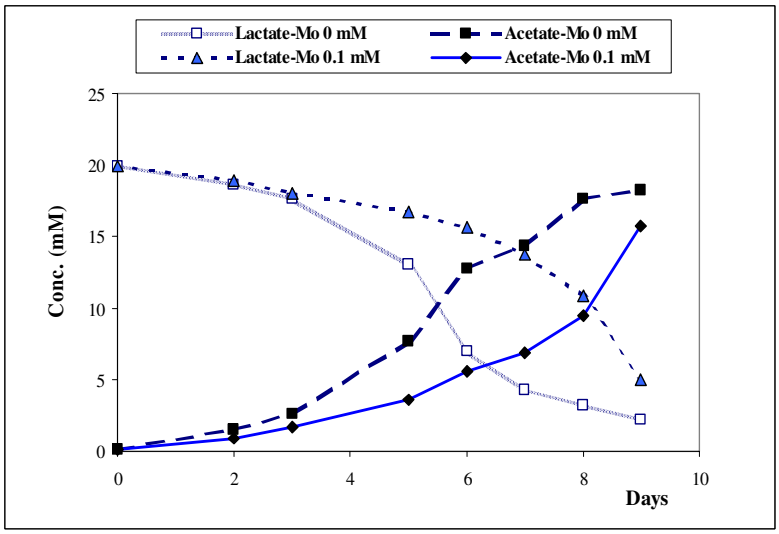


Figure 4.3.13 Lactate degradation and acetate production by *D. desulfuricans* in samples with and without molybdate.

Test 3: ***Effect of molybdate and the temperature as stress factors for growth of D. desulfuricans***

The pure culture studies revealed that a dosage of 0.1 mM molybdate slightly decreased growth of SRB. In this test the temperature as another stress factor was superimposed. The purpose of these experiments was to determine the effect of molybdate on sulfur isotope fractionation and growth of *D. desulfuricans* at two temperatures. The bacteria were cultured at 15 and 37 ^{o}C with and without 0.1 mM of molybdate.

Sulfate reduction rates of *D. desulfuricans* at 15 ^{o}C in the absence or presence of molybdate were investigated. The addition of 0.1 mM molybdate slightly inhibited growth of the SRB when compared with non-inhibited controls (Fig. 4.1.14). The lag phase of growth of bacteria was prolonged to a few days, followed by log phase growth until about day 8 (hour 200, with molybdate). In assays without molybdate the log-phase lasted from day 2 to day 7 (hour 180). After that the stationary phase was reached. The orange coloration of interaction between sulfate and molybdate could clearly be seen in the samples treated with molybdate as well as recognized by the smell of hydrogen sulfide.

The SRR with and without molybdate was 10.5 µM/hr (1.0 mg/L·hr) and 10.8 µM/hr (1.04 mg/L·hr), respectively. Approximately 9.5 and 14.5 % of the sulfate in the medium were reduced in MoO_4^{2-} -treated and untreated samples, respectively. The lag phase lasted a few days and then the log phase started until day 8 or 9 (Fig. 4.1.14). The population density of SRB could be correlated to the sulfate reduction rates (Fig. 4.1.14). The numbers of bacteria in the non-inhibited cultures increased from 3.9 x 10^6 to 1.25 x 10^8 cell/mL (day 9). Growth of the cultures treated with MoO_4^{2-} proceeded from an initial population density of 3.1 x 10^6 to finally 0.91 x 10^8 cell/mL (day 8). Molybdate, a specific inhibitor for SRB could explain the slight decline of bacterial biomass and lower SRR. The effect of molybdate on SRR or bacterial cell growth at 15 ^{o}C was, however, not so clearly visible as expected from previous experiments at 37 ^{o}C. This might be due to the temperature optimum being in the range of 37 ^{o}C. Lactate decreased from an initial concentration of 21.5 mM to 16.9 mM on day 8 and from 21.7 mM to 18.2 mM on day 9 in assays without and with molybdate, respectively. Vice versa, acetate accumulated from 0.8 mM to 3.4 mM and

from 0.5 mM to 4.0 mM in assays with and without molybdate, respectively (Fig. 4.1.14).

The stoichiometry of sulfate and lactate utilization by *D. desulfuricans* for the formation of acetate and hydrogen sulfide was (22, 23):

$$1.3\ SO_4^{2-} + 4.6\ \text{lactate} \rightarrow 3.5\ CH_3COOH + \textit{1.3}\ H_2S\ (\text{no Mo}) \quad (22)$$

$$0.7\ SO_4^{2-} + 3.5\ \text{lactate} \rightarrow 2.6\ CH_3COOH + \textit{0.7}\ H_2S\ (\text{Mo}) \quad (23)$$

The SRR of bacteria at 37 ^{o}C with and without molybdate was investigated (Fig. 4.1.15). The presence of 0.1 mM molybdate was slightly decreasing the SRR of the bacteria compared to the non-inhibited control cultures (absence molybdate). After approximately one-day lag phase, logarithmic growth of bacteria began and lasted to day 7 in the non-inhibited cultures. Logarithmic growth of the molybdate-inhibited cultures occurred between day 2 and day 5. The SRR with and without molybdate were 69.1 µM/hr or 6.6 mg/L^{-}hr and 88.9 µM/hr or 8.5 mg/L^{-}hr, respectively. Approximate 80 % of the sulfate in the medium was reduced in both cultures. No significant difference was seen in the extent, however the velocity of sulfate reduction was reduced by molybdate. The population density in the cultures without molybdate increased from 5.4 x 10^6 to 2.6 x 10^8 cell/mL (day 5). The added molybdate in parallel cultures reduced the growth rates only slightly. The population increased from 2.3 x 10^6 to 1.2 x 10^8 cell/mL in 5 days and to 2.3 x 10^8 cell/mL in 7 days. The concentrations of lactate and acetate correlated with each other. The initial lactate concentration (20.1 mM) was used up completely to zero in 5 days in the culture without molybdate and to 0.52 in 6 days in the culture with 0.1 mM molybdate. The consumption of lactate by the bacteria proceeded and vice versa the acetate concentration increased. The stoichiometry of the sulfate reducers in the experiments with and without molybdate is presented in equation 24 and 25.

$$8.2\ SO_4^{2-} + 20.1\ \text{lactate} \rightarrow 20.1\ CH_3COOH + \textit{8.2}\ \textit{H}_2\textit{S}\ (\text{no } MoO_4^{2-}) \quad (24)$$

$$7.0\ SO_4^{2-} + 19.9\ \text{lactate} \rightarrow 19.1\ CH_3COOH + \textit{7.0}\ \textit{H}_2\textit{S}\ (\text{with Mo } O_4^{2-}) \quad (25)$$

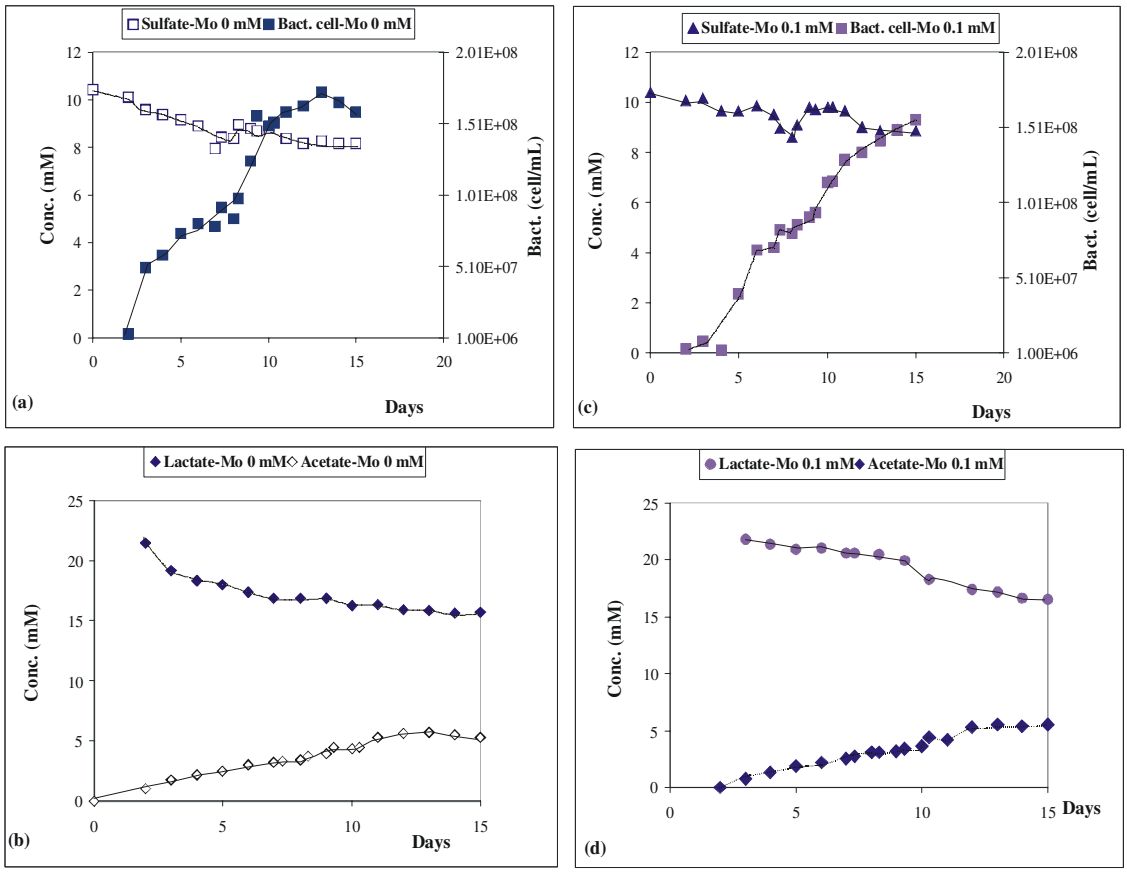


Figure 4.1.14 Change of sulfate, lactate and acetate concentration during growth of *D. desulfuricans* at 15 °C in the absence of molybdate (a, b) and in presence of 0.1 mM molybdate (c, d)

Molybdate reduced the SRR at both temperatures when compared to non-inhibited controls. In addition, the temperature itself influences growth and the SRR of *D. desulfuricans*. The higher temperature (37 °C versus 15 °C) resulted in higher degradation rates at both conditions, with or without molybdate. The Q_{10} was around 2. *D. desulfuricans* is a mesophilic bacterium with a temperature optimum between 25 - 40 °C. At 15 °C fermentation temperature, cultures were shocked, and grew slower than at 37 °C. The stoichiometry of sulfate reduction was, however close to theoretical expectations at both growth temperatures.

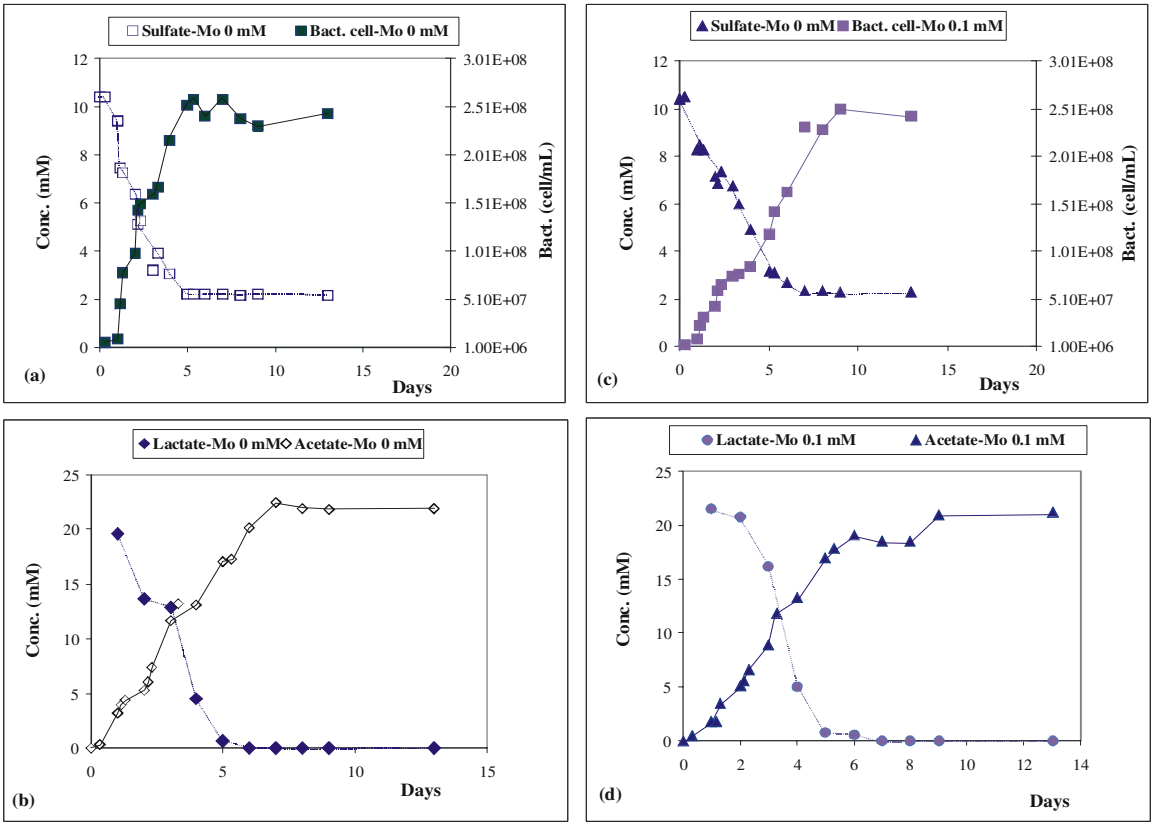


Figure 4.1.15 Change of sulfate, lactate and acetate concentrations during growth of *D. desulfuricans* at 37 °C in the absence of molybdate (a, b) and in presence of 0.1 mM molybdate (c, d)

Test 4: Influence of molybdate on the sulfate reduction rate of enrichment cultures from soil, taken below leaking sewers in Rastatt

The sulfate reduction rate of enrichment cultures from soil below leaking sewers in Rastatt was investigated in the absence or presence of 0.1 mM molybdate and in the presence of 0, 5, 10 and 20 mM sulfate at 37 °C (Table 4.1.2).

If no addition of sulfate in the assays but only lactate was added to the soil, either in the absence or presence of molybdate no sulfate reduction occurred, indicating that there was no sulfate available. Sulfate analyses revealed only 0.07 to 0.10 mM sulfate (Fig. 4.1.16a) and no smell of H_2S could be recognized. The lactate was utilized and converted to fatty acids such as acetate and propionate, presumably by other bacteria (Fig. 4.1.16b). The number of total bacteria increased with time (Fig. 4.1.16a). It was concluded that low concentrations of sulfate acted as the growth-

limiting factors of SRB, leaving the carbon source for other groups of bacteria, as indicated by H_2, CO_2 and methane production (Fig. 4.1.16c).

When 5 mM sulfate was supplied for growth of SRB sulfate reduction occurred until < 1 mM were left in the assay without molybdate and 2.8 mM were left in the assay with molybdate (Fig. 4.1.17). Thus, 68 % of the sulfate was reduced in the absence and only 43.4 % in the presence of molybdate. The SRB in the enrichment cultures belonged to the incompletely oxidizing group. They could use a variety of carbon sources as electron donors. As a result, they were divided in two incompletely oxidizing subgroups according to the spectrum of possible carbon sources.

The SRB could degrade lactate to acetate and also propionate to acetate. The sulfate reduction rates for lactate as a carbon source in samples with and without molybdate were 11.4 µM/hr (1.1 mg/L·hr) and 12.1 µM/hr (1.3 mg/L·hr), respectively. After two days no lactate could be found in both samples. The sulfate reduction rate with propionate as a carbon source was 16.5 µM/hr or 1.6 mg/L·hr and 31.0 µM/hr or 3.0 mg/L·hr in the samples with and without molybdate, respectively. The total number of bacteria increased from 2.0 x 10^5 to 1.7 x 10^8 cell/mL and from 1.9 x 10^5 to 1.7 x 10^8 cell/mL in sample without and with molybdate (Fig. 4.1.17a, d).

If 10 mM sulfate were supplied for growth of SRB without molybdate 1.5 mM and with molybdate 7.3 mM were left after incubation. Thus, 90 % of the sulfate was reduced in the absence and only 29 % in the presence of molybdate. The lactate conversion rates in samples with and without Mo were 13.9 µM/hr or 1.3 mg/L·hr and 16.2 µM/hr or 1.6 mg/L·hr, respectively. No lactate was left after the 2nd day. The conversion rate of propionate as a carbon source was 33.6 µM/hr or 3.2 mg/L·hr and 42.3 µM/hr or 4.1 mg/L·hr in samples treated with (day 2 - 5) and without Mo (day 7 - 10), respectively. The population density increased from 1.8 x 10^5 to 1.9 x 10^8 cell/mL and 1.9 x 10^5 to 1.5 x 10^8 cell/mL in sample without and with Mo (Fig. 4.1.18).

If 20 mM sulfate were supplied for growth of SRB, in the absence of molybdate 1.9 mM and in the presence of molybdate 14.3 mM were left after incubation for two days. Sulfate reduction was 90 and 27 %, respectively. During sulfate reduction in the absence or presence of molybdate, 40.1 µM/hr or 3.9 mg/L·hr and 24.0 µM/hr or 2.3 mg/L·hr lactate were degraded. After the 2nd day no lactate was

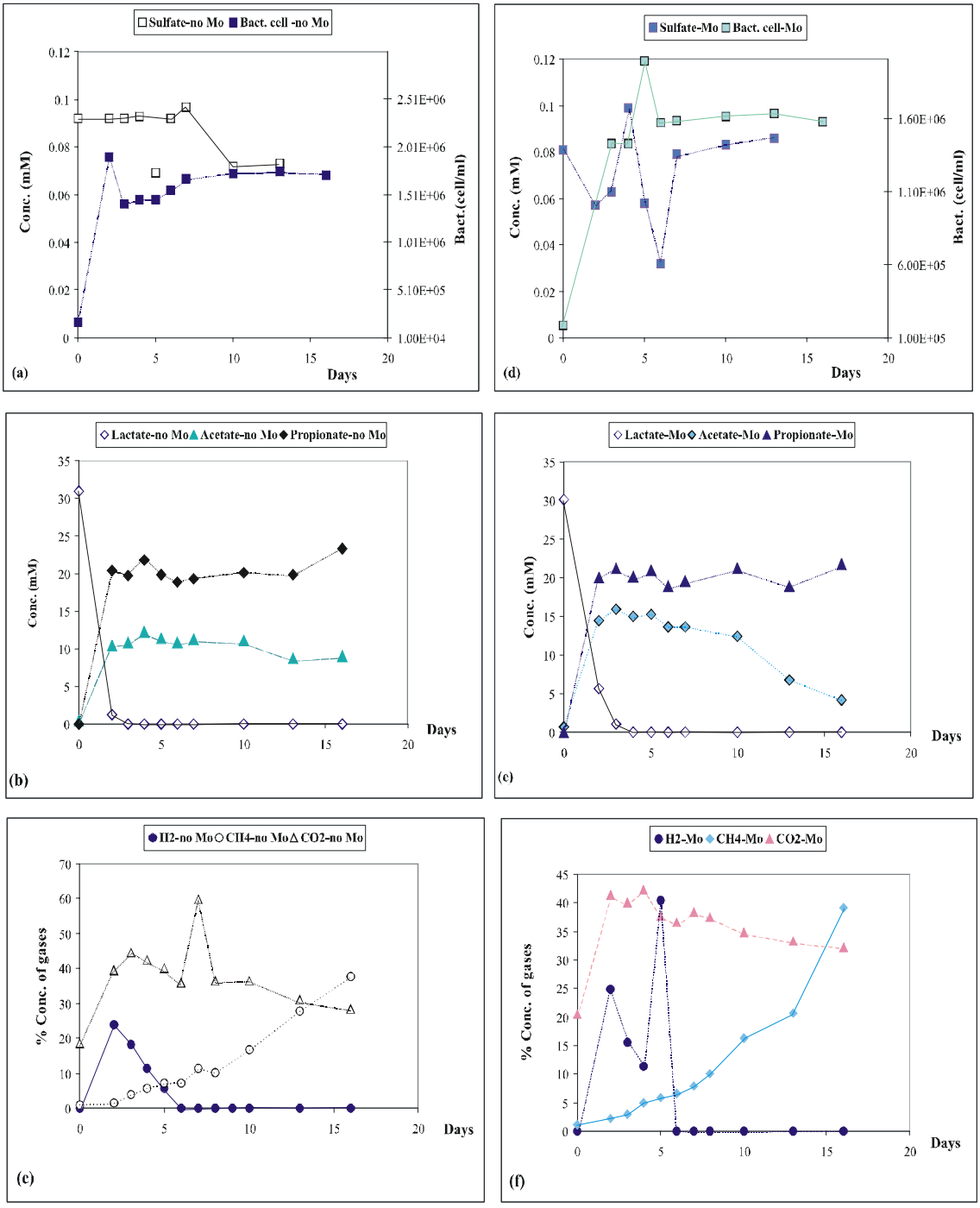


Figure 4.1.16 Degradation of sulfate and lactate by SRB in contaminated soil of Rastatt. Soil was incubated in Freshwater II medium (no sulfate added) with and without molybdate: a - c, no molybdate; d - f, 0.1 mM molybdate added.

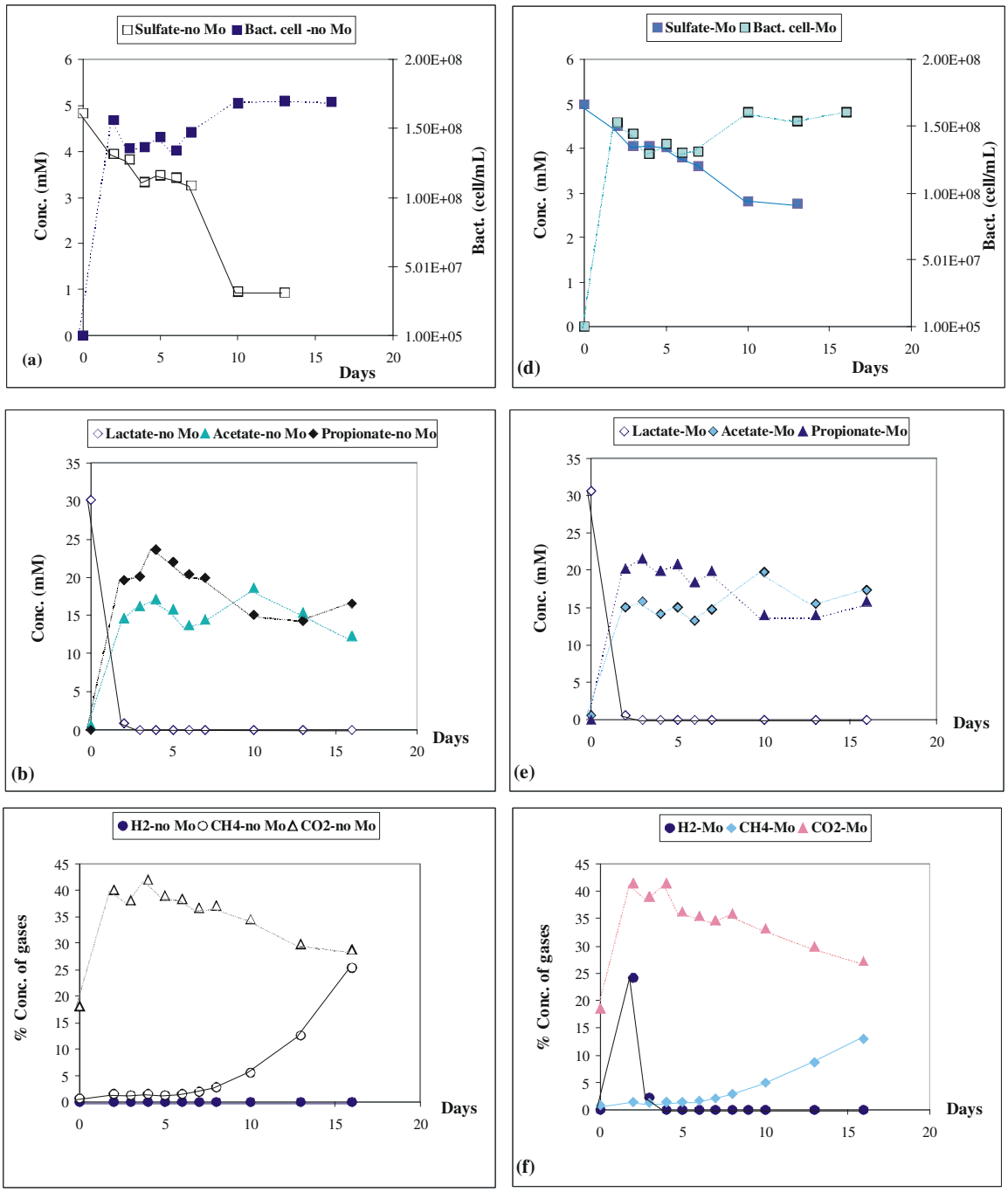


Figure 4.1.17 Degradation of sulfate and lactate by SRB in contaminated soil of Rastatt. Soil was incubated in Freshwater II medium (5mM sulfate) with and without molybdate: a - c, no molybdate; d - f, 0.1 mM molybdate added.

left in both samples. With propionate as a carbon source removal rates were 47.9 µM/hr or 4.6 mg/L·hr and 94.4 µM/hr or 9.1 mg/L·hr in the presence (day 7 - 10) and in the absence of molybdate, respectively. In both assays until day 8 of incubation some methane was generated (1 - 3 % in the gas phase) (Fig. 4.1.19).

The V_{max} and μ_{max} of SRB in the soil of Rastatt were calculated by the plot method of Hanes Woolf (Table 4.1.3)

Table 4.1.3 V_{max} and μ_{max} of SRB in enrichment cultures of soil from Rastatt

Test	V_{max} (µM/hr)	μ_{max} (cell/mL)	Temperature
Rastatt (no Mo)	400.0	2.7×10^8	37 °C
Rastatt (add Mo)	120.5	2×10^8	37 °C

Molybdate was an effective inhibitor of SRB from natural habitats. The enrichment started growth with a lag phase in the first day. The log phase could be divided into two periods, during which lactate and then propionate were used as carbon sources by the SRB. Lactate and propionate utilization was related to an increase of the population density. The light orange coloration of interaction between sulfate and molybdate could be clearly observed in samples containing molybdate. The number of total bacteria increased while the sulfate reduction proceeded. The inhibition of sulfate reducers by molybdate started after one day of incubation, when enough molybdate was taken up by the bacteria.

Microorganisms with the capability to degrade the supplied carbon compounds are found within four groups, the SRB, methane bacteria, propionate producing bacteria (PPB) and propionate degradation bacteria (PDB). The lactate in both samples was quickly utilized within two days. A comparison of the lactate utilization curve and the sulfate reduction curve revealed that sulfate reducing and propionate producing bacteria competed for lactate (e.g. Figure 4.1.16 and 4.1.17). The results showed that the propionate producing bacteria almost completely out-competed SRB in the utilization lactate if no or only little sulfate was present. Vice versa, the SRB out-competed the propionate degrading bacteria (e.g. Figure 4.1.18, 4.1.19) if sufficient sulfate was present. Acetate from propionate degradation could serve as a carbon source for MB.

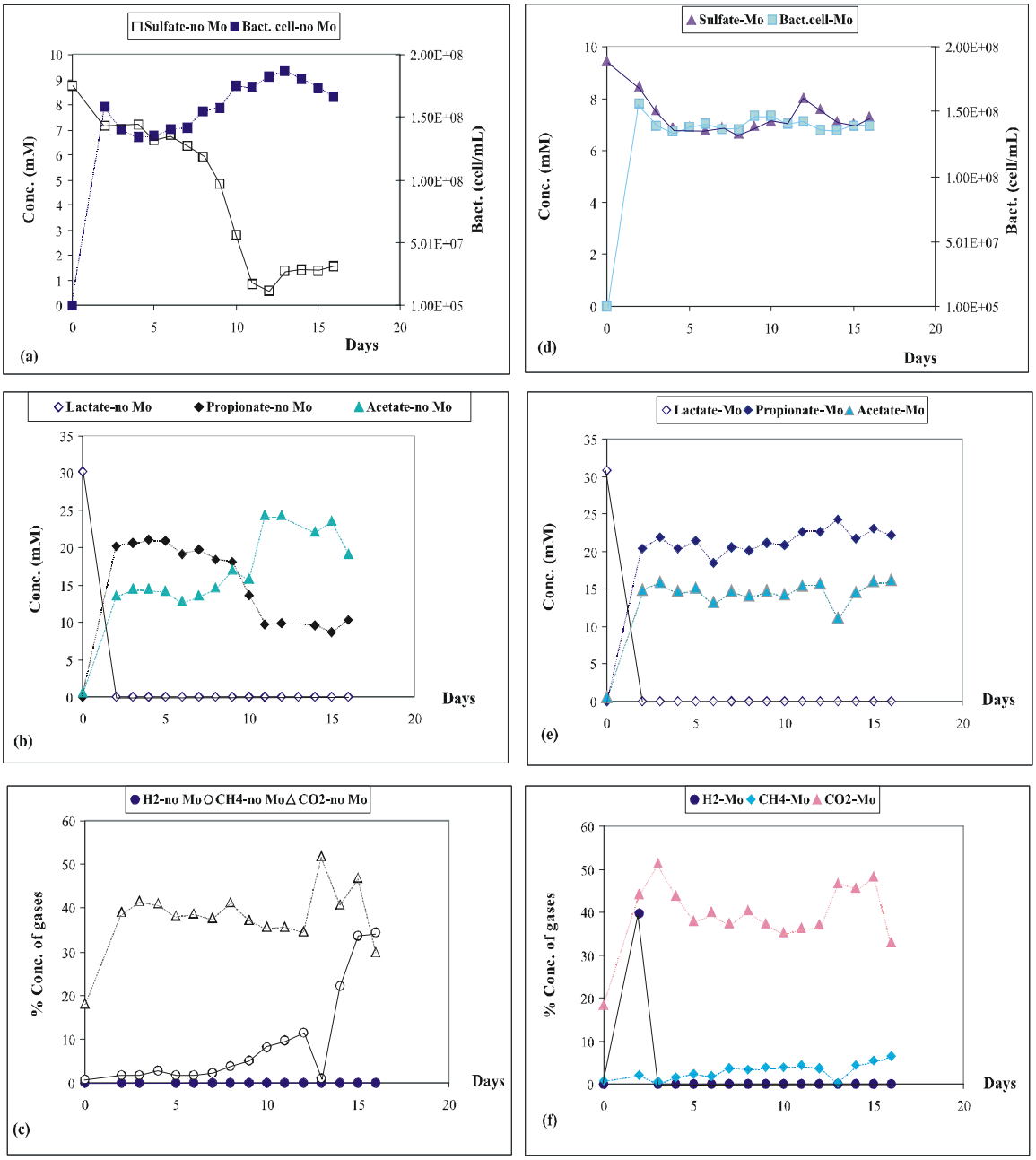


Figure 4.1.18 Degradation of sulfate and lactate by SRB in contaminated soil of Rastatt. Soil was incubated in Freshwater II medium (10 mM sulfate) with and without molybdate: a - c, no molybdate; d - f, 0.1 mM molybdate added.

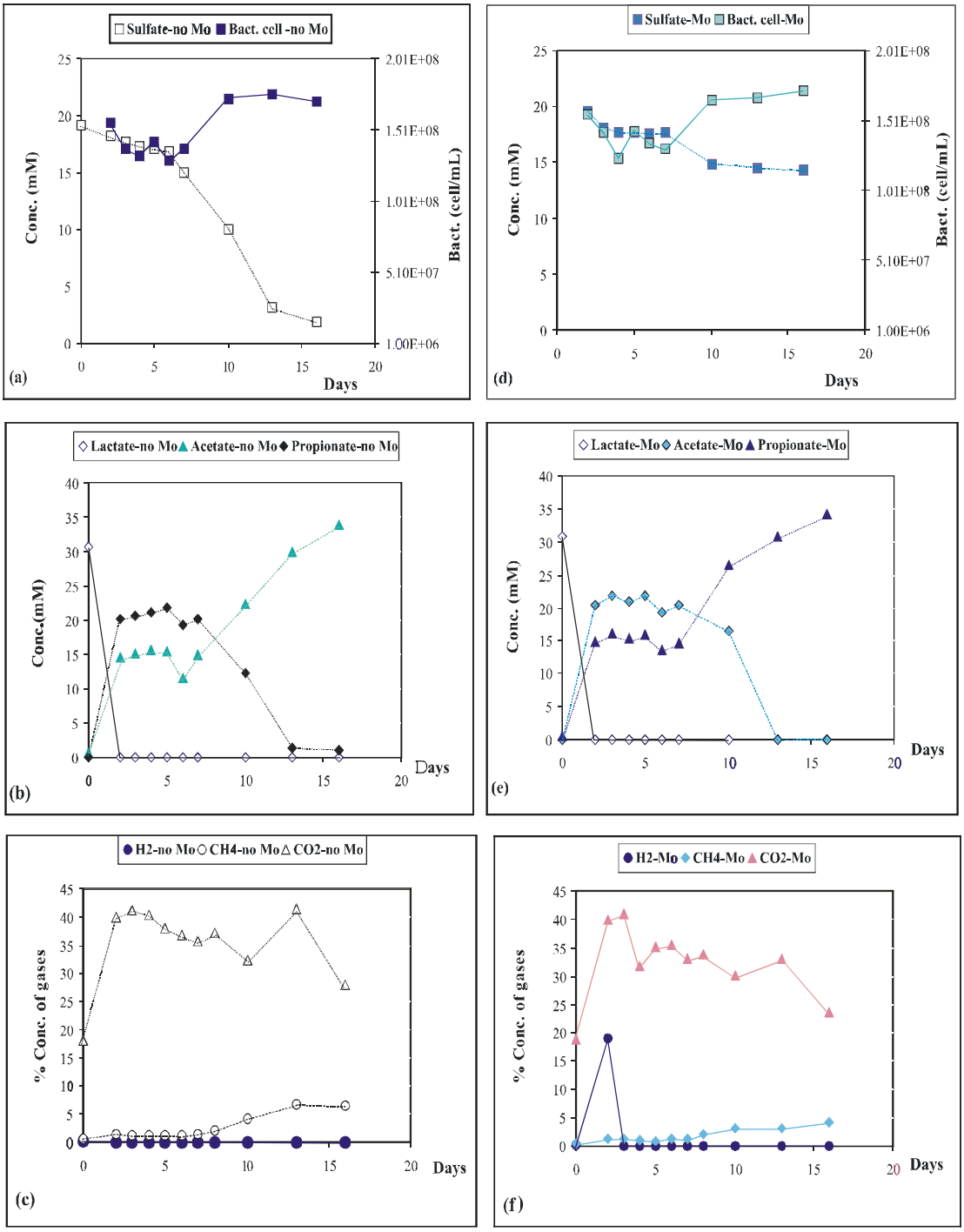


Figure 4.1.19 Degradation of sulfate and lactate by SRB in contaminated soil of Rastatt. Soil was incubated in Freshwater II medium (20 mM sulfate) with and without molybdate: a - c, no molybdate; d - f, 0.1 mM molybdate added.

4.1.5 Comparison of the capacity of organic matter in sewer wastewater to serve as a carbon source for growth of SRB from contaminated soil (column IBA 2).

Filtrated wastewater (0.2 μm filter pore size) was prepared as an initial solution. Some carbon or sulfate was added to find out the carbon sources of wastewater as nutrients for SRB. The inocula were sampled from soil at 10 cm depth of column IBA2 and cultured under anaerobic batch conditions for 2 months before use. Four different experiments were prepared: Filtrated wastewater (T1), filtrated wastewater + sulfate (5mM, T2), filtrated wastewater + sulfate (15 mM, T3) and filtrated wastewater + sulfate (15 mM) + propionate (T4).

The removal of sulfate: In the filtrated wastewater (assay T1), under strictly anaerobic conditions, 92 % of initial sulfate concentration in sewage was reduced and bacteria grew with organic matter from the wastewater. The gas phase contained 2.9 % of methane gas in the biogas, indicating some methanogenic activity.

The addition of different sulfate concentrations (assays T2 and T3) to induce activity of sulfate reducing bacteria was effective. More sulfate was reduced in assay T3 (3.8 mM) compared to T1 (1.3 mM) or T2 (1.0 mM) (Fig. 4.1.20, 4.1.21a). The SRB utilized 3.8 mM of sulfate by using organic substrate from wastewater as electron donors under strictly anaerobic conditions. Concerning the percentage of sulfate reduction in assay T1 a higher percentage was observed than in assay T2 and T3 due to lower sulfate concentrations (Fig. 4.1.21b). Sulfate reduction in the assay T4 with addition of propionate as an extra carbon source proceeded almost to completion (99 %). Methane appeared in each assays (Fig. 4.1.22).

Fatty acids: In all assays, an increasing acetate concentration was clearly observed, indicating the degradation of organic components in wastewater under anaerobic conditions. Methane bacteria used the acetate for methane production. In the assay T4 acetate accumulated due to propionate degradation by incompletely oxidizing SRB (Fig. 4.1.23).

Biomass: The biomass of bacteria increased in response to the decreasing of sulfate (Figure 4.1.20). Growths of bacteria in all assays began after 4 days lag phase except for assay T1 (2 days, Fig. 4.1.20).

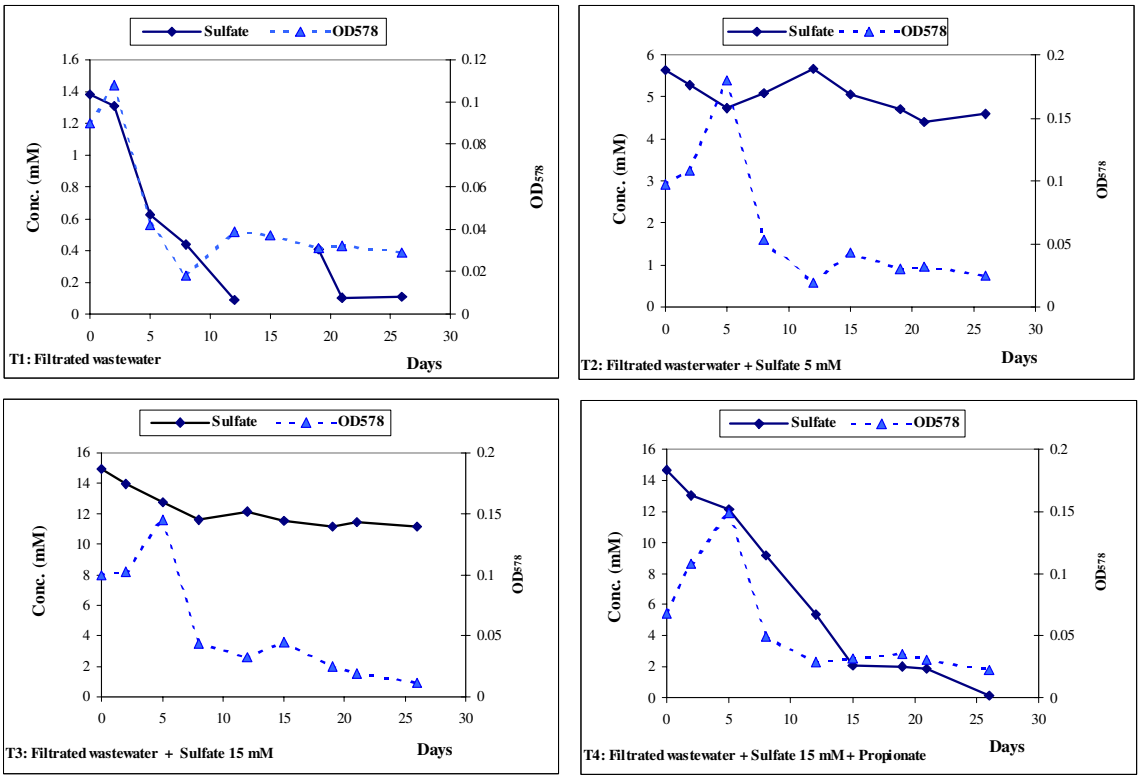


Figure 4.1.20 Change of sulfate concentration as related to biomass of assays T1-T4.

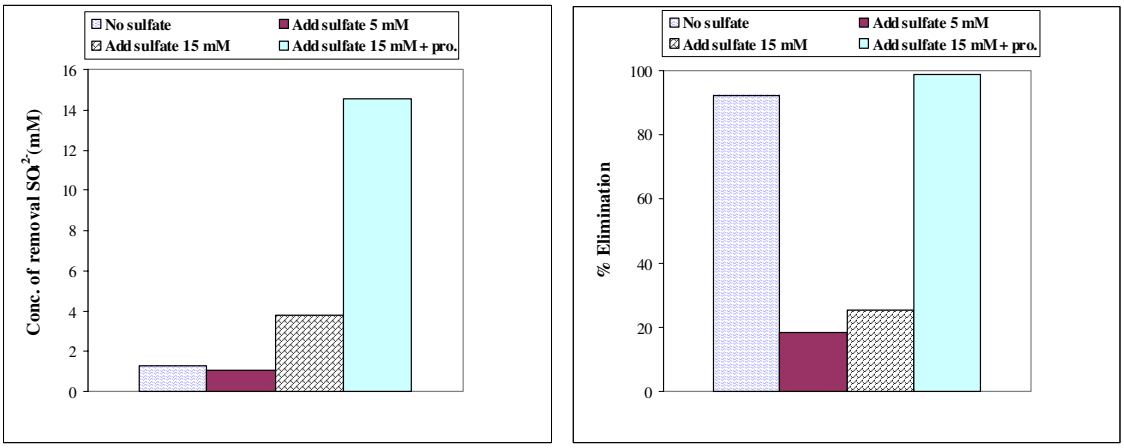


Figure 4.1.21 A comparison of sulfate removal (a) and % elimination of assays T1-T4 (b)

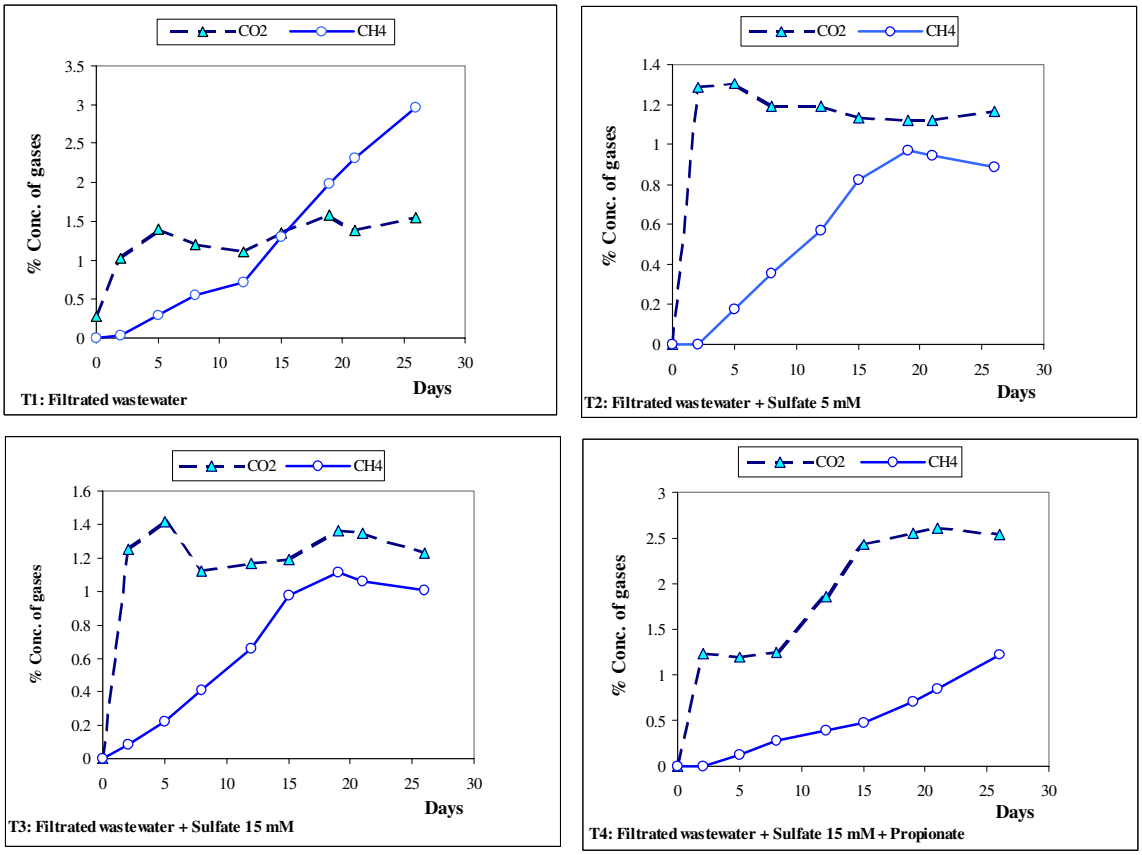


Figure 4.1.22 Concentrations of carbon dioxide and methane gas in assays of T1-T4

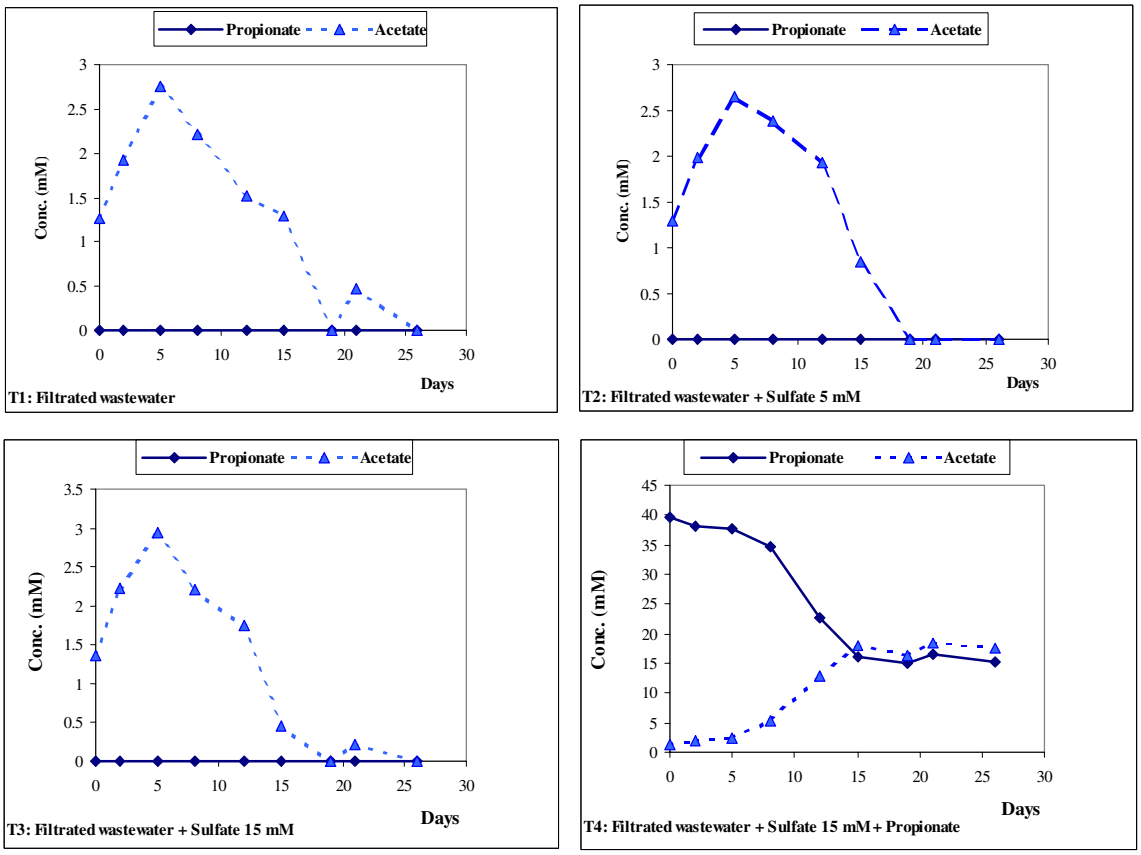


Figure 4.1.23 The concentration of fatty acids in assays of T1–T4

4.1.6 Comparison of the sulfate reduction rates (SRR) with lactate and with sewage components by enrichment cultures (batch culture) from contaminated soil of Rastatt.

Soil under leaking sewers in Rastatt was sampled and incubated in Freshwater medium II, which contained lactate as carbon source, at 37 °C. After 5 days of incubation the sample was withdrawn and used as initial inoculums for the experiment. To determine degradation rates 80 ml of sewage with supplementation of different carbon sources was inoculated (10 %) and incubated at 37 °C for 10 days. The following assays were run:

- Freshwater II (20 mM lactate and 10 mM sulfate, A1)
- Filtrated sewage through 0.2 µm filters (20 mM lactate and 9 mM sulfate, A2)
- Filtrated sewage through 0.2 µm filters (A3)

Sulfate reduction was found from the 2nd to the 10th day of incubation. The result showed that the 80 % of initial sulfate in the samples A1 and A2 were degraded at about the same velocity (Fig. 4.1.24). Only 57.6 % of the sulfate was degraded in

sample A3. The enrichment contained incompletely oxidizing sulfate reducing bacteria due to accumulation of acetate (Fig. 4.1.25). The bacteria could oxidize some carbon sources in sewage for growth but not all. No methane gas was found. The SRR was 82.4 µM/hr in A1, 79.6 µM/hr in A2 and 5.2 µM/hr in A3.

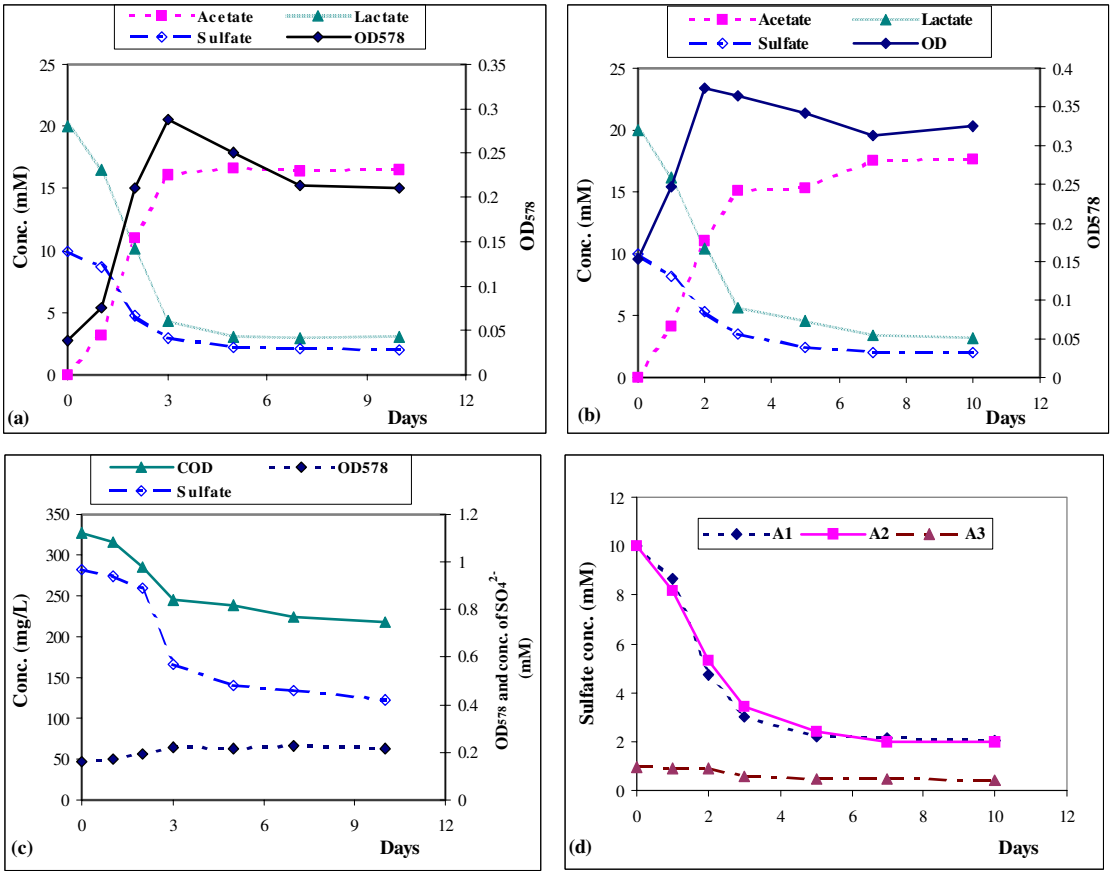


Figure 4.1.24 Sulfate reducing enrichment cultures in soil below leaking sewers were incubated after addition of lactate. Sulfate reduction was compared with sewage as a carbon source at 37 °C. (a) Freshwater II media (20 mM lactate and 10 mM sulfate, A1), (b) Filtrated sewage (0.2 µm filters, 20 mM lactate and 9 mM sulfate), (c) Filtrated sewage (0.2 µm filters, A3) and (d) comparison of the decreasing of sulfate in samples.

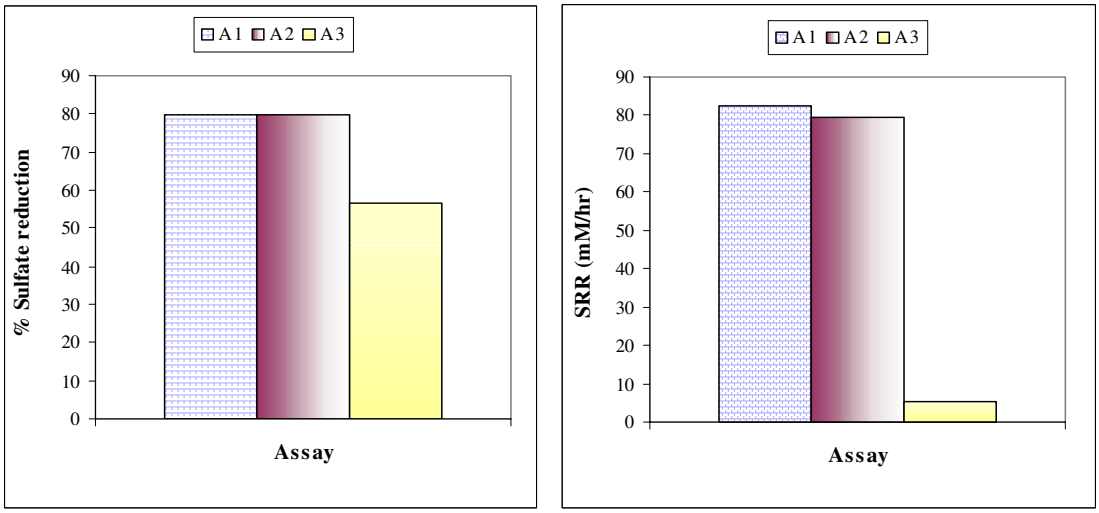


Figure 4.1.25 A comparison of percent of sulfate reduction and SRR by sulfate-reducing enrichment culture from contaminated soil of Rastatt.

4.2 Fed-batch and continuous cultures of sulfate reducers enriched from soil

4.2.1 Fed-batch sulfate reducing enrichment cultures from soil that was taken below leaking sewers in Rastatt

Soil samples under leaking sewers from Rastatt were collected and incubated at 37 ^{o}C in Freshwater medium II, that contained lactate as a carbon source. After 5 days of incubation samples were withdrawn and used as initial inocula for the experiment. To find out the sulfate reducing potential of bacteria in the soil, 10 % (v/v) of inoculum was transferred into a series of 500 ml-Schott flasks, containing 3 different media. The suspensions were incubated at 37 ^{o}C. Media used in this experiment were:

- **A1**: Freshwater medium II (20 mM lactate and 10 mM sulfate)
- **A2**: Filtrated sewage through 0.2 µm filter pore size (20 mM lactate and 9 mM sulfate)
- **A3**: Filtrated sewage through 0.2 µm filter pore size, no additions.

After 3 days of incubation the samples were fed 40 ml fresh medium per day. The cultures were flushed weekly with N_2 gas under atmospheric pressure to strip out H_2S. The assay A1 began to reduce sulfate after 4 days of incubation. Maximally 43 - 50 % of the available sulfate was reduced on day 22. After that no sulfate was reduced (Fig. 4.2.1). An accumulation of acetate was found (data not showed) and the gas

contained 7 - 19 % CO_2. Only in the first week, the gas phase contained little methane (0.5 - 1 %). The average sulfate reduction rate was 6.1 µM/hr. The assay A2 began to reduce sulfate after the 4th day and reached the maximum elimination on the 18th day (Fig. 4.2.1) with a total elimination of 45 – 55 %. Thereafter the sulfate concentration was more or less constant. Acetate accumulated in the medium due to the enrichment of incompletely oxidizing SRB. Little methane gas was found only during the first 14 days in the order of 0.3 - 1.2 %. The gas contained 15 - 20 % of CO_2. The sulfate reduction rate was 7.2 µM/hr.

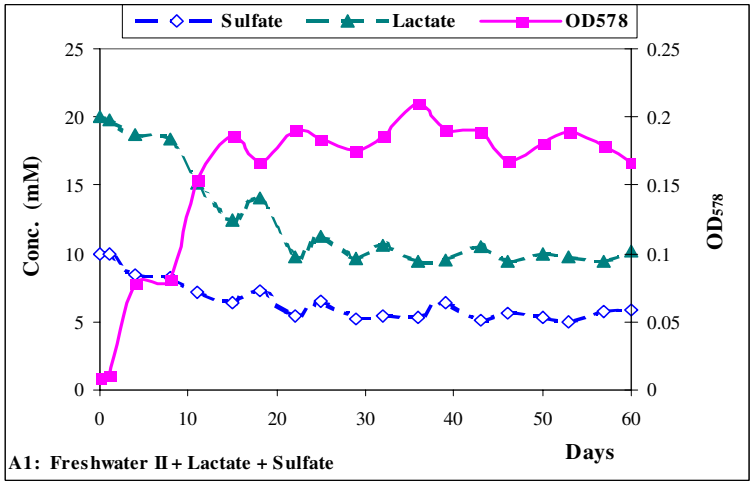
In the assays A3 sulfate reduction was found after the 8th day of incubation and the degradation continued until the 18th day (Fig. 4.2.1). From the available sulfate around 34 % was reduced 3.5 - 7.2 % of CO_2 were detected in gas phase. The SRR was 3.1 µM/hr.

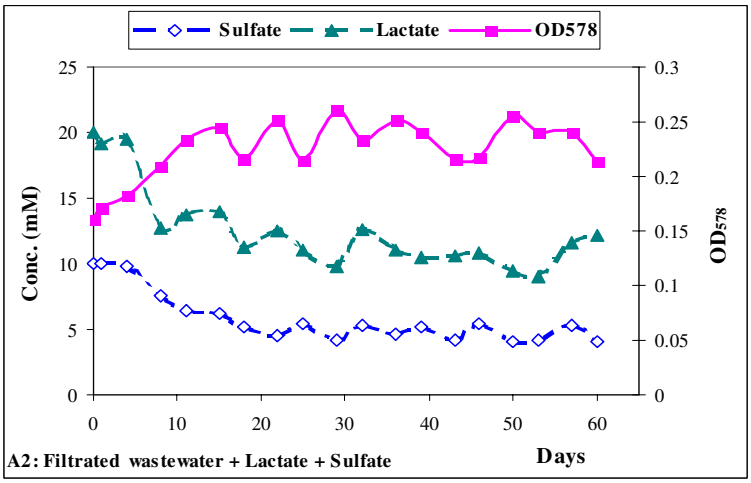
4.2.2 Continuous culture of Desulfovibrio desulfuricans in an Infors laboratory fermenter

A continuous culture of *D. desulfuricans* was cultured in non-sterile DSM medium Nr. 63 in an Infors fermenter (total volume 2.5 liter, working volume 2 liter) with 40-rpm stirring at room temperature. The medium, which contained 20 mM initial sulfate concentration and 20 mM lactate was feed into the reactor with a flow rate of 100 mL/day, equivalent to 20 dHRT). During the experiments fermenters were continuously flushed with nitrogen gas.

For start-up 25 % inoculum was added into the reactor and incubated as a batch culture for 3 days. Then the continuous feeding with a flow rate of 100 mL/day (equivalent to 20 dHRT) was started. Sulfate and lactate solutions were injected to reach the mentioned initial concentrations. After start up of the reactor for 9 days about 43 % of the sulfate and 45 % of the COD were reduced within the first month. In the second month sulfate removal reached 64 % and DOC removal 55 % (Fig. 4.2.2).

The extended incubation did not lead to a significantly increased of sulfate removal, presumably due to the fact, that the conditions were no longer suitable for bacterial growth or due to the accumulation of toxic substrates such as high amounts of H_2S.





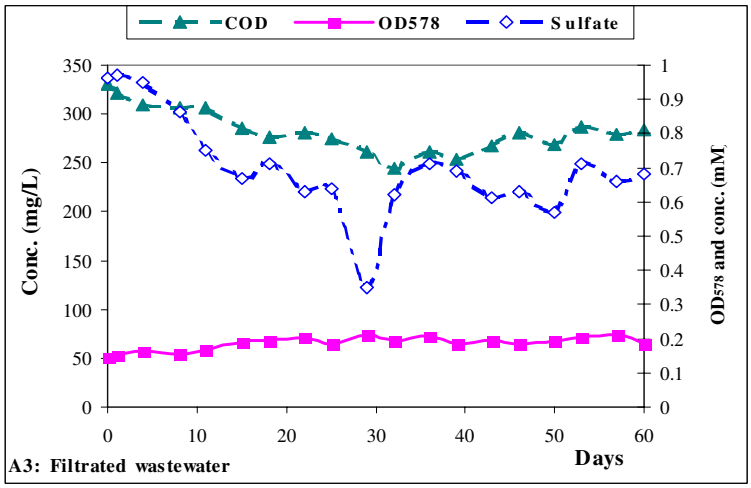


Figure 4.2.1 Concentration of sulfate, lactate, COD and biomass in media. (A1) Freshwater II medium, (A2) Filtrated wastewater plus lactate and sulfate, (A3) Filtrated wastewater. The incubation was at 37 °C.

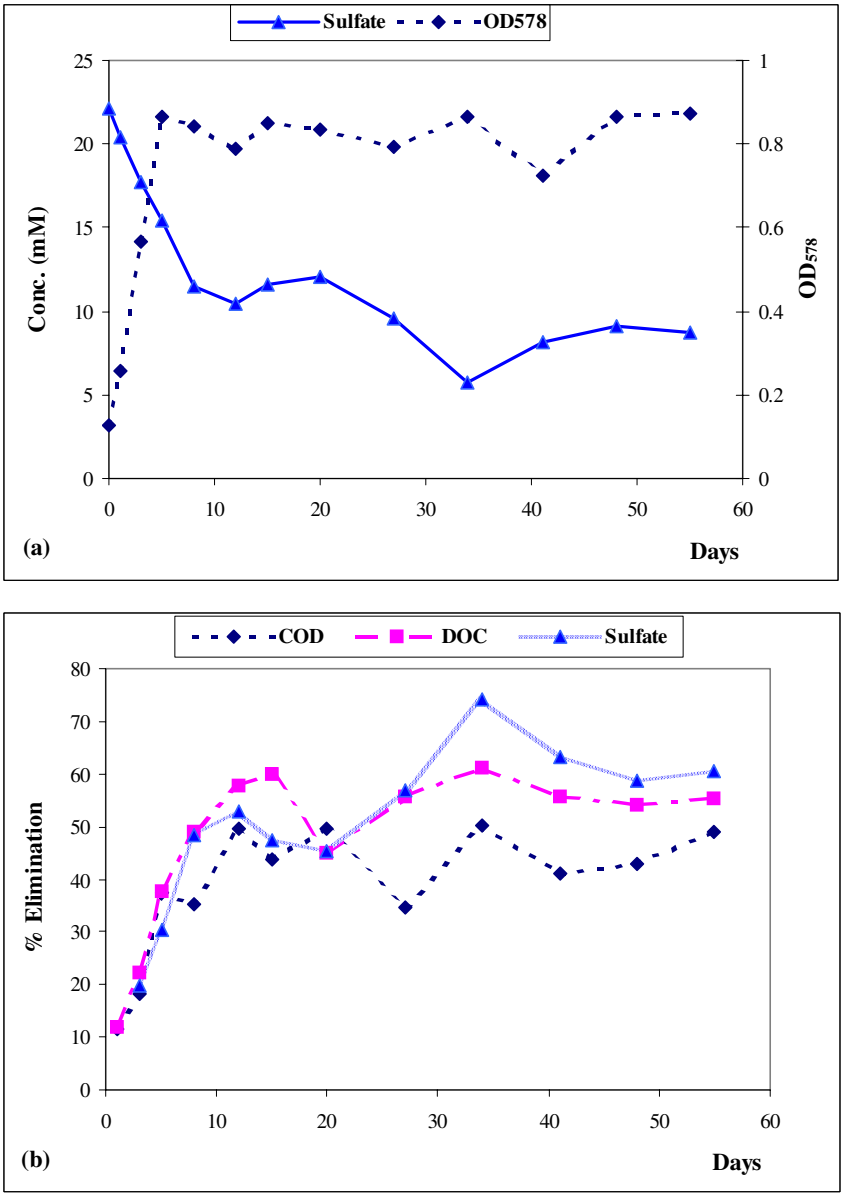


Figure 4.2.2 (a) Sulfate concentration and biomass in reactor, (b) % elimination of sulfate, DOC and COD.

4.2.3 The continuous culture of sulfate reducing enrichments from contaminated soil of Rastatt

Soil samples were taken below leaking sewers in Rastatt and were incubated in batch assays in Freshwater medium II at 20 ^{o}C. The inoculum was weekly subcultured for 4 weeks. The experiment was set up in a Sixfors fermenter (total volume 0.5 liter, working volume 0.4 liter). Homogenisation was obtained with a magnetic bar stirrer at 40 rpm and incubation was at room temperature (24.5 - 30 ^{o}C). Six reactors were run with 3 different types of influent (for each two parallel reactors) as follows:

T1 and T2: Filtrated sewage (0.2 µm filter pore size) plus 5 mM lactate and ca. 3 mM sulfate. The filtrated wastewater was freezed until use. Before use the required concentration of lactate and sulfate was added from stock solutions.

T3 and T4: Freshwater medium II plus 5 mM lactate and 4 mM sulfate

T5 and T6: Filtrated wastewater (filter pore size 0.2 µm).

25 % inoculum was added to each reactor and incubated under batch conditions for 3 days before starting the continuous feeding with a medium flow rate of 10 mL/day, equivalent to 40 d HRT. Sulfate and lactate solutions were injected into the reactor to obtain the desired initial concentration.

The reactors T1 and T2, which were fed with filtrated domestic wastewater, degraded increasing amounts of lactate and sulfate during 50 days and then reached a steady state (Fig. 4.2.3). Lactate and sulfate conversion was not complete.

After start up of the reactor T3-4 with fresh water medium for 60 days, sulfate removal had reached ca. 50 %, which did not increase upon further continuous cultivation. Some variation between 50 and 60 % occurred (Fig. 4.2.4). Although the COD removal increased, this did not influence the sulfate removal efficiency.

After start up of the reactor T3-4 for 60 days, the maximum sulfate removal reached ca. 50 %, which was constant during the rest of the observation time. COD removal reached its maximum after 50 days and varied from 55 – 60 % (Fig. 4.2.4).

In reactors T5-6 the maximum sulfate removal of 40 % was reached after 60 days of incubation and remained constant. The COD removal was 50 % after 71 days of incubation and remained constant (Fig. 4.2.5).

Extended incubations did not significantly increase removal percentages. This might be due to the competition of other bacteria for lactate, leading to depletion accumulation of toxic substrates such as H_2S.

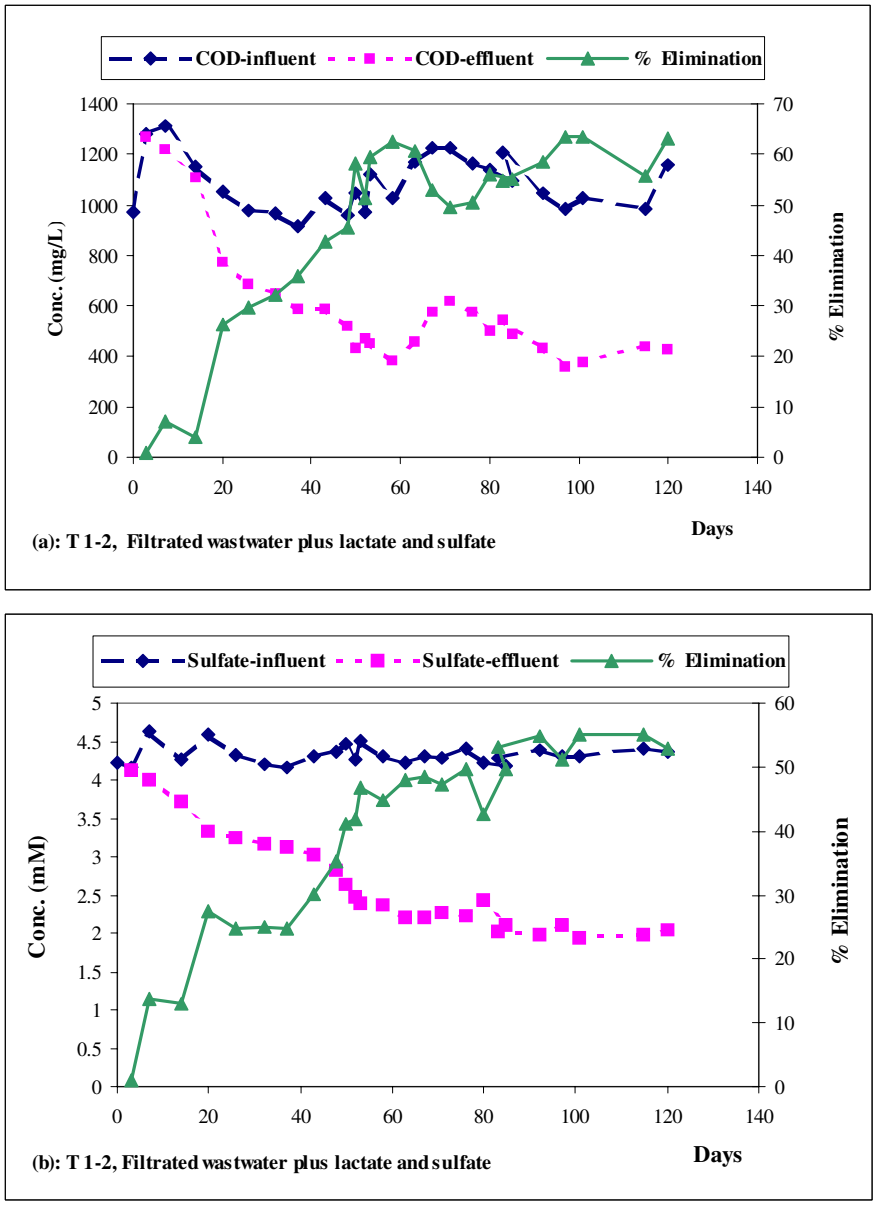


Figure 4.2.3 Average COD degradation and elimination (a), and sulfate concentration and % elimination (b) of fermenters T1 and T2 (average values).

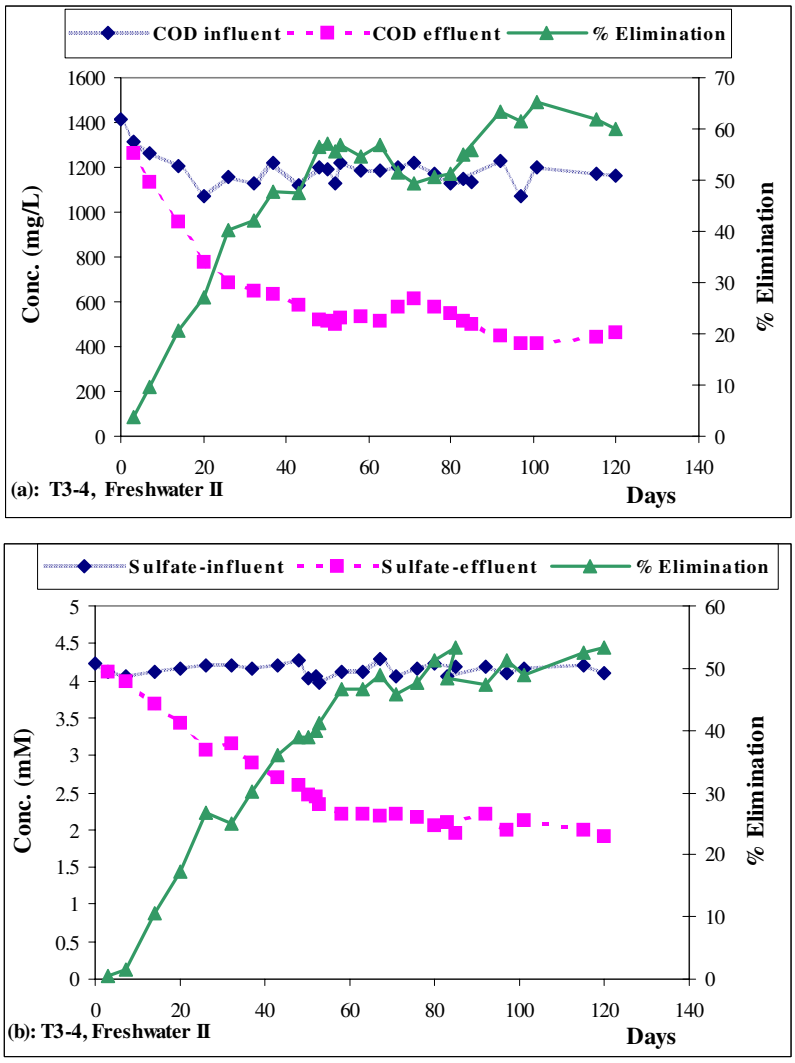


Figure 4.2.4 Average COD concentration and elimination (a), and sulfate concentration and % elimination (b) of fermenters T3 and T4 (average values)

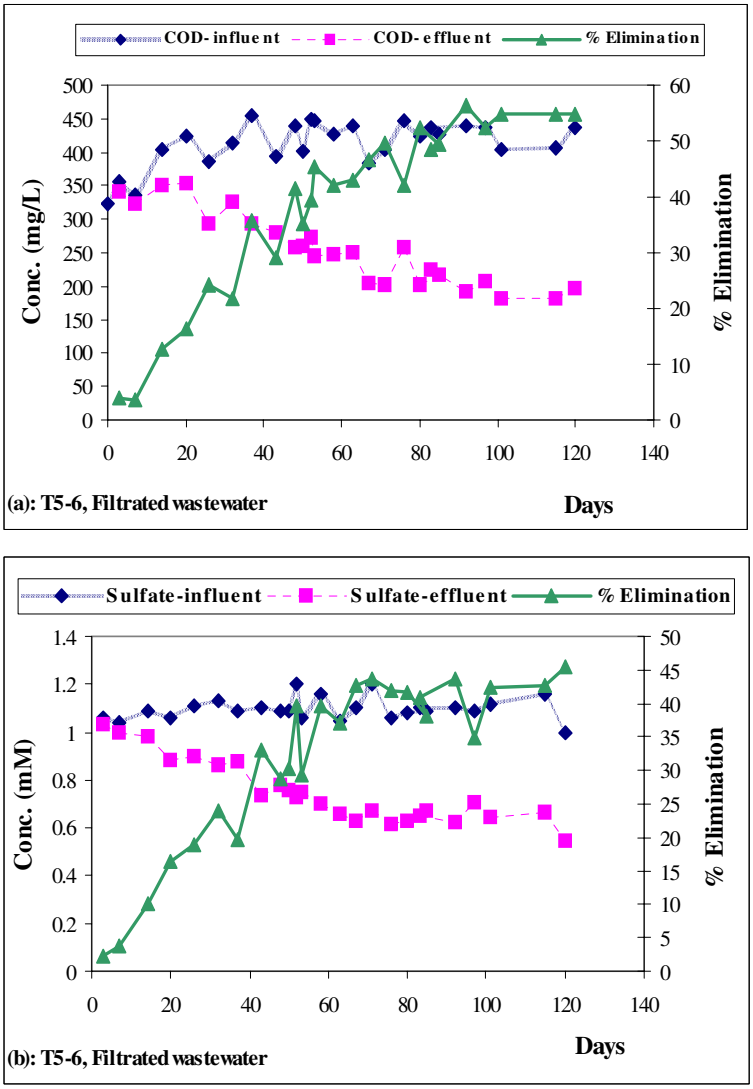


Figure 4.2.5 Average COD concentration and elimination (a), and sulfate concentration and % elimination (b) of fermenters T5 and T6 (average value)

4.3 Biofilm formation on sand particles after trickling of sewage

The laboratory sand columns were run as mentioned in Table 3.1. The degradation or retention of organic material was analyzed by chemical parameters, such as COD, BOD_5 or DOC. After several months of trickling of sewage through the sandy soil, a biofilm should have been developed on the grains. The columns were then unpacked and the bacterial population on the sand was examined. The soil profile was sampled from different depth (Fig. 4.3.1) for bacterial population studies. The coding of the samples was as listed below.

1IBA-S= top sludge sheath of column IBA1,

1IBA-0-5 = sand from 0 - 5 cm depth of column IBA1,

2IBA-110-120 = sand from 110 - 120 cm depth of column IBA2,

1NRT-S= top sludge sheath of column of column NRT1

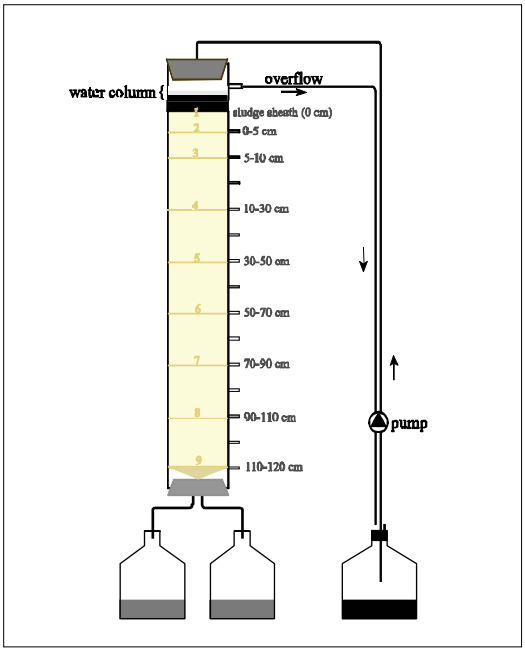


Figure 4.3.1 Sampling depths from soil columns

4.3.1 Distribution of different bacteria in soil of the sand columns.

When sewage was pumped on the column under anaerobic conditions (flushed with nitrogen to keep it anaerobic), the sandy soil in the column was expected to house an anaerobic ecosystem, fed with sewage components and the population present in the sewage. To enumerate the population of bacteria, which contributed to the activities in

the soil of the observed columns, the population of anaerobic, denitrifying, sulfate reducing, aerobic and coliforms bacteria in the observed columns which was immobilized as a biofilm on the sand grains was investigated by the spread plate method. In parallel, chemical components of soil, i.e. DOC, total carbon or total sulfur, were analyzed. The correlation between bacterial numbers and chemical parameters was found to follow linear, parabolic or exponential regression. The ratio of aerobic to anaerobic bacteria and their contribution to the total population in each profile was calculated for estimation of population shifts.

Soil column IBA1: Both, aerobic and anaerobic bacteria were discovered in the sludge level that accumulated above the sandy soil with time (Fitterer, 2001). The population density in the soil of column IBA1 was decreasing with depth. After start of the experiment the trickling sewage was not made anaerobic with nitrogen and thus in the column aerobic conditions predominated. High numbers of aerobic bacteria were determined after a running time of only 4 months. In addition, the bacterial numbers in soil were higher than in sewage (1.6×10^6 CFU/mL), indicating growth or biofilm formation of aerobic bacteria on sand particles.

Applying the plate count method, the number of aerobic bacteria was found to be 1.2×10^7 CFU/g dry soil in the sludge layer, 1.8×10^6 CFU/g dry soil in the sand at 0 – 5 cm depth and 5.2×10^5 CFU/g dry soil of column IBA1 at 110 – 120 cm. Fitterer (2001) did a comparison of the bacterial numbers that were determined by the plate count and MPN method, which resulted in higher numbers by the MPN method (Fig. 4.3.2).

The population density of anaerobic bacteria was 4.2×10^4 CFU/g dry soil in the sludge layer on top (sample 1IBA-S) and 1.2×10^4 CFU/g dry soil in the sand layer from 0 – 5 cm (sample 1IBA-0-5). At 110 – 120 cm depth, 4.2×10^3 CFU/g dry soil were culturable, indicating that only 10 % of the population density of the top region of the column was present as a biofilm on the sand grains close to the effluent (Fig. 4.3.2).

The enumeration of SRB from the biofilm was done with 3 different carbon sources: acetate, butyrate and lactate. After 4 months trickling of sewage through the sand column, the number of SRB was low. It was assumed that the conditions inside the column were still aerobic. The SRB decreased from 1.7×10^4 CFU/g dry soil in the sludge layer of the sand column 1IBA-S, to 4.4×10^2 CFU/g dry soil in 10 – 20 cm depth of column 1IBA-10-20, and further to 2.2×10^2 at 70 - 90 cm depth to finally

disappear completely in a depth of 110 - 120 cm (Fig. 4.3.3). This might have been due to oxygenation through the effluent tube, which would inactivate the sulfate reducers.

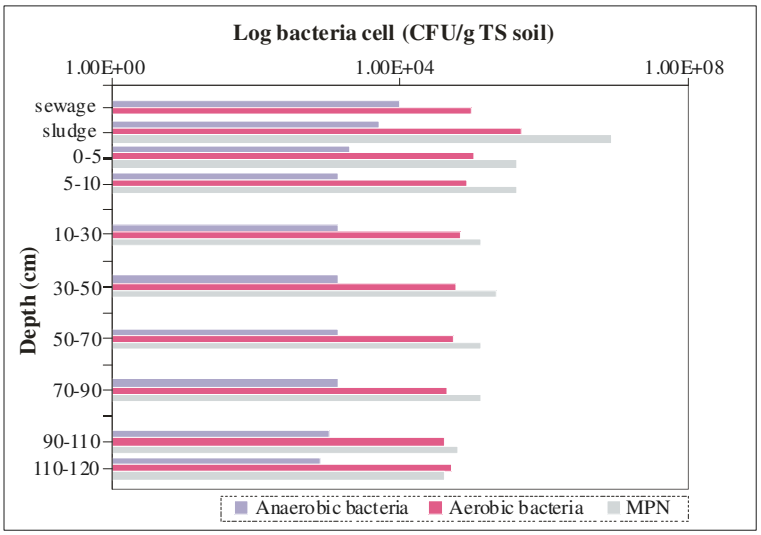


Figure 4.3.2 Comparison of CFU/g dry weight of soil for total aerobic and anaerobic bacteria) and of $MPN_{aerobic}$/g of soil cells of column IBA1 (Fitterer, 2001)

Since anaerobic bacterial numbers were particularly low in comparison with aerobic bacteria, the enumeration of aerobic bacteria and of total cells coincided.

The relationship between the total cells and TOC or total S and COD in the soil was shown to follow a linear regression with high $R^2 \geq 0.91$ - 0.97 (Fig. 4.3.4, Table 4.3.1). However, the correlation between total cells and total sulfur was negative. Applying a parabolic or exponential dependence, $R^2 < 0.5$ has been obtained. The correlation between SRB and total sulfur in soil provided a high correlation ($R^2 = 0.99$) both, by linear and parabolic regression.

The total cell density correlated with protein value in soil, oTS and dehydrogenase activity in form of a linear regression with $R^2 = 0.996$ - 0.999 (data not show).

The percentage of aerobic bacteria from the total population in each depth was > 98.7 % (Fig. 4.3.5). Anaerobic bacteria contributed only 0.35 - 1.22 %. Likewise, SRB were found to contribute less than 0.14 % to the total bacteria.

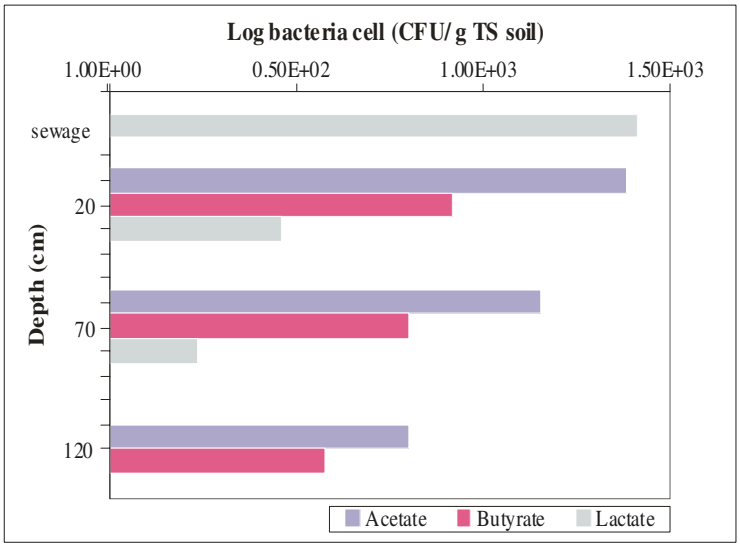


Figure 4.3.3 Population density of acetate, n-butyrate, lactate utilizing SRB at different depth of the soil column IBA1

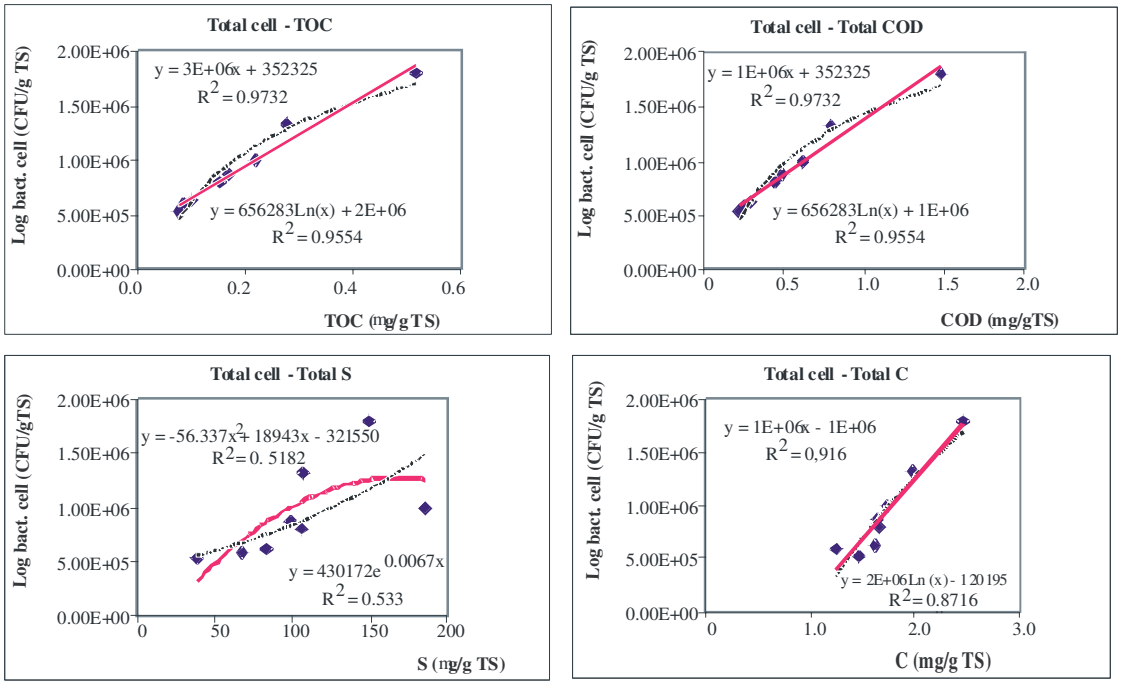
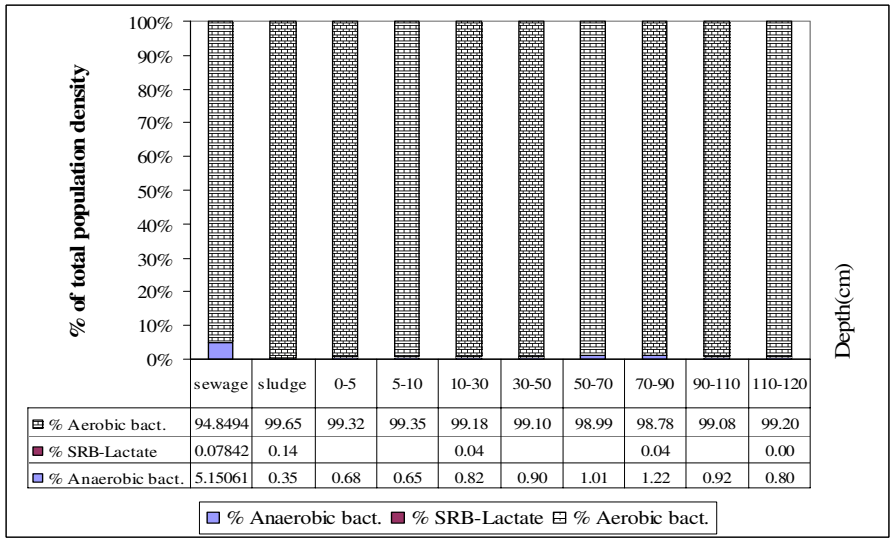


Figure 4.3.4 Correlation between total cells and TOC, total carbon, COD and sulfur in the soil column IBA1.

The ratio of aerobic and anaerobic bacteria was calculated and compared with the ratio of aerobic and anaerobic bacteria in raw sewage in order to predict the conditions inside a column. The results showed that the whole column was aerobic or at best anoxic (Fig. 4.3.6). For this reason, an enumeration of SRB resulted in very low numbers.



	sewage	sludge	0-5	5-10	10-30	30-50	50-70	70-90	90-110	110-120
% Aerobic bact.	94.8494	99.65	99.32	99.35	99.18	99.10	98.99	98.78	99.08	99.20
% SRB-Lactate	0.07842	0.14			0.04			0.04		0.00
% Anaerobic bact.	5.15061	0.35	0.68	0.65	0.82	0.90	1.01	1.22	0.92	0.80

Figure 4.3.5 Percentage of total anaerobic, total aerobic and sulfate reducing bacteria in different depth of the soil column IBA1.

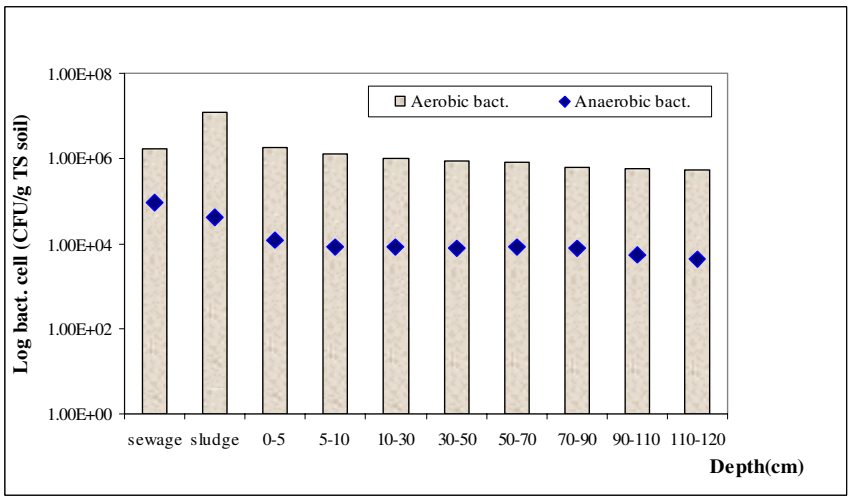


Figure 4.3.6 Population density of aerobic- and anaerobic bacteria in different depth of the soil column IBA1.

Table 4.3.1 The relationship of total-, anaerobic- and aerobic bacteria with composition of soil

Column, parameter	Equation	Correlation	Regression type	Remark
IBA1				Y = total cell
Total cell-total TOC	$Y= 3 E^{+06}X + 352325$	$R^2 = 0.9732$	Linear	X = analyzed
	$Y= 656283 \ln X + 2E^{+06}$	$R^2 = 0.9554$	Exponential	value
Total cell-total COD	$Y= 1 E^{+06}X + 352325$	$R^2 = 0.9732$	Linear	E = power of
	$Y= 656283 \ln X + 1E^{+06}$	$R^2 = 0.9554$	Exponential	
Total cell-total C	$Y= 1 E^{+06}X + 100000$	$R^2 = 0.916$	Linear	
	$Y= 2 E^{+06}\ln X - 120195$	$R^2 = 0.8716$	Exponential	
Total cell-total sulfur	$Y= -56.337X^2 + 18943 X –$ 321550	$R^2 = 0.5182$	Parabolic	
		$R^2 = 0.5330$	Exponential	
Total sulfur SRB	$Y= 430172 e^{0.0067x}$	$R^2 = 0.9874$	Parabolic	
	$Y=2.972X^2 - 423.97 X +14030$			
IBA2				
Total cell-total TOC	$Y= 39563 X - 607874$	$R^2 = 0.9993$	Linear	
	$Y= 2 E^{+07} \ln X – 1 E^{+08}$	$R^2=0.8393$	Exponential	
Total cell-total COD	$Y= 328258 X + 1 E^{+06}$	$R^2 =0 .9982$	Linear	
	$Y=1 E^{+07} \ln X + 6 E^{+06}$	$R^2 = 0.7056$	Exponential	
Total cell-total C	$Y= 218266X + 3E^{+06}$	$R^2 = 0.9926$	Linear	
	$Y= 1E^{+07}\ln X + 1E^{+07}$	$R^2 = 0.8505$	Exponential	
Total cell-total sulfur	$Y= 149737X + 7E^{+06}$	$R^2 = 0.8033$	Linear	
	$Y= 2E^{+07} \ln X - 8E^{+07}$	$R^2 = 0.6126$	Exponential	
Total sulfur SRB	$Y= 91154X + 16801$	$R^2 = 0.719$	Linear	
	$Y= 17235 \ln X - 40154$	$R^2= 0.7305$	Exponential	
NRT1				
Total cell- total TOC	$Y=32.912X^2 - 48241X + 2E^{+07}$	$R^2 = 0.6721$	Parabolic	
Total cell- total COD	$Y=4E^{+06}X^2 - 2E^{+07}X + 2E^{+07}$	$R^2 = 0.6721$	Parabolic	
Total cell- total C	$Y=2E^{+07}X^2 - 7E^{+07}X + 6E^{+07}$	$R^2 = 0.633$	Parabolic	
Total cell- total sulfur	$Y=236.93X^2 + 13910X + 4E^{+06}$	$R^2 = 0.7903$	Parabolic	
SRB-total sulfur	$Y=1.988X^2 - 232.79X + 6468.8$	$R^2 = 0.9475$	Parabolic	

Soil column IBA 2: The population density decreased with the depth of the soil profile. The conditions inside the column were aerobic and/or anoxic.

The number of anaerobic bacteria decreased from 1.1 x 10^8 CFU/g dry soil in the sludge layer of column IBA 2 (sample 2IBA-S) to 2.1 x 10^6 CFU/g dry soil at 0 – 1 cm depth of column IBA 2 (sample 2IBA-0-1). It slightly increased to 5.1 x 10^6 CFU/g dry soil at 1 – 5 cm depth of column IBA 2 (sample 2IBA-1-5) and decreased again to 3.0 x 10^3 CFU/g dry soil at 110 – 120 cm depth of column IBA 2 (sample 2IBA-110-120) (Fig. 4.3.7).

The denitrifying bacteria increased from 1.3 x 10^5 CFU/g dry soil in sample 2IBA-S, to 2.0 x 10^5 in sample 2IBA-0-1. Then they were decreased at a depth of 1 – 5 cm to 4.2 x 10^4 CFU/g dry soil (sample 2IBA-1-5) and to 9.3 x 10^3 CFU/g dry soil (sample 2IBA-110-115) (Fig. 4.3.8).

The sulfate reducing bacteria were slightly decreasing in the first 5 centimeter from 7.9 x 10^4 CFU/ g dry soil (sample 2IBA-S) to 5.4 x 10^4 CFU/g dry soil (sample 2IBA-0-1), to 4.4 x 10^4 CFU/g dry soil (sample 2IBA-1-5) and to 1.7 x 10^3 CFU/g dry soil (sample 2IBA-110-115) (Fig. 4.3.8).

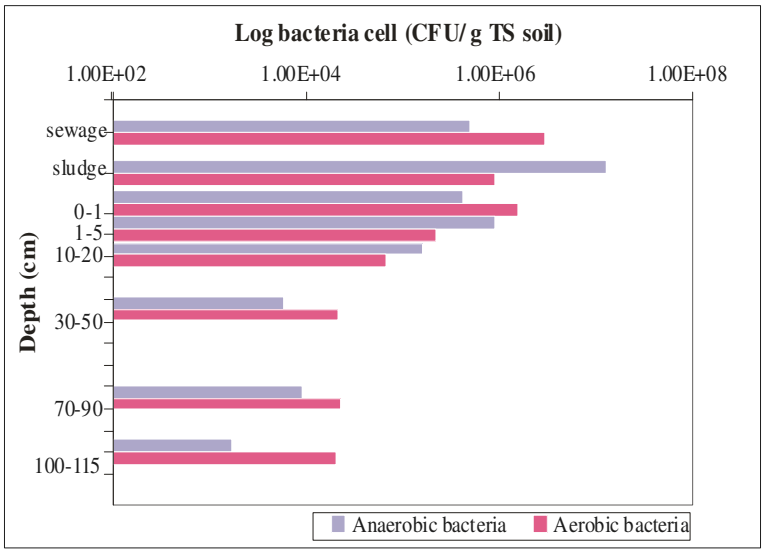


Figure 4.3.7 The distribution of total aerobic and total anaerobic bacteria (CFU/g-dry soil) with depth in soil column IBA2

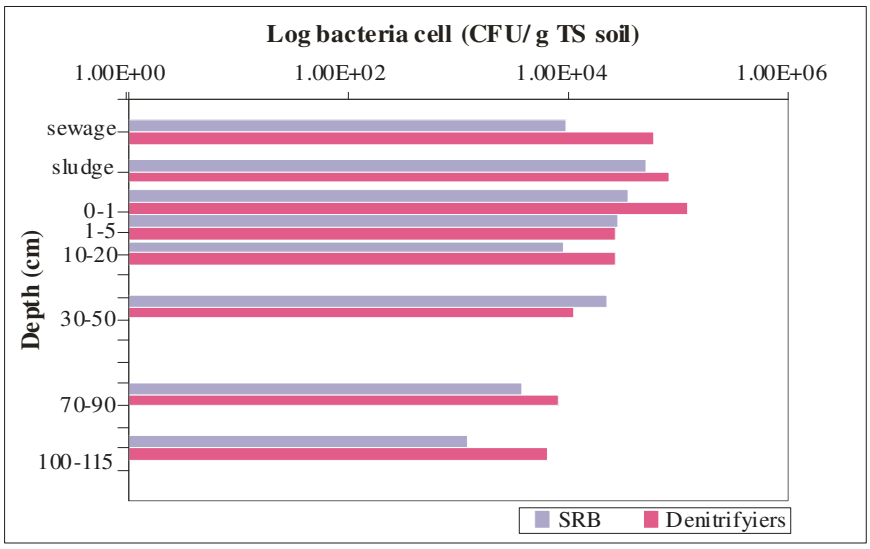


Figure 4.3.8 The distribution of SRB and denitrifiers (DN) (CFU/g-dry soil) in different depth of soil column IBA2

The population of aerobic bacteria was 4.9 x 10^6 CFU/g dry soil in the sludge layer of column IBA 2 (sample 2IBA-S) and increased to 9.8 x 10^6 CFU/g dry soil at 0 – 1 cm depth (sample 2IBA-0-1). After that the number decreased to 9.6 x 10^5 CFU/g dry soil in 1 – 5 cm depth (sample 2IBA-1-5) and to 5.6 x 10^4 CFU/g dry soil at 110 – 115 cm depth (sample 2IBA-110-115) (Fig. 4.3.7). The elimination of bacteria in the whole column was in the range of 98.86 %. Rosenbeiger-Ezzaoui (2002) determined the population density of aerobic bacteria in the same soil column and found higher numbers of aerobic bacteria with the MPN procedure (4.6 x 10^9 CFU/g dry soil at 2IBA-S, 1.4 x 10^8 CFU/g dry soil at 2IBA-0-1 and 4.4 x 10^7 CFU/g dry soil at 2IBA-1-5).

The ratio of aerobic and anaerobic bacteria changed with depth of the column (Fig. 4.3.9). At a depth 0 - 1 cm and after 30 cm, the numbers of aerobic bacteria were higher than those of anaerobic bacteria. Aerobic condition was prevailing on top and after 30 cm depth of the sand column to the outlet.

The correlation of total bacterial numbers with total carbon TOC and total COD data was determined. A positive correlation with linear regression was obtained (R^2 = 0.99). The same relationship between SRB and total sulfur in soil was found, i.e. R^2 = 0.72 by linear regression and R^2 = 0.73 by exponential regression (Table 4.3.1, Fig. 4.3.10).

The distribution of the different groups of bacteria is shown in Fig. 4.3.11. In the sludge layer, 95.8 % of the populations were anaerobic bacteria but only 5.1 % anaerobic bacteria were found in the effluent (sample 2IBA-110-115). The population densities of anaerobic bacteria were high between 1 - 20 cm depths. From 30 cm depth on until the outlet aerobic bacteria were predominant. The denitrifying bacteria contributed 0.1 - 13.5 %, the SRB contributed 0.07 - 28.1 %. Most SRB were found in sample 2IBA-30-50.

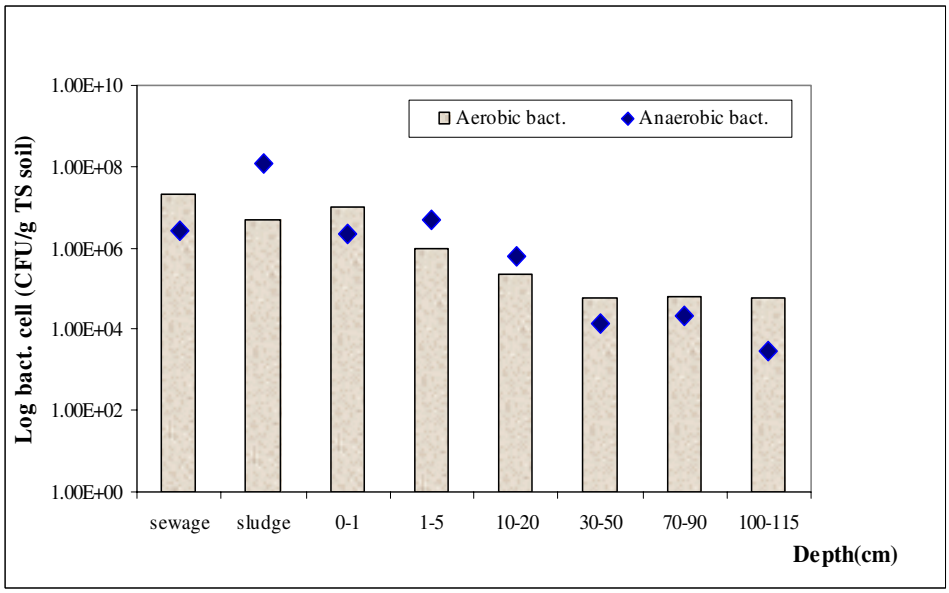


Figure 4.3.9 Population density of aerobic and anaerobic bacteria in soil of column IBA2

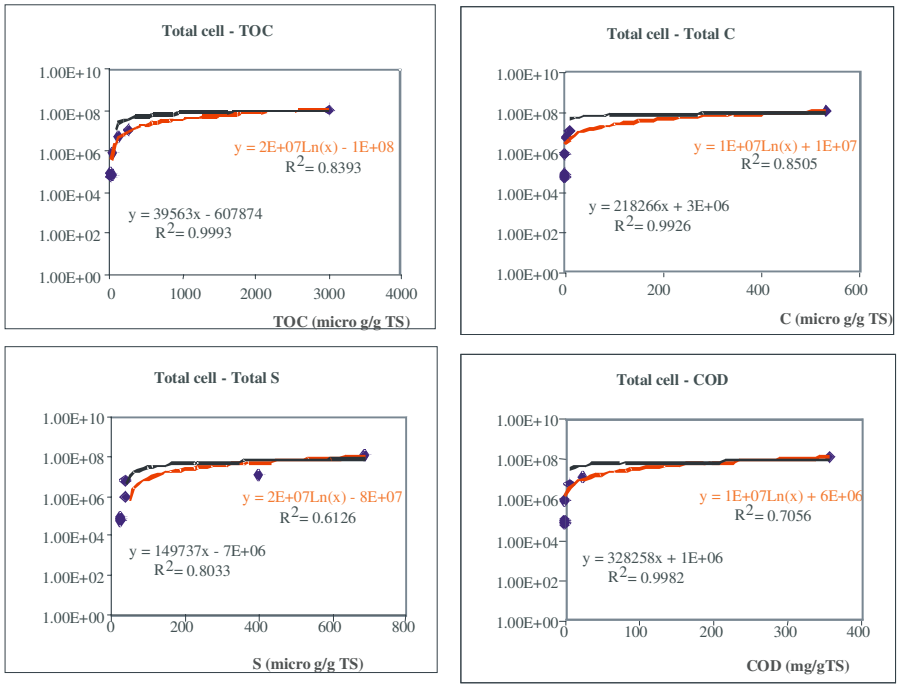
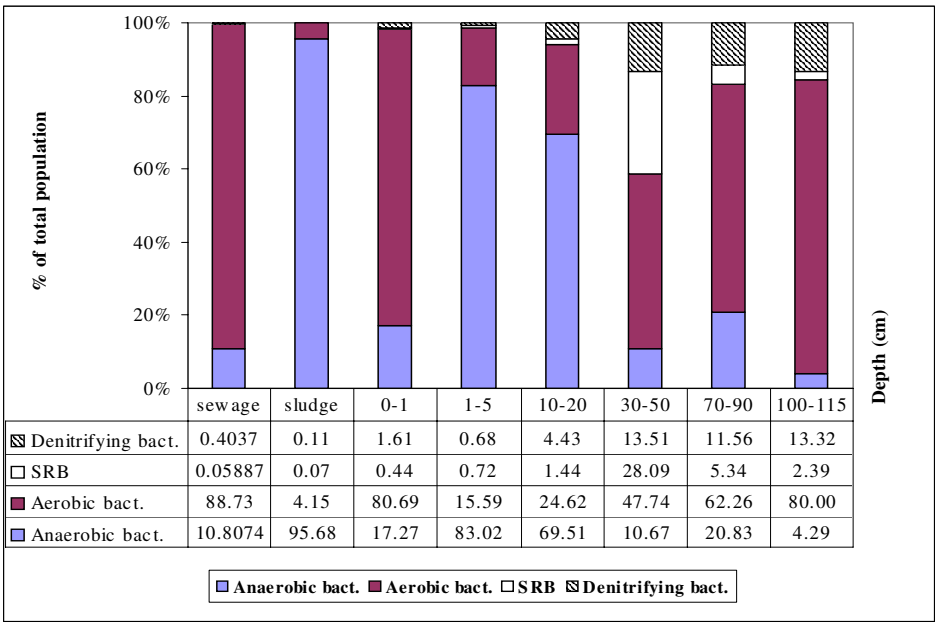


Figure 4.3.10 The correlation of total cells with TOC (a), total carbon (b), total sulfur (c) and COD (d) in soil of column IBA2



	sewage	sludge	0-1	1-5	10-20	30-50	70-90	100-115
Denitrifying bact.	0.4037	0.11	1.61	0.68	4.43	13.51	11.56	13.32
SRB	0.05887	0.07	0.44	0.72	1.44	28.09	5.34	2.39
Aerobic bact.	88.73	4.15	80.69	15.59	24.62	47.74	62.26	80.00
Anaerobic bact.	10.8074	95.68	17.27	83.02	69.51	10.67	20.83	4.29

Figure 4.3.11 The proportion of total anaerobic, total aerobic and sulfate reducing bacteria in soil of column IBA2

***The NRT1-column*:** Both, aerobic and anaerobic bacterial cells decreased with depth. Due to adsorption or biofilm formation, the number of bacterial cells at a depth of 0 - 40 cm was higher than in sewage (Fig. 4.3.12).

The numbers of anaerobic bacteria decreased from 8.9 x 10^7 CFU/g dry soil in sample 1NRT-S, to 8.3 x 10^6 CFU/g dry soil in sample 1NRT-0-5 and to 9.0 x 10^5 CFU/g dry soil in the effluent (sample 1NRT-110-120, Fig. 4.3.12a).

The denitrifying bacteria were eliminated from 3.4 x 10^5 CFU/g dry soil at 1NRT-S, to 1.9 x 10^5 CFU/g dry soil at 1NRT-0-5 and further to 2.6 x 10^4 CFU/g dry soil at 1NRT-110-120 (Figure 4.3.12c).

The sulfate reducing bacteria (SRB, Figure 4.3.12d) contributed 2.9 x 10^4 CFU/g dry soil in sample 1NRT-S and 3.9 x 10^4 CFU/g dry soil in sample 1NRT-0-5. However the number of SRB was decreasing with depth in the soil column. At the outlet the number of viable SRB was only 2.6 x 10^1 CFU/g dry soil, presumably due to aerobic conditions.

High numbers of aerobic bacteria of 8.1 x 10^8 CFU/g dry soil were found in the sludge layer on top of the sand (sample 1NRT-S), presumably originating from the sewage. The aerobic population decreased to 7.6 x 10^6 CFU/g dry soil in sample 1NRT-0-5 and to 2.0 x 10^6 CFU/g dry soil in sample 1NRT-110-120 cm (Fig. 4.3.12b).

The coliforms as indicators for pathogens were also determined. The population density was 1.2 x 10^6 CFU/g dry soil in sample 1NRT-S and 1.0 x 10^6 CFU/g dry soil in sample 1NRT-0-5. After 0 - 5 cm depth, the cell numbers were decreasing to 5.1 x 10^5 CFU/g dry soil in sample 1NRT-110-120 (Fig. 4.3.12e).

The ratio of aerobic to anaerobic bacteria as a rough indicator for the redox potential inside the column varied with depth (Fig. 4.3.13b). The numbers of aerobic bacteria were higher than those of anaerobic bacteria in the sludge profile and after 90 cm depth. However, at 0 - 5 cm depth the numbers of anaerobic and aerobic bacteria were almost equal. For the rest of the column (after 5 cm to 90 cm) anaerobic conditions were prevailing, according to the higher number of anaerobic bacteria in comparison to aerobic bacteria.

The relationship between the total bacterial cells and the chemical parameters, such as total carbon, total TOC, total S and total COD has been analyzed. A negative correlation by the normal x-y linear regression was obtained. Besides, the correlation of SRB with total sulfur in soil had shown a low coefficient in both types, namely

(R^2 = 0.47) by exponential and (R^2 = 0.67) by linear regression (data not showed). By using the parabolic regression as a calculation model a high correlation (R^2 > 0.6, see Table 4.3.1 and Fig. 4.3.14) in comparison to linear regression or exponential or logarithmic correlation has been found (cf. Table 4.3.1).

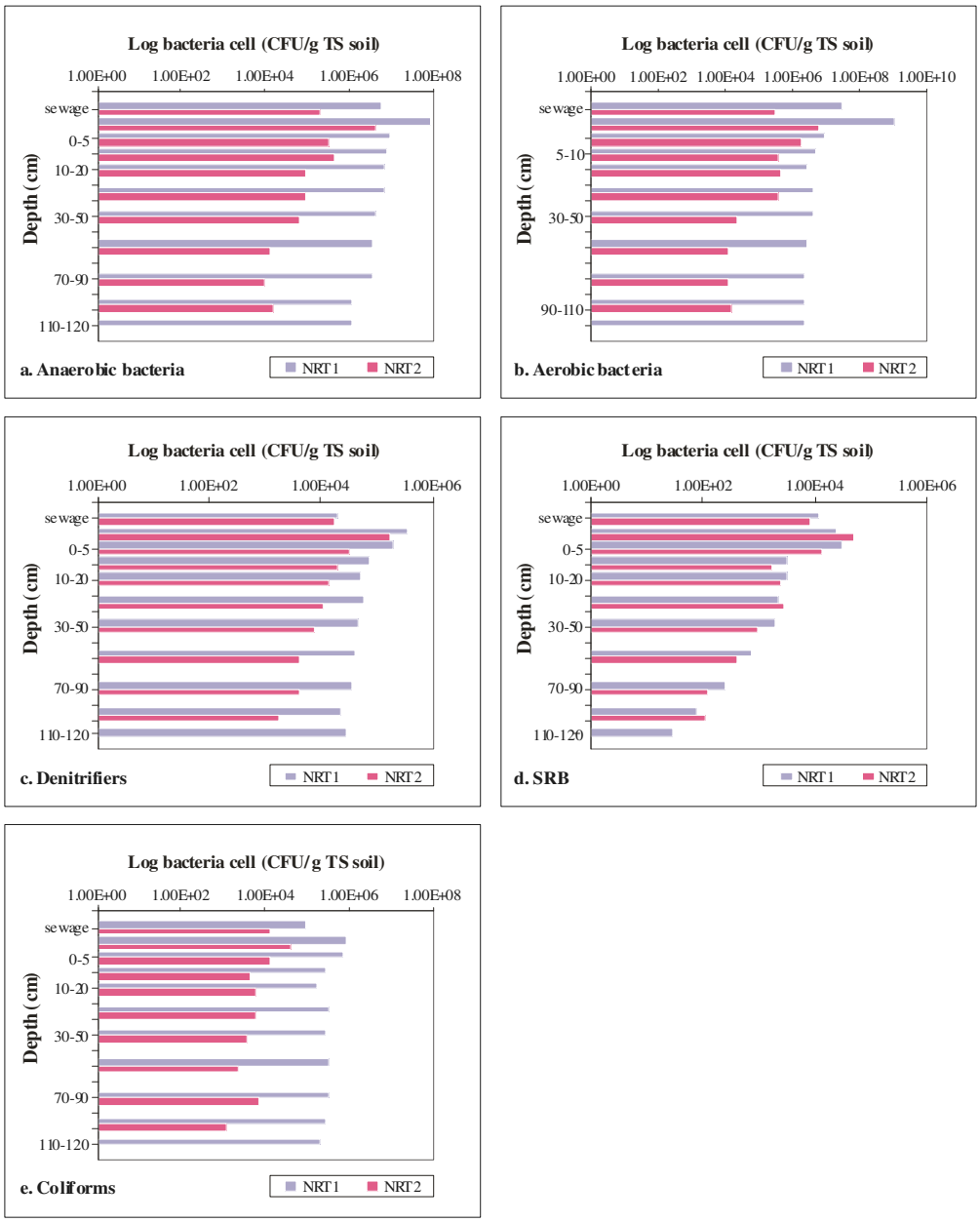


Figure 4.3.12 Distribution of (a) anaerobic, (b) aerobic, (c) denitrifying, (d) sulfate reducing bacteria and (e) coliforms (CFU/g dry soil) of the soil profiles of column NRT1 and NRT2 at different depth.

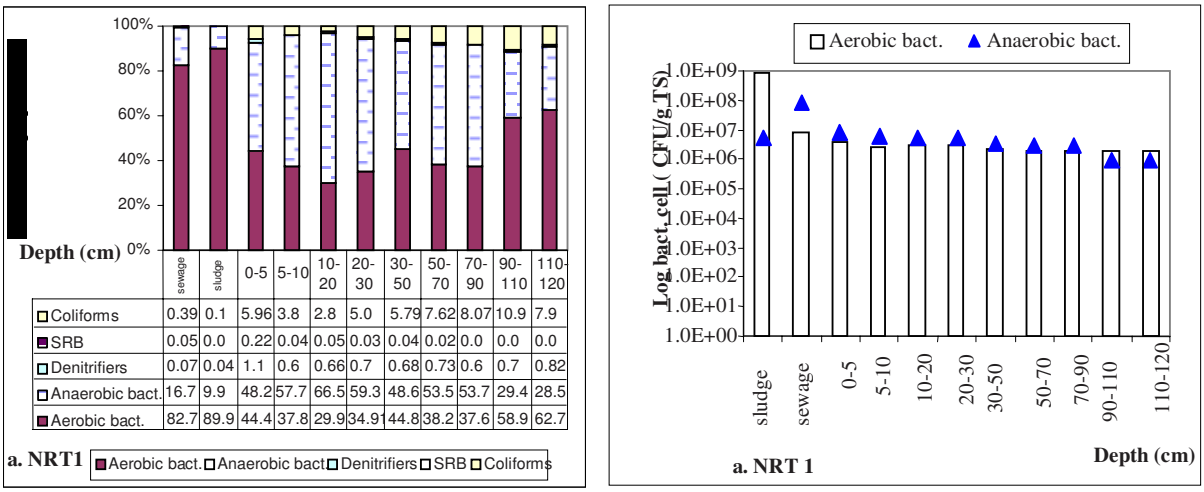


Figure 4.3.13 (a) Percentage of bacterial population and (b) population density of aerobic and anaerobic bacteria in column NRT1

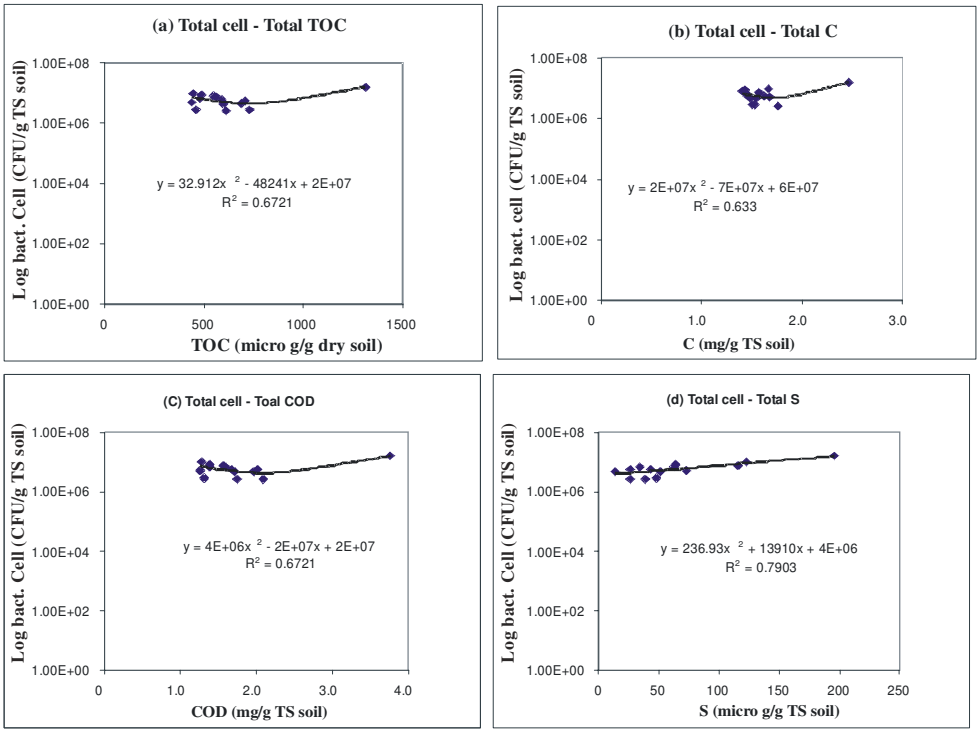


Figure 4.3.14 Correlation between total cells and total organic carbon (TOC; a), carbon (C; b), COD (d) or sulfur (S; d) in column NRT1.

The percentages of different bacterial groups in each depth of the sand column are presented in Fig. 4.3.13a. The percentage of aerobic bacteria was quite high (90 %) in sample 1NRT-S and after 90 cm depth until the outlet (> 58 %). At 5 - 90 cm depth, the percentage of anaerobic bacteria was higher than that of aerobic bacteria. The percentage of coliforms varied in the range of 0.1 to 10.9 %. The denitrifying bacteria were found in the range of 0.04 to 1.1 %. For the SRB only 0.2 % of the total bacteria were found in sample 1NRT-0-5 and < 0.05 % in the rest of the soil profile was determined.

As a result, aerobic conditions were assumed for the sludge layer and anoxic or anaerobic conditions started after 0 - 5 cm depth. This part of the sand column was suitable for bacterial growth, due to the high amount of dissolved organic carbon. However, the rest of the sand column profiles (until 90 cm depth) had a low concentration of dissolved organic carbon or hardly degradable substances at all, which might have caused the low number of SRB. Even if the SRB could use the residual substrates the possibilities of SRB to overgrow the other groups of bacteria were low, because SRB required strictly anaerobic conditions while the column was aerobic or anoxic. The low sulfate concentration in sewage (~ 1 mM) was another limiting factor for SRB.

The NRT 2 column: Domestic sewage was drained with a constant flow rate of 2 liter/day through the sand column, which was incubated at 17 ^{o}C, to simulate the in-situ soil temperature. Thus, the transformation rates of chemical components of sewage and bacterial activities represented in-situ conditions. Finally, the column was unpacked and the spread plate technique was used to determine the bacterial population. No sludge layer was present on top of the soil. It was found that the numbers of bacteria of all groups such as anaerobic, denitrifying, aerobic as well as coliform bacteria were decreasing with depth as well (Fig. 4.3.12a-d, Table 4.3.2).

99.9 % of *E. coli* (1.0 x 10^4 CFU/g dry soil) was eliminated. However, 15 CFU/g dry soil was still found at 110 - 120 cm (data not show), although the coliforms were rapidly decreasing with depth (Fig. 4.3.12e).

The ratio of aerobic to anaerobic bacteria varied at different depths. The environmental conditions 0 - 50 cm depth seemed to be aerobic. At a depth 50 - 70 cm the sand was anaerobic, as judged from a higher number of anaerobes than aerobes. In

all other parts of the sand column the ratio of aerobic and anaerobic bacteria was approximately 1 : 1 (Fig. 4.3.15b).

The SRB contributed 0.3 - 1.6 % of total bacteria, with the highest percentage found at 30 - 50 cm. At 0 - 50 cm depth, the denitrifying bacteria were present at a constant percentage (ca. 2 %) and after 50 cm depth until the outlet 6.6 - 15.3 % were found.

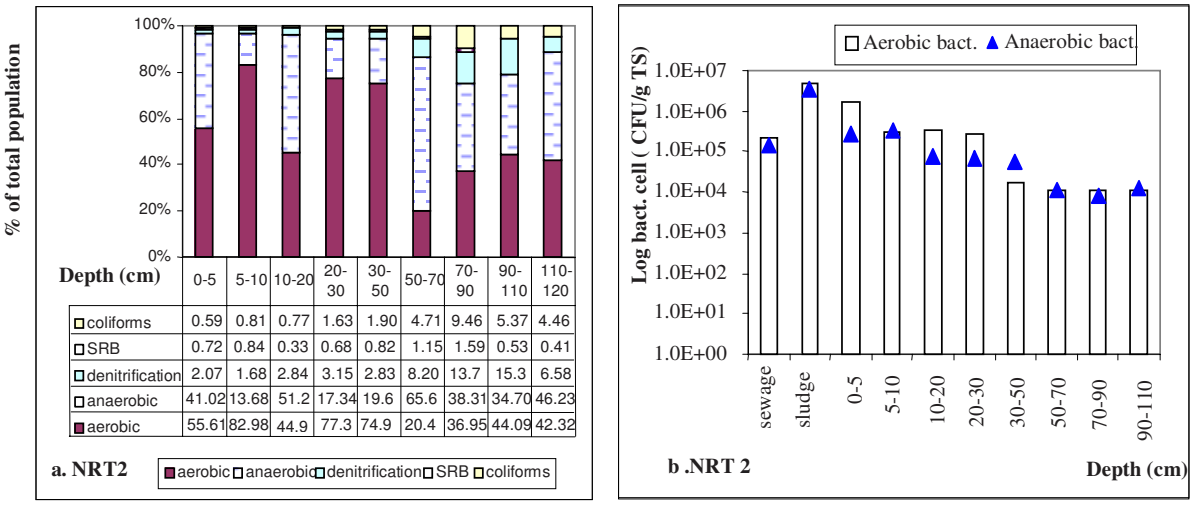


Figure 4.3.15 (a) Percentages of bacterial population and (b) population density of aerobic and anaerobic bacteria in column NRT1

Table 4.3.2 Bacterial numbers in soil column NRT2

	Number of bacteria (CFU/ g TS soil)			
Depth (cm)	Anaerobic bact.	Denitrifying bact.	SRB	Aerobic bact.
0-5	2.9×10^6	6.0×10^5	5.7×10^4	5.8×10^6
5-10	1.0×10^6	4.8×10^5	2.6×10^4	1.8×10^6
10-20	1.3×10^6	2.3×10^5	3.8×10^4	3.5×10^5
30-50	1.3×10^4	9.7×10^3	6.2×10^3	1.8×10^4
110-120	9.2×10^3	5.9×10^3	8.1×10^2	9.7×10^3

The SAW1 and SAW2 columns: Both columns were drained with an artificial model wastewater according to OECD guidelines. Both columns had a different experimental time span: SAW1 was run for 646 days and SAW2 was run for 245 days. After unpacking of the columns the bacterial number was examined. Much higher numbers were present on the soil as a biofilm than in the trickling wastewater in the pores. In SAW2 a sludge layer developed at the top of the column but not in column SAW1. The population of bacteria decreased exponentially with depth from 4.7 x 10^4 to 3.5 x 10^4 CFU/g dry sand at 5 - 10 cm and further to 2.0 x 10^3 CFU/g dry sand at the outlet (Fig. 4.3.16).

Anaerobic bacteria in the sand column *SAW 1* were present with 2.9 x 10^6 CFU/g dry soil at 0 - 5 cm, 1.0 x 10^6 CFU/g dry soil at 5 - 10 cm and 9.2 x 10^3 CFU/g dry soil at 90 - 110 cm. In the sand column *SAW2* 1.9 x 10^6 CFU/g dry soil accumulated in the sludge layer, 3.8 x 10^5 CFU/g dry soil at 0 - 5 cm depth and a further decline of the population density to 7.7 x 10^3 CFU/g dry soil at the outlet of the column was seen (Fig. 4.3.16a).

Denitrifying bacteria: in soil of *SAW1* were present with 5.1 x 10^5 CFU/g dry soil were found at 0 - 5 cm depth, 4.8 x 10^5 CFU/g dry soil at 5 - 10 cm depth and 5.9 x 10^5 CFU/g dry soil at 110 - 120 cm depth (Fig. 4.3.16d). *Soil of SAW2* contained 2 x 10^5 CFU/g dry soil in the sludge profile, 1.0 x 10^5 CFU/g dry soil at 5 - 10 cm depth and to 8.1 x 10^3 CFU/g dry soil at the outlet.

SRB: in 0 – 5 cm depth of column *SAW1* were present with 5.7 x 10^4 bacteria were enumerated, 3.8 x 10^4 at 10 - 20 cm and 8.1 x 10^2 at 110 - 120 cm depth (Fig. 4.3.16e). In column *SAW2* 1.2 x 10^5 bacteria were found in the sludge profile, 8.9 x 10^4 at 5 - 10 cm depth and 2.0 x 10^3 at 90 - 110 cm depth.

Aerobic bacteria: In soil of column *SAW1* were present with 2.9 x 10^6 CFU/g dry soil were found at 0-5 cm depth, which declined to 9.2 x 10^3 at 90 - 110 cm (Fig. 4.3.16b). Soil of *SAW2* contained 4.1 x 10^5 CFU/g dry soil in the sludge layer, which decreased to 2.5 x 10^5 CFU/g dry soil at 5 - 10 cm and further to 9.7 x 10^3 at the outlet (Figure 4.3.16b). Only ca. 40 % of total bacteria were removed at 0 - 10 cm depth and 97 % at 90 - 110 cm depth.

Figure 4.3.16 Decrease of bacterial cell numbers in soil of SAW1 and SAW2. (a) = aerobic. (b) = anaerobic, (c) = coliforms, (d) denitrifying bacteria and (e) SRB

Coliforms bacteria were present only at a depth of 0 - 20 cm in column *SAW1* with 20 - 52 CFU/g dry soil.

The ratio of aerobic and anaerobic bacteria inside the column was similar, except for soil from *SAW1* at 0 - 5 cm depth, which seemed to be aerobic. In soil column *SAW2* the sludge layer until a depth of 0 - 5 cm was anaerobic (Fig. 4.3.17).

Proportion of anaerobic to aerobic bacteria: Soil of SAW1, at 0 - 10 cm depth had a higher percentage of aerobic bacteria than of anaerobic bacteria. From 10 cm to 120 cm depth aerobic and anaerobic bacteria were present at a similar propotion. The SRB contributed 0.6 - 13.5 % of total bacteria with the highest percents found at 30 - 50 cm. The denitrifying bacteria in the whole column were present with constant population densities (14.5 - 26.6 %) except at 0 - 5 cm depth. As a result, in soil of SAW2 high numbers of anaerobic bacteria in the sludge layer and in the soil profile at 0 - 5 cm depth were found (Fig. 4.3.18). The SRB were found in a high percentage at 0 - 20 cm depth (11.3 - 13.6 %). Denitrifiers were found in high percentages in the sludge layer and at 90 - 110 cm depth

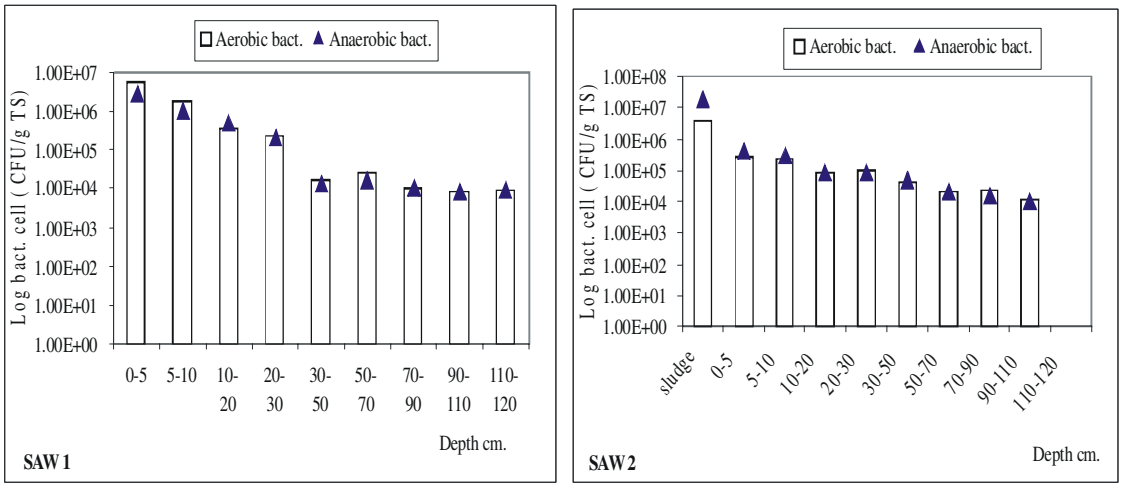


Figure 4.3.17 Ratio of aerobic and anaerobic bacteria in soil of SAW 1 and SAW 2

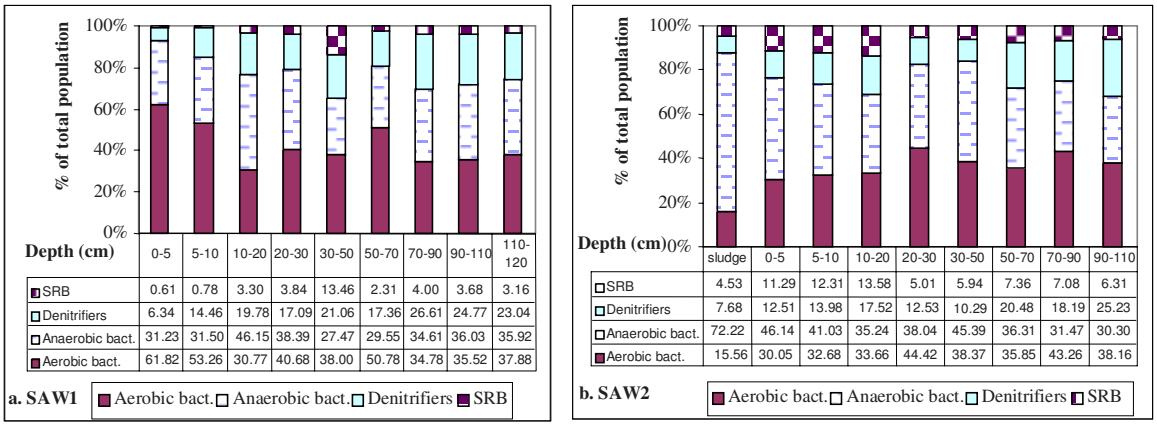


Figure 4.3.18 Percent of bacterial population at different depth of the soil profile in column SAW1 and SAW2.

Blank: The column was drained with normal tap water, which performed as a control column. The numbers of aerobic or anaerobic bacteria were low, only 10^3 CFU/g dry soil (Fig. 4.3.19). However, the soil contained higher bacterial numbers than tap water (< 10^2 CFU/mL).

High numbers of anaerobic bacteria were presented 2.5 x 10^3 CFU/g dry soil at 0 - 5 cm depth and slightly decreased until 1.9 x 10^2 CFU/g dry soil at the outlet (Fig. 4.3.19a).

The denitrifying bacteria were decreasing with depth. At 0 - 5 cm high numbers (1.4 x 10^2 cell/g dry soil) were present, but in the deeper soil layers less than 100 CFU/g were found (Fig. 4.3.19a).

Less than 100 CFU/ g dry soil of SRB at all depths were found.

A population of 1.8 x 10^3 CFU/g dry soil at 0 - 5 cm of aerobic bacteria was determined. After that, the numbers were declining with depth to 91 % removal at the outlet (Fig. 4.3.19a).

Coliform bacteria and *E. coli*, in all profiles were not found.

Higher numbers of aerobic than anaerobic bacteria were present (Fig. 4.3.19b). The condition inside the column was aerobic.

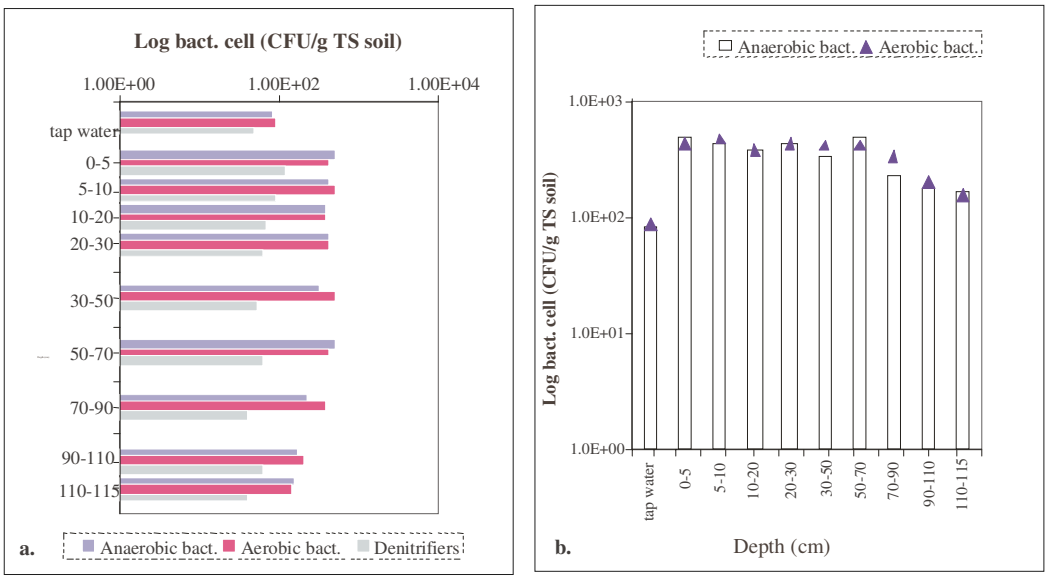


Figure 4.3.19 Bacterial cell numbers in soil of a blank column at different depth: (a) aerobic, anaerobic and denitrifying bacteria and (b) = ratio of aerobic and anaerobic bacteria.

4.3.2 The distribution of suspended bacteria in the water phase of the non-segmented and segmented soil column during trickling of sewage.

To test the trickling behaviour of sewage and the removal efficiency of sewage components two types of soil columns with a total length of 125 cm were run for more than two years. The first column was a one-piece column and the second column was segmented into 5 sub-columns, each upper sub-column connected with the lower sub-column by a siphon-like tubing. (Fig. 3.1 and 3.2). All columns were incubated in a thermostated room at 20 ^{o}C and continuously supplied with domestic wastewater. Samples of the effluent of the one-piece-column and of each sub-column of the segmented column were monthly withdrawn with sterile syringes and the population density was investigated by plating of 100 µL on the surface of agar plates. By variation of the incubation conditions or of the media the following types of bacteria were distinguished after growth on agar plates: Anaerobic bacteria, denitrifying bacteria, sulfate-reducing bacteria, aerobic bacteria and coliform bacteria. Chemical parameters, i.e. DOC, COD, SO_4^{2-} or different species of nitrogenous compounds and physical parameters, e.g. the flow rates were analysed in parallel (Hua, unpublished, Uni-Karlsruhe). There was a reasonable correlation between bacterial numbers and

chemical parameters. From the ratio of aerobic and anaerobic bacteria within the total population the conditions inside each segmented column (presence or absence of oxygen) could be deduced.

4.3.2.1. The segmented column

Effluent of the 5 segments of the segmented column (length: 25 cm soil layer/segment) was collected and analysed for the different microbial and chemical parameters of trickling sewage.

The correlation between chemical parameters and bacterial numbers

♦***Flow rate***: During 50 – 100 days, the column was drained with an increasing flow rate from 1 liter to 4.5 liter per day by increasing the pump speed. After the maximum flow rate was reached and the pump was set to a constant speed, the daily flow rate still varied between much lower and higher values than those that were adjusted on the pump. During 100 - 200 days of observation (July 2001 - November 2001), the flow rate was 0 - 500 mL/day and increased to 700 - 800 mL/day (December 2001 - April 2002, Fig. 4.3.20). Obviously, during September to November, the flow rate was relatively constant at ca. 300 mL/day.

♦***The population density in sewage*** (Fig. 4.3.21) varied slightly in correlation to the amount of available organic matter (i.e. $R^2 = 0.54$). The raw data indicated that in sewage with a high content of organic matter (COD = 647 - 1246 mg/L), high numbers of bacteria were found (September 2001 and January 2002). During this period, the DOC as a carbon source for bacterial growth was more than 100 mg/L, except for December 2001, where it was very high (ca. 538 mg/L). The DOC of sewage was positively correlated with the total bacterial cell number (with $R^2 = 0.9$). The correlation between DOC and COD in wastewater was also calculated and resulted in a positive correlation (Fig. 4.3.22), indicating that DOC or COD directly contributed as carbon source for the bacteria.

The main portion of the DOC was degraded already at 25 cm depth (1st segmented column), in other words the removal of DOC was 60 - 80 %. Most of the DOC was apparently an easily utilizable carbon source for bacterial growth. In the subsequent 4 sub-columns of the segmented column (totally 125 cm) only slightly more COD or DOC in the order of 5 - 15 % was removed. Degradation of the residual COD or DOC was not complete, either due to its chemical origin or due to its complex biological nature, that would require - if degradable at all - an extended hydraulic

retention time for the slow-growing bacteria to multiply and to express the necessary enzyme machinery.

The sewage contains both, suspended solids and dissolved organic compounds. To investigate the contribution of the amount of suspended solids on bacterial degradation activity, the solid organic matter of sewage was separated by centrifugation, leaving back only the dissolved proportion of the COD. The average ratio of total COD : dissolved COD was 1.7 : 1. In other words, total COD of sewage contained about 60 % dissolved COD.

- The elimination of ***total COD and DOC*** was in a similar range, reaching 60 - 80 % removal in the first 25 cm soil, leaving behind 8 - 15 % in the effluent of the fifth column.

- Some ***nitrogen*** was eliminated during the first 50 cm, presumably some ammonia was used for bacterial growth. Generally the concentration of ammonia was increasing during about 75 cm trickling stretch and then decreased. Nitrate was found after 75 cm trickling stretch at increasing concentrations. Protein degradation during bacterial growth increases NH_4^+/NH_3 and nitrification under aerobic conditions inside the column decreases NH_4^+/NH_3.

- Sulfate reduction in the order of 40 - 55 % of the initial amount of sulfate was analyzed in 25 cm soil depth, and another 1- 8 % of the residual sulfates were reduced in the 2nd sub-column. After 50 cm of trickling stretch no sulfate reduction was observed.

- ***Total bacteria***: The population density was not correlated with the flow rate, TKN and ammonium at each depth. It was, however, correlated to the DOC and COD. Particulate and dissolved COD revealed a positive correlation with bacterial numbers at each depth (Fig. 4.3.23).

- ***Aerobic bacteria***: Flow rate, TKN and ammonia were negatively correlated with the number of aerobic bacteria, whereas the COD revealed a positive correlation with bacterial cell densities (Fig. 4.3.24).

- ***Anaerobic bacteria***: The COD correlated directly with the bacterial number (Figure 4.3.25) only at 1st and 2nd segment.

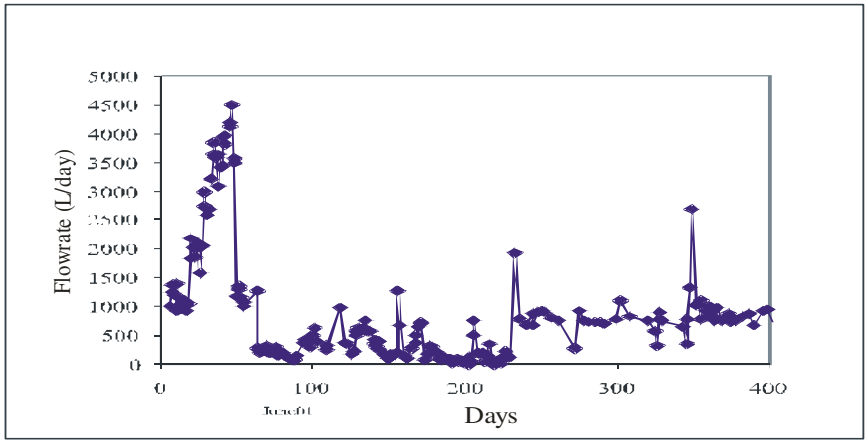


Figure 4.3.20 Sewage flow rates in the segmented column, April 01-April 02

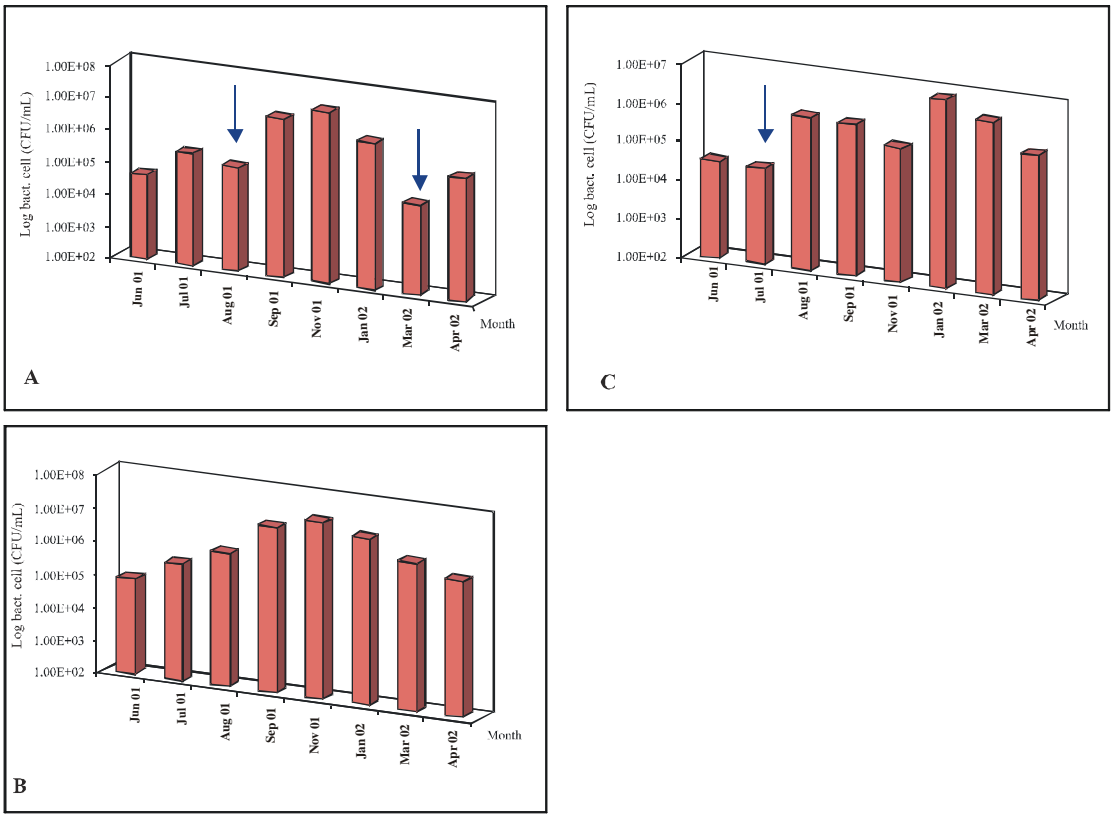


Figure 4.3.21 The number of total aerobic (a), anaerobic (b) and the total bacteria (c) in sewage from the sewage treatment plant in Neureut.

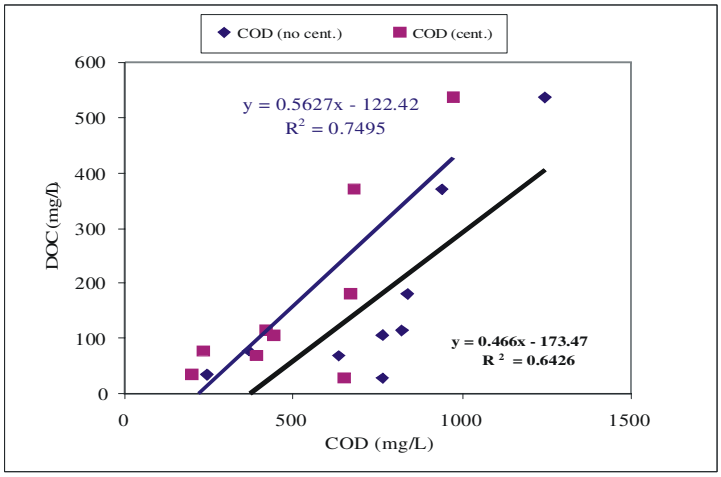


Figure 4.3.22 The correlation between total COD or dissolved COD (centrifuge, supernatant) with DOC from the sewage treatment plant in Neureut.

♦***% Elimination:*** The removal of different types of bacteria was compared to the removal of other parameters with depth or flow path. After the 25 cm of the segmented column, the population density of bacteria was reduced by the filtration effect to a similar extend as the chemical parameters COD or DOC.

Total aerobic bacteria: A varying initial bacterial number in sewage had only a restricted effect on the percent of elimination. During the observed time, the total removal of bacterial numbers in sewage was 68 – 99 %. The main retention of bacteria was observed after only 25 cm trickling stretch and only slightly more bacteria were eliminated in the following 4 sub-columns. The population density in the effluent after 125 cm passage of sewage through sandy soil was increasing and sometimes higher than in the influent (Table 4.3.3, Fig. 4.3.26). This could have been due to biofilm dissolution or growth of e.g. nitrifiers in the sand column after COD-removal.

Total anaerobic bacteria: The elimination of anaerobic bacteria from sewage after trickling through sandy soil during one year was checked. Similar to the filtration effect that was observed for aerobic bacteria, 71- 97 % retention of total anaerobic bacteria was found after 25 cm trickling stretch. The elimination was improved to 86 - 99.7 % after a total trickling stretch of 125 cm depth. In March 2002 a high number of anaerobic bacteria was leaving the 5th segment of the segmented column after a total

trickling stretch of 125 cm depth, as observed above for aerobic bacteria (Table 4.3.3, Fig. 4.3.26).

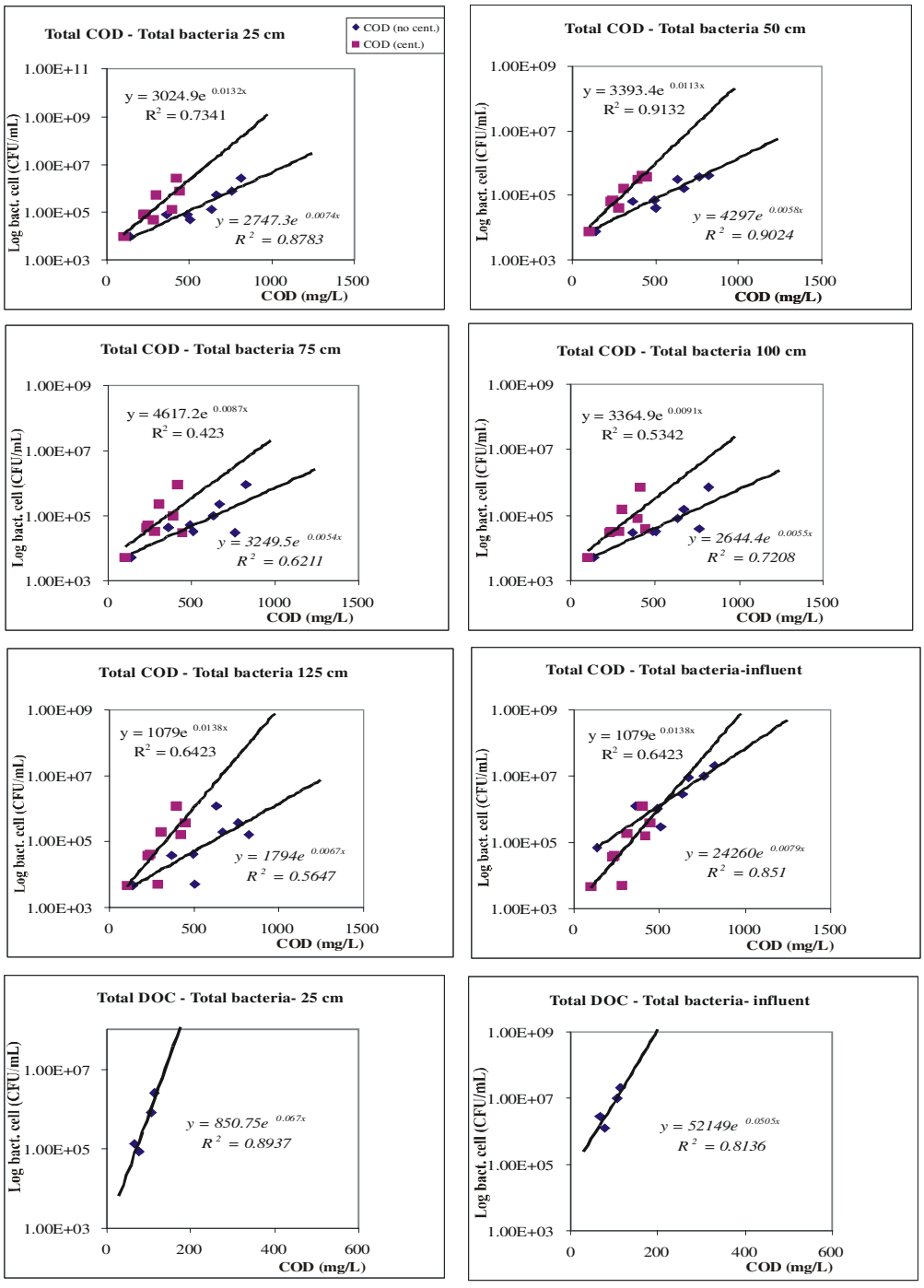


Figure 4.3.23 Correlation of total bacterial cells and COD, DOC at different depth in the segmented soil column during trickling of sewage.

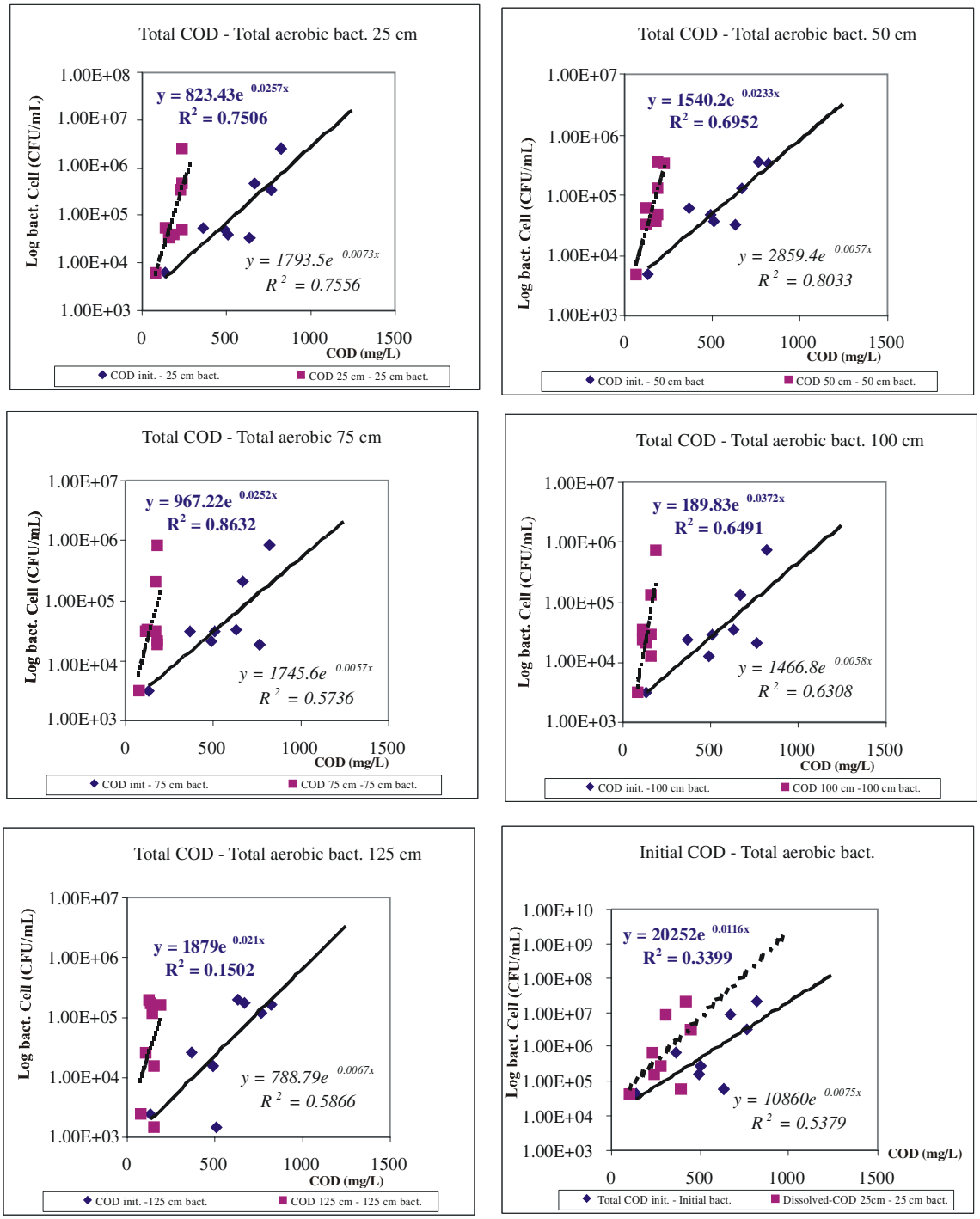


Figure 4.3.24 Correlation of aerobic bacterial numbers and COD at different depth in the segmented soil column during trickling of sewage.

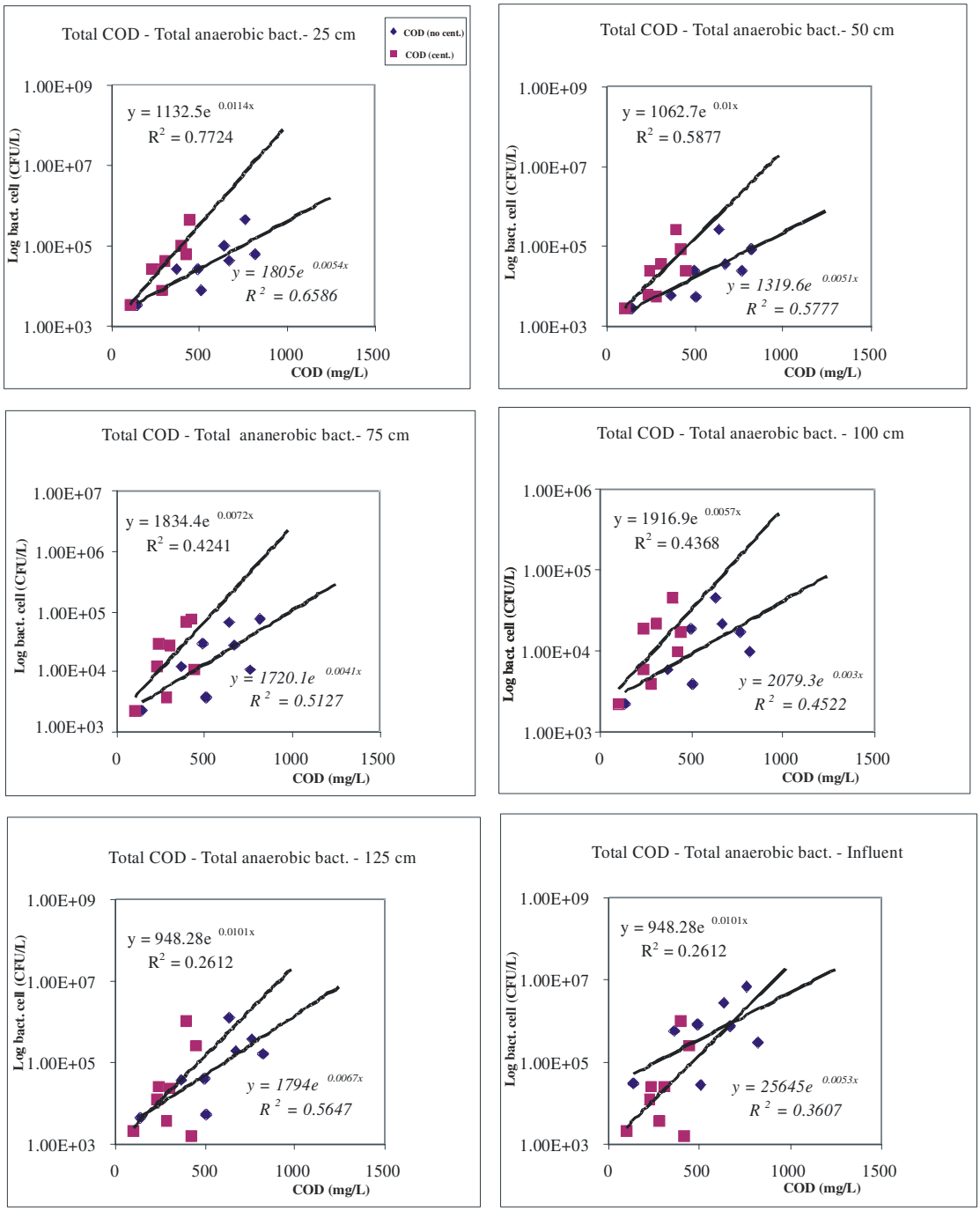


Figure 4.3.25 Correlation of total anaerobic bacteria and COD at different depth in the segmented soil column during trickling of sewage.

The total number of culturable bacteria (Figure 4.3.27): was removed in the range of 83 - 95 % during trickling of sewage through 25 cm sandy soil. The removal efficiency increased to 85 – 98 % after trickling for 125 cm .

Denitrification: The activity of denitrifying bacteria varied corresponding to the conditions inside the column (Table 4.3.3, Fig. 4.3.26). At 25 cm, elimination was 20 – 99 %, apparently depending on the availability of oxygen. In its absence almost all of the nitrate was denitrified, whereas in the presence of oxygen only little nitrate was denitrified. In the sub-columns 4 and 5 some oxygen was diffusing backwards from time to time from the effluent tube (if it was not ending below the surface of the effluent) and thus temporarily aerobic conditions were prevailing. Since more nitrate could be detected in the 4^{th} or 5^{th} segment than above it, enough oxygen for nitrification seemed to be available. Denitrifying bacteria were anoxic bacteria that could grow in the presence of nitrate.

Acetogenic and methane bacteria: The population density during trickling through 25 cm of sand decreased between 24 – 77 % and during the following 35 cm by another 20 % (Table 4.3.3 and Fig. 4.3.26). Subsequently, the population density of anaerobes increased again. At a depth of 75 until 125 cm no reduction of the population density of anaerobes was observed.

SRB: During June 2001-September 2001, the bacterial numbers of sulfate reducing bacteria in sewage were low, only 3 x 10^3 cell/mL. In a dry season (November-April) higher numbers were detected. The removal efficiency of sandy soil for sulfate reducers was only 26 – 60 % during June-July. It was low when compared to the time period from August - April, (Table 4.3.3, Fig. 4.3.27). The biggest decrease was found at 25 cm depth.

E. coli: *E. coli* was chosen as an indicator for pathogenic bacteria. The population density declined with depth. The majority of the bacteria were eliminated in the first segmented column and almost not further removal was seen in deeper layers (Table 4.3.3, Fig. 4.3.27). The removal efficiency was 81-97 %. However, even after 125 cm trickling stretch *E. coli* could be detected and might effect the quality of the groundwater, if used as a source of drinking water. In EU countries *E. coli* must be absent in drinking water.

Table 4.3.3 The % elimination of bacteria with depth of the segmented column.

Total bacteria	Jun 01	Jul 01	Aug 01	Sep 01	Nov 01	Jan 02	Mar 02	Apr 02
Influent								
Depth 25 cm	86.67	83.52	92.29	94.44	87.37	92.31	95.37	93.33
Depth 50 cm	89.44	85.82	93.10	98.23	97.92	96.29	89.59	94.54
Depth 75 cm	92.71	88.66	95.10	97.52	95.48	99.72	96.53	96.50
Depth 100 cm	92.64	89.11	96.87	98.32	96.40	99.63	97.23	97.56
Depth 125 cm	93.68	98.22	96.05	97.90	99.21	96.35	57.34	96.91
Anaerobic bact.	Jun 01	Jul 01	Aug 01	Sep 01	Nov 01	Jan 02	Mar 02	Apr 02
Influent								
Depth 25 cm	88.71	71.07	96.75	94.67	78.28	93.52	96.45	95.36
Depth 50 cm	90.81	79.46	97.06	95.48	71.38	99.63	90.53	98.91
Depth 75 cm	92.90	86.43	96.54	96.35	73.45	99.85	97.63	97.86
Depth 100 cm	92.74	85.89	97.77	97.21	96.55	99.76	98.39	98.93
Depth 125 cm	93.06	86.43	97.11	96.86	99.78	96.34	63.12	97.86
Aerobic bact.	Jun 01	Jul 01	Aug 01	Sep 01	Nov 01	Jan 02	Mar 02	Apr 02
Influent								
Depth 25 cm	85.12	84.81	68.98	94.41	87.50	89.70	45.05	91.64
Depth 50 cm	88.41	86.48	72.41	98.48	98.30	89.09	45.54	90.90
Depth 75 cm	92.56	88.89	87.59	97.63	95.80	99.45	45.05	95.37
Depth 100 cm	92.56	89.44	92.17	98.42	96.40	99.36	43.63	96.42
Depth 125 cm	94.15	99.44	90.48	97.99	99.20	96.36	-211.88	96.12
E. coli	Jun 01	Jul 01	Aug 01	Sep 01	Nov 01	Jan 02	Mar 02	Apr 02
Influent								
Depth 25 cm	95.83	58.33	46.24	86.73	80.63	85.24	94.52	93.14
Depth 50 cm	95.83	58.33	66.67	91.78	93.75	88.57	97.07	95.10
Depth 75 cm	95.83	58.33	98.92	99.56	95.00	98.05	95.24	96.08
Depth 100 cm	95.83	58.33	95.94	99.89	94.69	98.29	96.43	96.67
Depth 125 cm	95.83	58.33	81.18	99.93	97.81	98.41	97.14	96.27
SRB	Jun 01	Jul 01	Aug 01	Sep 01	Nov 01	Jan 02	Mar 02	Apr 02
Influent								
Depth 25 cm	0.00	26.89	88.91	83.72	99.26	99.26	99.49	86.74
Depth 50 cm	32.26	13.45	71.36	75.04	88.48	99.42	98.97	99.26
Depth 75 cm	29.03	30.25	89.07	86.67	99.43	99.81	98.97	98.89
Depth 100 cm	37.10	45.38	98.18	94.68	96.74	99.73	98.40	99.20
Depth 125 cm	59.19	39.50	99.34	96.08	99.30	99.39	98.23	99.65
AcB - MB bact.	Jun 01	Jul 01	Aug 01	Sep 01	Nov 01	Jan 02	Mar 02	Apr 02
Influent								
Depth 25 cm	30.95	28.87	68.06	51.00	76.79	23.81	36.62	27.38
Depth 50 cm	33.33	39.18	86.45	86.50	87.14	94.57	92.82	83.33
Depth 75 cm	32.62	35.05	79.03	87.00	88.39	91.55	90.99	91.67
Depth 100 cm	30.95	37.01	80.81	90.20	95.89	96.79	93.66	92.86
Depth 125 cm	34.29	42.27	89.84	92.20	92.50	95.95	96.48	93.57
Cellulose bact.	Jun 01	Jul 01	Aug 01	Sep 01	Nov 01	Jan 02	Mar 02	Apr 02
Influent								
Depth 25 cm	30.56	43.21	96.36	93.31	56.60	91.31	46.91	29.69
Depth 50 cm	38.89	72.14	97.06	95.71	69.81	94.10	49.38	42.19
Depth 75 cm	41.67	73.57	99.23	98.99	91.51	98.36	54.32	59.38
Depth 100 cm	33.33	69.64	99.72	99.42	90.38	98.90	95.80	98.89
Depth 125 cm	38.89	84.64	99.72	99.50	92.87	99.11	92.47	90.47
Denitrifying bact.	Jun 01	Jul 01	Aug 01	Sep 01	Nov 01	Jan 02	Mar 02	Apr 02
Influent								
Depth 25 cm	19.05	25.83	34.66	53.20	57.14	96.00	99.75	53.79
Depth 50 cm	33.33	36.67	-39.78	33.30	63.57	97.83	99.28	74.24
Depth 75 cm	38.10	40.00	47.89	52.07	92.86	99.50	99.94	96.82
Depth 100 cm	38.10	40.83	41.94	52.94	-78.57	98.97	99.63	90.91
Depth 125 cm	49.21	46.67	-3.39	43.95	98.00	51.67	90.88	84.09

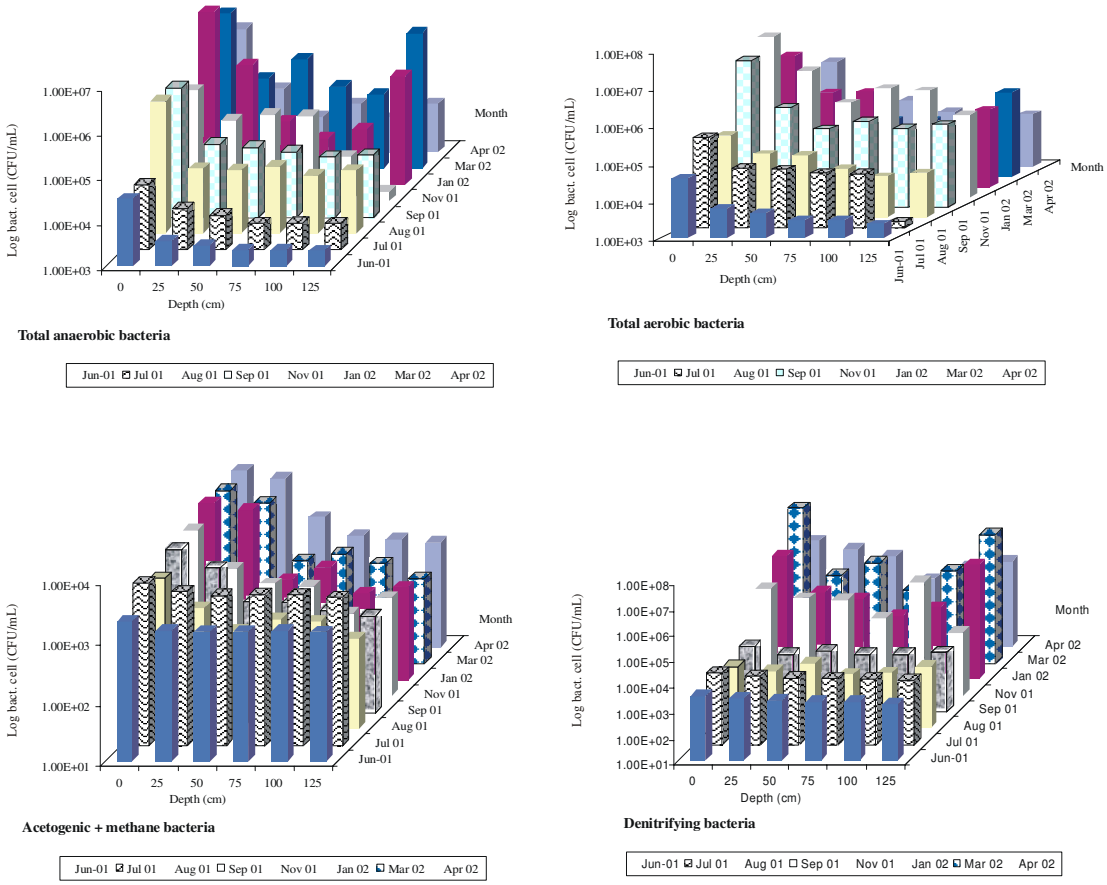


Figure 4.3.26 Changes of the population density within one year in the effluent of the 5 subcolumns of the segmented column.

Cellulose-degrading bacteria: The bacteria in this group were investigated cause in nature these bacteria contribute a major portion to the autochthonic population of soil. In sewage they were not a major portion of the population. The removal was 45 - 99 %. After 50 cm (1st and 2nd column), the numbers were rarely changed (Table 4.3.4 and Fig. 4.3.27).

♦Population of bacteria**:** The culturable population in each segment of the soil column was determined. If population densities were related to metabolic activities during trickling of sewage then the contribution of each of the sub-columns of the segmented column could be estimated (Table 4.3.4).

Aerobic bacteria contributed the highest proportion to the bacterial population in fresh trickling sewage. The proportion was more than 50 %, except in August, January and March. The proportion of anaerobic bacteria was slightly increasing with depth until 75 cm. After 75 cm the main population was aerobic. The SRB contributed only a few percent to the total culturable population (0.1 – 7 %), except for April, when they contributed 24 % to the total population. Denitrifying, acetogenic and methanogenic bacteria were present in a similar ratio. The total population contained 0.1 – 10 % *E. coli.*

Ratio aerobic : anaerobic bacteria*:* To predict the conditions inside the column, the numbers of aerobic and anaerobic bacteria were calculated (Fig. 4.3.28). The ratio of aerobic : anaerobic bacteria was > 1 during the observed time, except for August 2001, January 2002 and March 2002. The reaction conditions within the column apparently were not always the same. This result on bacterial numbers could well be related to chemical data, e.g. the increase of nitrifiers in the 4^{th} and 5^{th} segment of the segmented column, indicative for aerobic conditions.

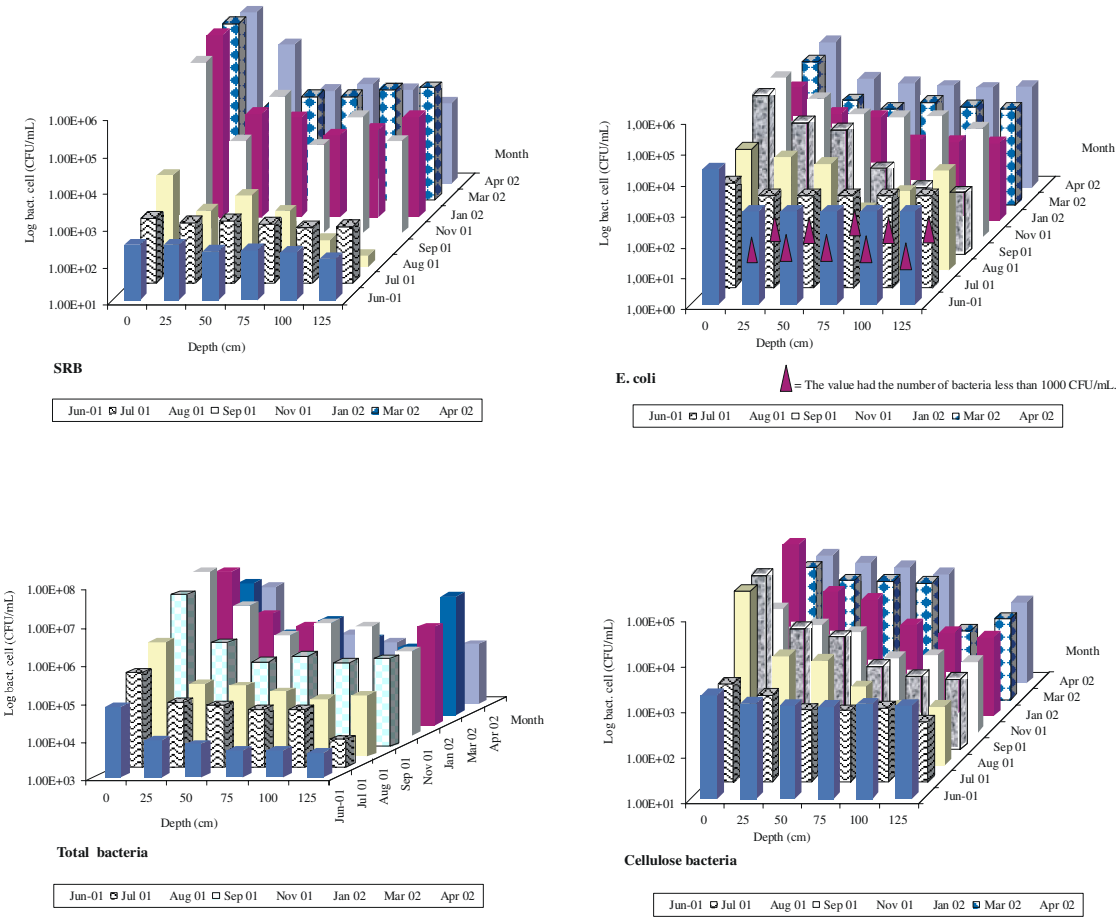


Figure 4.3.27 Changes of numbers of bacteria in trickling sewage with trickling depth within one year of the 5 subcolumns of the segmented column.

Table 4.3.4 The percentage of population of different bacteria in different depth in the segmented column.

Depth		Jun 01	Jul 01	Aug 01	Sep 01	Nov 01	Jan 02	Mar 02	Apr 02
Influent	Aerobic bact.	39.67	86.19	14.82	89.51	94.96	27.57	1.31	35.49
	Anaerobic bact.	29.99	8.94	77.34	8.21	1.38	59.31	60.80	29.66
	Denitrifying bact.	3..05	1.92	0.22	0.04	0.66	5.01	22.64	6.99
	Cellulose bact.	1.74	0.45	6.25	0.71	0.03	0.51	0.17	0.34
	AcB-MB	2.03	1.55	0.28	0.05	0.03	0.07	0.15	0.44
	SRB	0.30	0.19	0.27	0.03	2.18	7.35	14.02	24.37
	E. coli	23.22	0.77	0.83	1.44	0.76	0.18	0.91	2.70
	Total bacteria	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
25 cm	Aerobic bact.	37.75	69.22	57.18	87.33	93.95	40.22	19.09	25.68
	Anaerobic bact.	21.66	13.68	31.21	7.63	2.37	54.42	57.33	11.92
	Denitrifying bact.	15.78	7.51	1.75	0.31	2.25	2.84	15.31	27.97
	Cellulose bact.	7.74	1.34	2.83	0.83	0.09	0.63	2.47	2.06
	AcB - MB bact.	8..97	5.82	1.10	0.45	0.05	0.76	2.58	2.80
	SRB	1.92	0.73	0.37	0.10	0.13	0.77	1.91	27.97
	E. coli	6.19	1.69	5.55	3.35	1.16	0.37	1.32	1.60
	Total bacteria	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
50 cm	Aerobic bact.	35.42	71.70	56.49	70.81	63.04	87.69	8.50	54.42
	Anaerobic bact.	21.25	11.30	31.39	19.30	15.39	6.33	68.76	5.44
	Denitrifying bact.	15.66	7.46	4.17	1.30	9.46	3.17	19.52	30.33
	Cellulose bact.	8.20	0.77	2.54	1.59	0.30	0.88	1.06	3.30
	AcB - MB bact.	10.44	5.80	0.52	0.37	0.13	0.11	0.13	1.25
	SRB	1.57	1.01	1.07	0.44	9.83	1.24	1.72	3.03
	E. coli	7.46	1.96	3.82	6.18	1.85	0.58	0.32	2.23
	Total bacteria	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
75 cm	Aerobic bact.	28.02	70.86	38.52	86.11	89.49	50.25	27.82	53.82
	Anaerobic bact.	20.21	8.98	56.10	12.15	8.20	30.71	55.73	20.83
	Denitrifying bact.	17.91	8.50	2.36	0.73	1.07	8.38	5.57	7.29
	Cellulose bact.	9.65	0.87	1.01	0.29	0.05	2.79	3.09	4.51
	AcB -MB bact.	13.00	7.44	1.22	0.28	0.07	1.98	0.53	1.22
	SRB	2.02	0.98	0.62	0.18	0.28	4.75	5.57	8.85
	E. coli	9.19	2.36	0.19	0.26	0.85	1.14	1.67	3.47
	Total bacteria	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
100 cm	Aerobic bact.	27.49	69.84	37.22	84.50	71.70	43.84	26.07	49.93
	Anaerobic bact.	20.28	9.68	55.26	13.69	1.00	35.49	34.72	12.48
	Denitrifying bact.	17.58	8.70	4.02	1.05	24.89	12.94	29.53	24.96
	Cellulose bact.	10.82	1.04	0.56	0.25	0.05	1.40	0.26	0.15
	AcB - MB bact.	13.07	7.49	1.70	0.31	0.02	0.56	0.34	1.25
	SRB	1.76	0.80	0.16	0.11	1.49	5.01	7.94	7.70
	E. coli	9.01	2.45	1.08	0.09	0.85	0.75	1.14	3.54
	Total bacteria	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
125 cm	Aerobic bact.	24.60	11.65	34.66	86.17	93.05	17.74	8.59	40.85
	Anaerobic bact.	22.04	29.51	54.85	12.35	0.96	38.43	47.26	18.85
	Denitrifying bact.	16.40	24.85	5.48	1.01	1.63	42.86	43.53	32.99
	Cellulose bact.	11.27	1.67	0.43	0.17	0.22	0.08	0.03	0.96
	AcB - MB bact.	14.14	21.75	0.69	0.20	0.24	0.05	0.01	0.85
	SRB	1.30	2.80	0.04	0.06	1.86	0.80	0.52	2.51
	E. coli	10.25	7.77	3.84	0.05	2.04	0.05	0.05	2.99
	Total bacteria	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

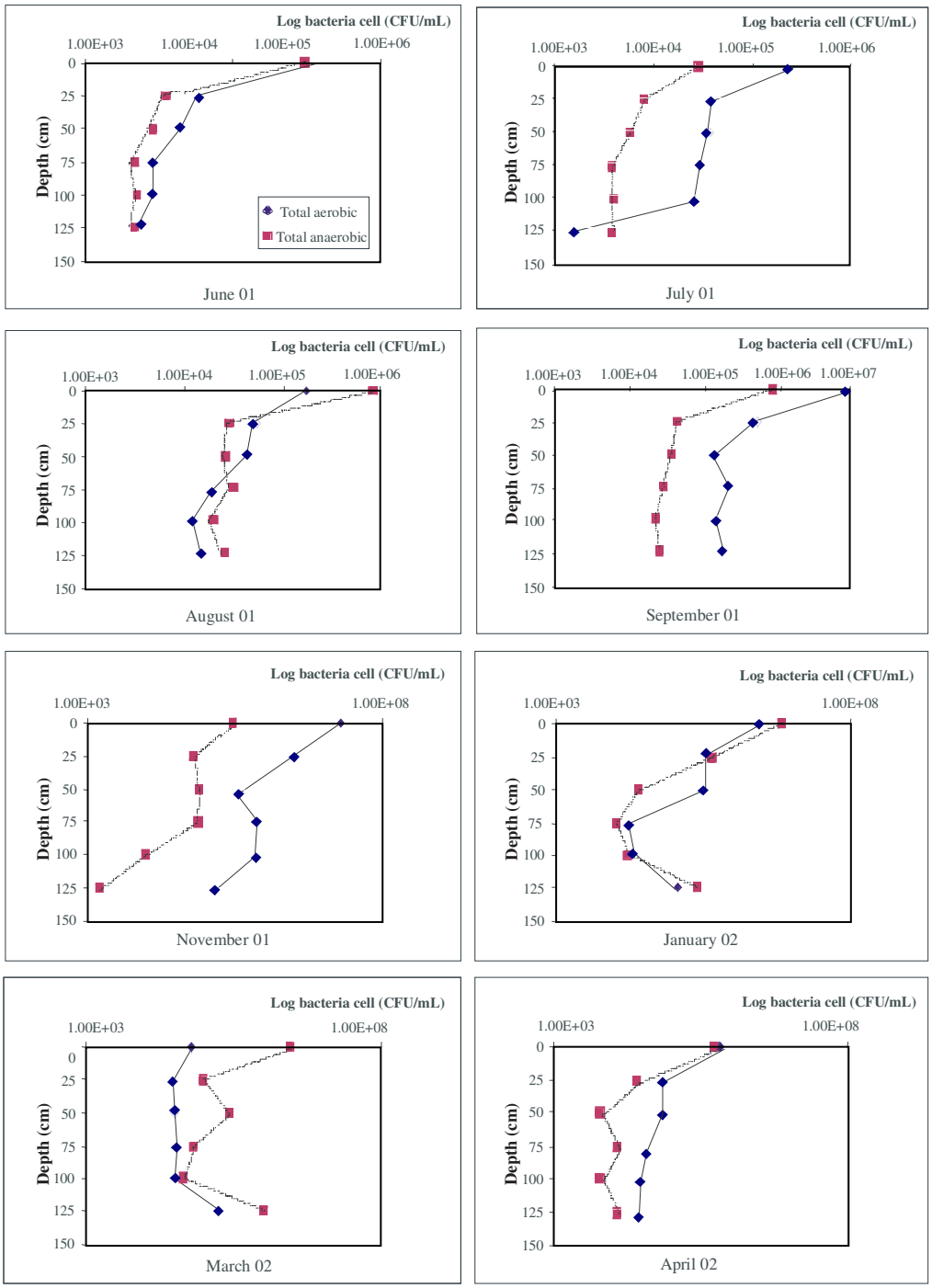


Figure 4.3.28 The ratio of aerobic-to-anaerobic bacteria as an indicator to predict the growth conditions inside the column.

4.3.2.2. Results of trickling of sewage through a one-piece 125 cm soil column

Two columns were investigated that were run for a different time span. In June 2001, the soil column IBAI was run for 6 months and the soil column IBA2 was run for 14 months already. From this time on, the columns were investigated during the next 8 months.

The bacterial number: In effluent it has been found that, ca. 90 % of all aerobic and anaerobic bacteria have been eliminated (Table 4.3.5). The different time span of both columns had no influence on bacterial removal.

E. coli: These bacteria served as indicators for the removal of pathogens. They were still found after 125 cm of sewer trickling. The percent removal was 57 - 99 % (Table 4.3.5).

SRB: The removal of SRB during June 2001 and September 2001 was only 25 - 53 % but increased to 80 - 97 % elimination from November 2001 until January 2002 (Table 4.3.5).

Table 4.3.5 Bacterial cell numbers in effluent after trickling through 125 cm soil and % removal.

Total cell	Jun. 01	% Elimi.	Jul. 01	% Elimi.	Aug. 01	% Elimi.	Sep. 01	% Elimi.	Nov. 01	% Elimi.	Jan. 02	% Elimi.
Influent	7.20E+04		2.98E+05		1.03E+06		9.30E+06		2.03E+07		1.04E+07	
IBA1-effluent	5.05E+03	*92.99*	7.25E+03	*97.57*	5.35E+04	*94.82*	6.29E+05	*93.24*	2.91E+05	*98.57*	4.90E+05	*95.29*
IBA2-effluent	4.20E+03	*94.17*	8.45E+03	*97.16*	4.32E+04	*95.81*	1.42E+05	*98.47*	1.94E+06	*90.44*	3.10E+05	*97.02*
Aerobic bacteria												
Influent	4.10E+04		2.70E+05		1.66E+05		8.52E+06		2.00E+07		3.30E+06	
IBA1-effluent	2.00E+03	*95.12*	3.25E+03	*98.80*	2.10E+04	*87.35*	5.96E+05	*93.00*	2.80E+05	*98.60*	2.30E+05	*93.03*
IBA2-effluent	1.50E+03	*96.34*	2.00E+03	*99.26*	2.09E+04	*87.41*	1.13E+05	*98.67*	1.80E+06	*91.00*	1.80E+05	*94.55*
E.coli												
Influent	1.06E+04		5.70E+03		2.80E+03		<1000		6.10E+03		8.00E+03	
IBA1-effluent	4.60E+03	*56.60*	1.45E+03	*74.56*	<1000	*n.d.*	<1000	*n.d.*	3.00E+01	*99.51*	2.60E+03	*67.50*
IBA2-effluent	1.50E+03	*85.85*	1.15E+03	*79.82*	<1000	*n.d.*	<1000	*n.d.*	2.50E+03	*59.02*	1.30E+03	*83.75*
Anaerobic bact.												
influent	3.10E+04		2.80E+04		8.66E+05		7.81E+05		2.90E+05		7.10E+06	
IBA1-effluent	3.05E+03	*90.16*	4.00E+03	*85.71*	3.25E+04	*96.25*	3.25E+04	*95.84*	1.10E+04	*96.21*	2.60E+05	*96.34*
IBA2-effluent	2.70E+03	*91.29*	6.45E+03	*76.96*	2.23E+04	*97.43*	2.90E+04	*96.29*	1.40E+05	*51.72*	1.30E+05	*98.17*
Denitrifying bact.												
Influent	4.05E+03		6.95E+03		3.30E+03		3.53E+03		7.20E+04		7.30E+04	
IBA1-effluent	1.85E+03	*54.32*	1.25E+03	*82.01*	2.00E+03	*39.39*	2.50E+03	*29.18*	1.10E+04	*84.72*	6.40E+03	*91.23*
IBA2-effluent	1.95E+03	*51.85*	1.01E+03	*85.47*	2.00E+03	*39.39*	2.48E+03	*29.75*	2.10E+04	*70.83*	2.40E+03	*96.71*
Cellulose bact.												
Influent	2.75E+03		1.10E+03		1.45E+03		5.00E+03			*n.d.*		*n.d.*
IBA1-effluent	1.10E+03	*60.00*	6.60E+02	*40.00*	1.25E+03	*13.79*	1.30E+03	*74.00*		*n.d.*		*n.d.*
IBA2-effluent	1.15E+03	*58.18*	6.00E+02	*45.45*	1.31E+03	*9.66*	1.01E+03	*79.80*		*n.d.*		*n.d.*
Aceto - MB bact.												
Influent	2.15E+03		5.95E+03		1.90E+03		4.45E+03					
IBA1-effluent	1.30E+03	*39.53*	3.25E+03	*45.38*	2.35E+03	*-23.68*	3.15E+03	*29.21*		*n.d.*		*n.d.*
IBA2-effluent	1.30E+03	*39.53*	3.65E+03	*38.66*	3.30E+03	*-73.68*	3.11E+03	*30.11*		*n.d.*		*n.d.*
Total cell												
Influent	3.50E+02		6.50E+02		2.50E+03		2.90E+03		2.20E+05		1.90E+04	
IBA1-effluent	2.10E+02	*40.00*	4.90E+02	*24.62*	1.34E+03	*46.40*	1.38E+03	*52.41*	2.20E+02	*99.90*	1.16E+03	*93.89*
IBA2-effluent	1.90E+02	*45.71*	4.60E+02	*29.23*	1.17E+03	*53.20*	1.56E+03	*46.21*	2.80E+04	*87.27*	3.70E+03	*80.53*

4.3.3 The sulfate reduction rate (SRR) in soil of IBA2

The potential of sulfate reduction in sandy soil of column IBA2 was examined in 120 ml serum bottles by incubating soil samples from the column in Freshwater medium II which had lactate as a carbon source and 10 mM sulfate as an electron source (n = 2), at 20 ^{o}C under dark atmosphere.

The sulfate reduction occured mainly from the 2nd week to the 4th week of incubation. After that the residual sulfate concentration was no longer decreasing. The SRR was calculated by using data during log phase growth. The result was that the SRR was decreasing with the depth of the column (Fig. 4.3.29). The highest SRR was found in the sludge layer on top of the sand, 23.5 µM/hr and it decreased to 1.2 µM/hr at a depth of 110 - 115 cm. Although there were higher numbers of SRB at 30 - 50 cm, they contributed less to the SRR as compared to the lower numbers of sulfate reducing bacteria at 1 - 20 cm depth with a higher SSR. According to this result, it can be assumed that the highly active sulfate reducing bacteria were found at a depth of 1 - 20 cm, due to the high amount of organic matter and sulfate.

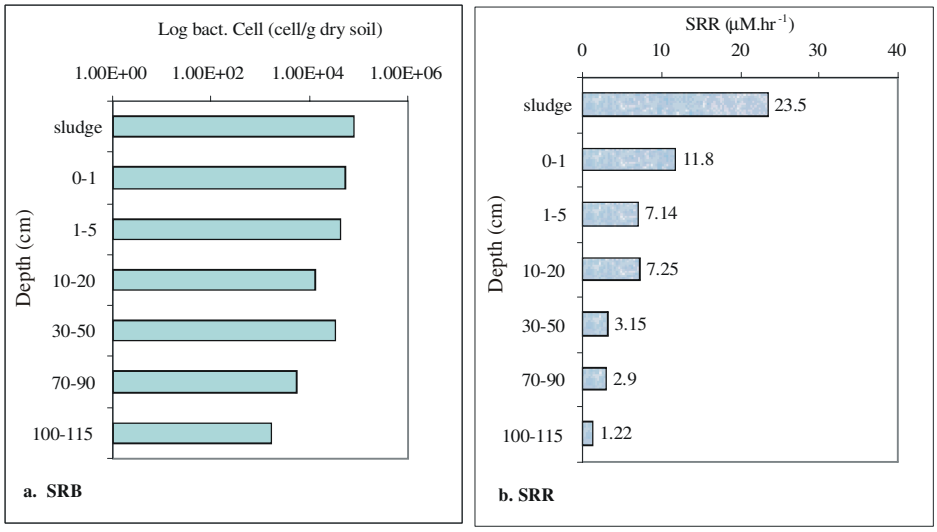


Figure 4.3.29 Bacterial numbers of SRB (a) and SRR (b) in soil of IBA2 column.

5 Discussion

5.1 Batch culture experiments

Investigations to determine sulfate reduction rate

Sulfate reducing bacteria were found in different environments including sludge or soil under leaky sewers. Almost all of them belong to the incompletely oxidizing mesophilic group of SRB. Under suitable conditions like high sulfate concentration and/or strictly anaerobic atmosphere, sulfate reducers will significantly contribute to carbon removal (Kleikemper et al., 2002). Sulfate reduction is one of main bacterial activities in many reduced environments, such as marine sediments (Jϕrgenson, 1977), anaerobic sludge (Manz et al., 1998) and contaminated aquifers (Lovley, 1997).

At certain conditions SRB can outcompete methane bacteria. The successful competition of sulfate reducers with methane bacteria for mineralization of simple organic matter has been reported (Kazumi and Capone, 1995). However when sulfate reducing enrichment cultures from contaminated soil samples of Mannheim were inhibited with bromoethane sulfonic acid (BESA) as an inhibitor of methane bacteria the sulfate reduction rate or % sulfate reduction was the same as compared with the control (Table 5.1.1). Methane gas was produced in the control. The addition of BESA seemed not significantly favor the activity of sulfate-reducing bacteria. High concentrations of sulfate or high sulfide concentrations may become toxic for methane bacteria (Isa, 1986). Furthermore sulfate reducers often had a higher affinity for different carbon sources and thus were more competitive than methane bacteria.

The temperature influenced bacterial growth and the velocity of sulfate reduction in enrichment cultures and pure cultures (Table 5.1.1). At lower temperature conversion rates decreased as predicted by the Vant-Hoff-law. Pfennig (1986) reported that the Q10 for sulfate reduction in marine sediments ranged between 2.0 and 3.9 (i.e. if the growth temperature was increased by 10 ^{o}C this stimulated the sulfate reduction rates 2 until 3.9 fold).

Table 5.1.1 Comparison of sulfate reduction rates by enrichment cultures from sewage sludge or contaminated soil

Test	Temp. (^oC)	C-source	SRR ($\mu mol/L \cdot hr$)	COD : SO_4^{2-} mg/L : 1 mM	Remark
4.1.1	4 15 37	Acetate, butyrate	8.6 11.7 21.7	45.4 51.9 251.9	Sludge of Neureut
4.1.2	15	Lactate	22.4 13.9	38.2 46.3	+BESA, Soil samples of Mannheim -BESA
	15	Acetate	10.7 11.0	63.1 72.3	+BESA, Soil samples of Mannheim -BESA
	15	Butyrate	8.7 8.7	41.2 56.6	+BESA, Soil samples of Mannheim -BESA
4.1.2	37	Lactate	53.8 34.7	36.4 31.2	+BESA, Soil samples of Mannheim -BESA
	37	Acetate	12.6 11.6	51.3 74.2	+BESA, Soil samples of Mannheim -BESA
	37	Butyrate	23.3 20.2	45.6 46.7	+BESA, Soil samples of Mannheim -BESA

Carbon sources. The sulfate reducing enrichment cultures utilized butyrate as a carbon source and accumulated acetate due to incomplete oxidation. The most favourite carbon source of enrichment culture in soil of Mannheim was lactate in both assays (with or without BESA) follow by butyrate and acetate. Kleikemper et al. (2002) assumed that the dominant groups of SRB were the incomplete oxidizing SRB. The freshwater SRB were able to utilize various carbon sources. Fukai (2000) reported that the activity of SRB and methane bacteria showed coexistence at 1.2 to 2.7 mM of sulfate. Sulfate did not show any inhibiting effect on methane bacteria but SRB utilized propionate, not acetate and hydrogen, which would be substrate for both groups of bacteria. The addition of molybdate did not enhance the activity of methane bacteria.

The stoichiometric ratio of COD/sulfate, DOC/sulfate and the *SRR* for contaminated soil and sewage sludge were calculated and are shown in Table 5.1.1. From the result it was concluded that the SRB belonged to the incompletely oxidizing group except for those in the soil sample of Mannheim, which showed the activity of completely oxidizing SRB. The ratio of carbon source to sulfate was higher than theoretically expected due to the enrichment culture, where the SRB had to share the

carbon source for growth with other bacteria. The data of stoichiometries or SRR of previous reports are presented in Table 5.1.2 and 5.1.3.

Table 5.1.2 The stoichiometric ratio of carbon source and sulfate in contaminated groundwater

Carbon source mM	Actual mM- C : mM sulfate	Stoichiometric of incomplete oxidation mMC : mM Sulfate	Stoichiometric of complete oxidation mMC : mM Sulfate	Remark	Reference
Propionate	1.51	1.33	0.57	Groundwater	Kleikemper et al (2002)
Butyrate	1.63	0.66	0.40	contaminated	
Lactate	0.97	2.00	0.66	petroleum	
Acetate	3.49	-	1.00	hydrocarbon	

Table 5.1.3 The sulfate reduction rates by SRB in different environments

Source of SRB	C-source	SRR (µmol/L·hr)	Reference
Salt pond mats of Guerrero Negro		126 - 270	Canfield and DesMarais (1991)
Temperate intertidal mats		13 - 26	Uria-Nickelson et al. (1993)
Stromatlite mat	Acetate	127	Visscher et al. (1999)
Salt pond mat	Acetate	201	
Salt march mat	Acetate	184	
Stromatlite mat	Lactate	79	
Salt pond mat	Lactate	153	
Salt march mat	Lactate	177	
Stromatlite mat	Ethanol	64	
Sediment slurry	Lactate	40.83	Vester and Ingvorsen (1998)
Kysing Fjord slurry	Lactate	67.92	
activated sludge	Lactate	39.16	
Desulfobulbus propionicus	DSM medium 194	14.17	
Control digestor	SO_4^{2-} 0.1 mM	13 µmol/L·day	Peterson and Ahring (1992)
Sulfate-adapted digestor	SO_4^{2-} 0.3 mM	17 µmol/L·day	
	SO_4^{2-} 0.5 mM	17 µmol/L·day	
	SO_4^{2-} 0.2 mM	580 µmol/L·day	
	SO_4^{2-} 0.6 mM	980 µmol/L·day	
	SO_4^{2-} 1.0 mM	910 µmol/L·day	
Thermodesulfobacterium sp.	µm/g dry cell hr^{-1}	96 ± 1.1	Sonne-Hansen et al (1999)
Thermodesulfovibrio sp.	µm/g dry cell hr^{-1}	59 ± 7	

5.1.4 Growth of sulfate reducing bacteria under stress conditions: Molybdate as a stress substrate or inhibitor.

In previous investigation the conditions that completely prevented activity of sulfate reducing bacteria were investigated to avoid corrosion of pipelines. With a purpose to find a suitable chemical that effectively inhibited SRB and had no effect on the environment molybdate was found and was recommended as an inhibiting chemical for SRB (Oremland and Capone, 1988). Molybdate can be found in bacterial cells as a compound of coenzymes and thus should be a minor component of bacterial media. Molybdate should not have a side effect on the environment. Recently, anthraquinones were introduced as highly effective inhibitors for SRB. Aim of this research was to find out the sulfate reduction rate or growth of bacteria, under stress conditions of samples from contaminated soil under leaky sewers. Low concentrations of molybdate were used instead of anthraquinones to distrub sulfate reduction. Molybdate may be a geological or anthropogenic compound of the respective habitat. Inhibiting concentration of molybdate had to be determined for the respective culture conditions.

In earlier reports on inhibition of sulfate reduction by molybdate different concentrations in the range of 1 - 50 mM were applied for pure or enrichment cultures (Taylor and Oremland, 1979; Yadav and Archer, 1989; Lie et al., 1999). No sulfate reduction was obtained at > 1 mM Mo with our samples, due to a complete inhibition of sulfate reduction. When ≤ 0.1 mM of molybdate was added it partially inhibited sulfate reduction and growth of sulfate reducers. 66.7 % of sulfate was degraded in a sample containing 0.1 mM molybdate compared to a control assay where 88.4 % of the sulfate were reduced. This was similarly observed by Chen et al. (1998), where 0.1 mM of molybdate inhibited sulfate reduction partially, but 5 mM of molybdate led to a complete inhibition. Contrary, Lovely et al. (1982) could not find a toxic effect of ~ 2 mM molybdate. Ye et al. (1999) reported that 1 mM of molybdate had partially inhibited dechlorinating SRB but ≤ 0.5 mM molybdate did not cause any inhibition.

The inhibitory effects of molybdate may be delayed for up to 4 days due to restrictive uptake of molybdate into cells. In our tests it was shown that molybdate had little effect on resting cells, but efficiently inhibited SRB that were in the log phase of growth. This result was consistent with the pioneer research of Taylor and Oremland (1979). They commented that bacteria at log phase growth have less resistance to

inhibitors than stationary stage cells. The inhibition actively interfered with metabolic reduction. In assays with sulfate reducing consortia from soil or slurry, molybdate had less inhibiting capacity than in pure culture studies. Since clay or soil particles absorbed part of the molybdate it was no longer bioavailable (Phelan and Mattigod, 1984).

The effect of molybdate on the growth of pure cultures of *D. desulfuricans* was studied by adding 0.1 mM of sodium molybdate. This amount of molybdate was slightly inhibiting bacterial growth as previously shown by Hsieh et al. (1998). Less biomass was formed and less sulfate was reduced due to a depletion of the ATP-pool.

Normally, sulfate was taken up by single cells of bacteria, reacted with ATP sulfurylase and was converted to APS. There sulfate was reduced to sulfite and to sulfide. Sulfide was released into the medium.

Molybdate in samples competed with sulfate for attachment at the active site of ATP sulfurylase, resulting of a non stable analogue-AMP complex which hydrolyses to AP-molybdate and pyrophosphate. ATP in the cells will be depleted. At the same time, intermediate products such as sulfite will increase (Chen et al, 1998; Taylor and Oremland, 1979). In non-inhibited assay of *D. desulfuricans* IC1 (Lie et al, 1999), 80 % remaining ATP (control) were found in cell suspensions compared to only 2 % ATP in suspension in the presence of 5 mM molybdate. Molybdate had also effected the bacterial size, from 3.5 ± 0.6 µm (without molybdate) to 3.8 ± 0.9 µm (with molybdate) (Lie et al, 1999). In our cultures no clearly change of the bacterial size was observed.

According to our results, 0.1 mM molybdate had inhibited the sulfate reduction rate at both temperatures (15 and 37 ^{o}C). However the temperature itself should influence only the velocity of sulfate reduction. At 15 ^{o}C in the assay with addition of molybdate the percentage of sulfate reduction was approximate 1.53 fold less than without molybdate. At 37 ^{o}C no different percentage of sulfate reduction was seen.

Consistent with the research of Chen et al. (1998), the quantity of orange coloration between sulfate and molybdate was related to the concentration of molybdate in the assay and was slightly affected by the OD_{600}. According to results of Lie et al. (1999) addition of molybdate in the range of 8 - 15 mM had no effect on the coloration. In our assays in the presence of 0.1 mM molybdate a pale yellow coloration appeared but no nuisance effect on the OD_{578} measurement was observed. SRB in

enrichment cultures were less resistant to molybdate than *D. desulfuricans* in pure culture if incubated at the same concentration of molybdate (0.1 mM). In the pure cultures, molybdate had influenced only the decline of the growth rates but not the percentage of sulfate reduction. In enrichment cultures from soil, molybdate affected the velocity of reactions and the percentage of sulfate reduction (Table 4.1.3) at each sulfate concentration. The presence of 0.1 mM molybdate had however no effect on other bacteria. As reported by Yadah and Archer (1989), 10 mM of molybdate was inhibitory for both SRB and methane bacteria. The addition of 0.1 mM molybdate had a significant influence on the growth of lactate consuming SRB if the bacteria were in the lag phase or beginning log phase. The inhibiting effect of molybdate was less pronounced in the lag phase than in the log phase cultures. If 0.1 mM molybdate was added to cultures of the propionate consuming SRB group, molybdate caused about 2.3 fold (4.8 - 5 mM sulfate), 3.0 fold (8.9 - 9.5 mM sulfate) and 4.4 fold (19.1 - 19.9 mM sulfate) less sulfate reduction than in control. This means that under stress condition in enrichment cultures growth of SRB may occur but with a 2.3 - 4.4 fold slower sulfate reduction rate than in the assay without molybdate.

The sulfate reducing bacteria can be divided into 2 groups due to the consumption of different electron donors. When SRB were grown with lactate as the carbon source they had to compete with other bacteria for the carbon source, especially with propionate forming bacteria. The SRB had to compete with all other anaerobic lactate consuming bacteria. This was indicated by a stoichiometric ratio of sulfate and lactate that was higher than in theory (data in Table 5.1.4). The enrichment cultures belonged to the incomplete SRB since the stoichiometric ratio is far from complete oxidation that of completely oxidizing sulfates reducers.

In the enrichment cultures lactate was rapidly degraded in only two days, but the quantity of sulfate was not dramatically decreased. By theory, 2 mM of lactate required one mole of sulfate for conversion of lactate to acetate. The rapid consumption of lactate could be catalysed by PFB (propionate fermented bacteria) and/or other bacteria. The main carbon source for SRB in these assays was thus propionate. The quantities of propionate decreased in correlation with the amount of sulfate that was reduced.

Table 5.1.4 Stoichiometry of SRB from contaminated soil in Rastatt (Test 4)

SO_4^{2-} (mM)	% SO_4^{2-} reduction	Carbon	Actual ratio	Remark
4.8	21.1 % 58.9 %	Lactate Propionate	29.5 1.8	Without molybdate
5.0	18.5 % 25.9 %	Lactate Propionate	33.2 5.9	With molybdate
8.8	18.2 % 72.4 %	Lactate Propionate	19.0 1.6	Without molybdate
9.5	10.4 % 24.6 %	Lactate Propionate	31.3 nc	With molybdate
19.1	5 % 85 %	Lactate Propionate	32.0 1.2	Without molybdate
19.9	1.4 % 19.5 %	Lactate Propionate	110.4 3.9	With molybdate
Theoretical ratio		*Lactate* *Propionate*	*0.7* *0.6*	*Complete SRB*
Theoretical ratio		*Lactate* *Propionate*	*2.0* *1.3*	*Incomplete SRB*

The bacteria, which participated in the degradation of originally available or intermediary formed carbon sources in the assay, belonged to four groups: lactate-utilizing sulfate-reducing bacteria (SRB) in a foodchain with methane bacteria, propionate forming bacteria (PFB) and propionate degrading bacteria like *Syntrophobacter*, in syntrophy with methane bacteria. Some SRB may have been growing syntrophically competing with other bacterial groups by sharing the carbon sources. The initial bacteria were SRB and PFB. PFB were converting most of the lactate to propionate and SRB, which utilized the residual lactate decarboxylated it to acetate. Following must a major population of SRB, which consumes propionate and released acetate as an end product. No further degradation of acetate by sulfate reducing bacteria was found, if the amount of sulfate was considered. The SRB belonged to *Desulfobulbus* sp. and *Desulfovibrio* sp. due to the fact that they were using propionate as a carbon source.

Methane bacteria utilized the accumulated acetate and may have competed for $CO_2 + H_2$ with the sulfate reducers. Methane gas was detected in both samples (with or without molybdate). After extended incubation of cultures methane bacteria played a major role according to increasing percentages of methane gas.

Under stress conditions, the pure cultures of SRB were more resistantly degrading sulfate than enrichment cultures. In pure cultures, molybdate reduced the sulfate reduction rate at 37 ^{o}C and slightly affected the sulfate reduction rate at 15 ^{o}C.

In enrichment cultures, molybdate had more pronounced inhibition effect. It may have been not only the inhibition effect of molybdate but SRB in enrichment cultures must compete with other bacteria for the electron donor. 0.1 mM of molybdate is recommended as an approximate concentration to study growth of SRB under stress conditions.

5.1.5-5.1.6 Batch cultures, which used organic matter from domestic wastewater as a carbon source for sulfate reduction.

Result of sulfate reduction in enrichment cultures from natural sources like contaminated soil (sand column or soil under leaking sewers) demonstrate the potential of mineralization of organic matter by sulfate reducers in domestic wastewater. The carbon sources in wastewater were utilized by these bacteria. However, a small part of this organic matter cannot be degraded, since it is not biodegradable or because the HRT in the experiment was too short. Alternatively, the presence of inhibiting substances or the complex structures of humic acid-like residues may have prevented degradation. Normally these SRB grow with a different type of carbon sources. The potential of these bacteria to utilize organic matter in domestic wastewater was even lower in continuous cultures (data in part 4.2). For sulfate reduction anaerobic conditions must be maintained. Many previous reports were mainly focused on industrial wastewater sources, which contain more sulfate than domestic wastewater. However SRB were accounted for up to 50 % of the mineralization of organic matter even in aerobic wastewater treatment (Kühl and Jorgensen, 1992). This must be a matter of sludge floc formation, which will provide anaerobic condition immediately below the surface.

Part 5.2 Fed batch and continuous cultures of sulfate reducers enriched from soil

Only 50 % of initial sulfate concentration, which was feed to pure or enrichment cultures in a fed batch culture were degraded. Several reasons, such as a high concentration of H_2S, residual O_2 in influent, variation of the room temperature (24.5 – 30 ^oC) wash out of biomass may cause restricted sulfate usage. The H_2S (part 4.2.3) in culture was 67 - 112 mg/L. Although the gas phases in reactors were continuously purged with N_2 gas, high concentrations of H_2S accumulated. Huezeler (1990) reported that 380 mg/L of H_2S at pH 7.7 - 7.8 has not dangerous effect on bacterial growth. But most previous reports mentioned that around 50 - 200 mg/L of H_2S has an influence on growth of these bacteria.

Under continuous culture conditions the SRB were capable to utilize organic matter in domestic wastewater as a carbon source for growth. According to result of Agrawal et al. (1997) lactate was selected as a carbon source in this research. Apparently lactate utilizing SRB were the dominant group of bacteria in wastewater followed by propionate utilizing SRB (Schonheit et al., 1982; Isa et al. 1986).

The addition of higher sulfate concentrations than stoichiometrically required was selected with the purpose to repress growth of methanogens like mentioned in earlier investigations (Ingvorsen et al., 1984; Fang et al., 1994; Goosen et al., 1988). In fed batch cultures only 0.5 - 1 % of methane gas were detected. According to results part 4.2.3 SRB could utilize organic matter from domestic wastewater as a carbon source. This was evidence that a variety of carbon sources must be suitable for sulfate reducers and that they presumably can use different carbon sources consecutively.

Previous investigators (Harada, 1994; Hickey et al., 1991; Fang et al., 1994; Harada, 1990; Fang, 1995; Dolfing, 1985) informed that the density of SRB and their activities was greater in granules that developed on the low strength wastewater. SRB might also play a significant role in the degradation of various organic contaminants, which are present in sewage. Sulfate reducers produced sulfide as end product, which precipitate with metal ions to form metallic sulfide (26) and prevent hazard of heavy metal form environment.

$$Fe^{2+} + HS^{-} \rightarrow FeS + H^{-} \qquad (26)$$

5.3 Biofilm formation on sand particles and the distribution of suspended bacteria in the water phase of the non-segmented and segmented soil column during trickling of sewage.

A. Elimination of coliforms or E. coli as pathogenic bacteria that affected the quality of groundwater:

Leaky sewer pipes or leaking sewage endangers the quality of groundwater. Since groundwater is a source of drinking water contaminated groundwater bears the risks of diseases. To study transformation processes during the passage of sewage through the unsaturated underground model sand columns were run with trickling sewage. Several parameters were analysed and checked for functioning as tracers to predict a danger of this process. Commonly bacteria were used to study contamination at sites such as leaky sewer. Because of their size (1- 2 µm), they can easily pass

through soil porosity compared to fungi (3 - 7 µm), however a little less than viruses (0.02 - 0.4 µm). Especially, coliforms or *E. coli* as a pathogenic indicator organism of the area contaminated by leaking sewage was considered.

In the United State, approximately 25 % of water is groundwater and 50 % of the population in USA use groundwater as a principal source of drinking water (Bitton and Gerba, 1984). In Germany, more than 66 % of drinking water is prepared from groundwater (Haupt, 2000). The main sources of groundwater contamination are industrial wastes and also the discharge of sewer wastes from septic tanks into the groundwater. In addition natural factors that affect the quality of groundwater such as chemical weathering, agriculture, soil leaching etc., which diminish the purity of groundwater. Due to the contamination risk for the consumers, standards of drinking water have been established, e.g. by the US environmental protection agency (www.epa.gov). In the European countries and throughout the world, contaminated soil and groundwater are a main concern since the middle 1980. With different tracers and methods, scientists tried to analyse processes and flow behaviour in the underground and in the groundwater.

Many of water borne diseases were caused by biological contamination of drinking water. Although a certain population density of heterotrophic bacteria has no health effect, a lower number means a better water quality. No coliforms (including faecal coliforms and *E. coli)* are permitted to be in drinking water. In Mexico (Orpeza et al., 2001), only 5 % of raw water samples in a month are permitted to contain coliforms if less than 40 routine water samples per month were collected. Every sample that contained coliforms must additionally be analysed for either *E. coli* or faecal coliforms. If its result is positive, the system has a higher level of contamination that is allowed in drinking water. In Germany, the number of total bacteria may not exceed 100/mL in drinking water at 36 ^{o}C (Drinking water standard criteria, www.trinkwasser.de) and no coliforms or *E. coli* are allowed. The standard criteria in each country are different; c.f. the effluent standard of Mexico allows 1000 MPN/100 mL as a maximum limit for faecal coliforms in treated wastewater (Orpeza et al., 2001). In one case in Finland, contaminated groundwater, which was used as a source of drinking water contained higher numbers of bacteria (1000 CFU/ mL) than the surface water (40 CFU/mL). The AOC (organic carbon) had no correlation with the microbial growth potential, (R^2 = -0.46, $P \leq 0.004$, N = 28, Miettinen *et al.*, 1997).

Previous publications, using the pathogenic bacteria such as coliforms or *E. coli* as indicators of fecal contamination at different sites were summarized in Table 5.3.1. In wastewater higher numbers of coliform bacteria than in the river water samples were found (Table 5.3.2).

Skraber et al. (2002) found that environmental parameters had a significant impact on the number of bacteria. The flow rate for instance had a higher correlation with thermo-tolerant coliforms than water temperature and bacterial population. The number of total coliforms and *E. coli* correlated also with rainfall (Auckenthaler et al., 2002). In the report of Rångeby et al. (1996), the retention filter effect is the most important factor on the bacterial removal.

- *The Suspended bacteria in trickling sewage*:

Unsegmented column (125 cm): After trickling of sewage through 125 cm of a sand column, a number of *E. coli* or total coliforms in effluent were found. A 125 cm soil column represented the vadose zone or unsaturated zone, respectively and normally retained more than 99 % of the bacteria from sewage. The non-retained bacteria would be drained into the groundwater and contaminated the drinking water source. In the developed countries the presence of *E. coli* in water that it is a source of drinking water is not allowed. The ratio of total coliforms to *E. coli* in our experiments was 2.1 - 8.6 (average = 4 .1), which is the same as in previous studies (Table 5.3.2) After passing through the sand column, the portion decreased to 1.1 - 2.8 (average 2.1).

Segmented column: After 25 cm, the main portion of both coliforms and *E. coli* were eliminated. With every 25 cm trickling stretch that followed the percentage of removal increased in range of 5 - 10 % until the outlet segment (Table 4.3.5).

According to this result, 125 cm trickling depth is not enough for bacterial straining or adsorption into inter-surface soil. In previous studies Martin and Noonan (1977) used faecal coliforms as a tracer in a land disposal site over a distance of 900 m in the groundwater in New Zealand. The bacteria moved approximately 150 m/day. Faecal coliforms dispersed on average 2.5 km from a discharge source. Normally, faecal coliforms moved 900 m in horizontal and 15 - 22 m in vertical direction. *E. coli* (various strains) was reported to be capable of movement of around 15 - 920 m in horizontal and 0.6 - 18 m in vertical direction. Auckenthaler et al. (2002) studied bacteriophages in the artificial tracer tests. Urine transported bacteriophages 846 m/d and 770 m/d, respectively. The recovery rate of the bacteriophages was only 2.5 %.

After passing through a sand column, the ratios were decreased to 1.1 - 2 (average 2.3). In each segment, the ratios were almost constant in the same month.

Table 5.3.1 Numbers of pathogenic bacteria that were detected in different sources

Type of bacteria	Source	Number (cell/mL)	References
Somatic coliforms (*E. coli*)	Raw wastewater Treated wastewater River	1.86×10^4 9.12×10^1 1.78×10^1	Skraber et al. (2002)
Thermo-tolerant Coliforms (not total Coliforms)	Raw wastewater Treated wastewater River	2.63×10^4 1.66 x1 0^2 2.95	Skraber et al. (2002)
Total coliforms	Wastewater Agriculture site: horse : cattle Mixed sites: Dock :Intake Urban site	4.27×10^5 1.15 3.8 2.0×10^2 *CFU/L* 2.09×10^2 *CFU/L* 1.82	Brion et al. (2000)
Total coliforms	Raw wastewater	10^4 - 10^6	George et al. (2001)
Faecal coliforms	Seine River	ca. 3.16×10^2	George et al. (2001)
Faecal coliforms	Wastewater	2.2×10^5 CFU/mL 8.8×10^4 CFU/mL	Turner et al. (1997)
Faecal coliforms	Rivers in Austria	min 6.7×10^1 CFU/100 mL max 1.6×10^5	Farnleitner et al. (2002)

Bioflim formation after trickling with wastewater: The numbers of total coliforms and those of *E. coli* in soil decreased responding to the depth. Within the total accumulation of bacteria inside a sand column more than 90 % of *E. coli* was retained in the sand of columns NRT1, NRT2 and IBA column. Some *E. coli* bacteria were still present in the effluent. Apparently, the bacteria can move well in both directions, as shown by Martin and Noonan (1977) in previous work.

The US Public Health Service recommended that the approximate depth of septic tanks is at least 120 - 150 cm above the groundwater level for the protection of groundwater. According to our results, it must be assumed that leaking sewers can be a risk for human health in areas, which have a high groundwater table of less than 125

cm under surface. The drinking water must be treated if groundwater is the raw water source.

Table 5.3.2 Ratio of total coliforms to coliphages or human coliforms in different sources

Type of bacteria	Ratio	Source	Reference
Total coliforms: coliphages	8.7 : 1 0.15 : 1	Wastewater River	Bell (1976)
Total coliforms: coliphages	0.19 : 1	River	Borrego *et al.* (1987)
Thermo-tolerant coliforms: Somatic coliforms	1.41 : 1 0.17 : 1	Wastewater River	Skraber *et al.* (2002)
Total coliforms:faecal coliforms	20.83 1.35 10.47 2.95 2.24 4.90	Wastewater Agriculture site : horse : cattle Mixed sites: Dock : Intake Urban site	Brion *et al.* (2000)
Faecal coliforms*: E. coli*	2.5 : 1	Wastewater	Turner *et al.* (1997)

B. Removal of bacteria through sand column:

In the last ten years several groups were interested in the bacteria in a variety of subsurface environments or vadose zones (Table 5.3.3). The vadose zone is defined as a three phase system, consisting of solid, gas, fluid and located above the groundwater zone (Kieft, 1999; Fletcher and Murphy, 1999). Normally, this area has little bacterial activity. If it is supplied with organic matter and the environment has an artificial recharge, high population densities of bacteria are found. The bacteria in these areas tend to be Gram positive. The distribution of bacteria and of nutrients was controlled by the thickness of the water film between the particles. If the flow was minimal, the transport was controlled by diffusion (Kieft, 1999), at higher flow rates convective processes were superimposed.

For protecting groundwater, bioremediation in the vadose zone is an important process. Contaminants or toxic substrates are transformed in this zone by

biotic processes. The biomass in this zone can increase a lot above the normal level of oligotrophic environmental.

Bacterial formation of biofilm in soil

The number of bacteria in soil, which grow as biofilms or adsorb or interact at the soil surface or with soil pores in different environments were, investigated (Table 5.3.3). The parameters, which influence the formation of biofilms, bacterial transport and interaction between bacteria and soil surface are numerous. Complex processes such as moisture, temperature, component of soil, porosity, light and organic carbon are determining factors. According to the result of Randjard and Agnes (2001), most of the the microbial life occurs in soil pores accounting for up to 50 % of the total soil volume. Kilbertus (1980) estimated that the mean diameter of pores, mainly colonized by bacteria, was 2 μm, while no bacteria could be observed in pores less than 0.8 μm in diameter. Hassink et al. (1995) found a positive correlation between the quantity of bacterial biomass and the volume of the pores with a mean diameter of 1.2 μm in different soil. The maximum diameter of the pores most frequently colonised by bacteria was estimated in a range from 2.5 to 9 μm for fine and coarse texture soil, respectively. About 40 - 70 % of total bacteria is located in 2 - 20 μm cavernes.

In both laboratory columns (ϕ 10 and 20 cm), the highest numbers of bacteria were found in the first 30 cm. The population densities in the sand at first 5 cm depth were almost higher than the number of bacteria in sewage. In the sludge layer at the soil surface the biomass increased after passage of wastewater through the columns, which was in accordance with earlier observations by Sandaa et al. (1999). They studied bacterial distribution in contaminated soil with low and high heavy metals concentration from sewage sludge. It was found that the bacterial diversity increased by ca. 60 % in low contaminated soil compared with the control and by around 90 % in soil with high heavy metals concentration. Nevertheless, the long-term contamination had effected the composition of the bacterial communities. Normally, after receiving the artificial discharge of sewage, the number of soil bacteria increased according to the flux of pollutants and the kind of native bacteria as well as the bacteria in the pollutant.

Hence, the high numbers of bacteria in the first 30 cm of sand in the laboratory columns indicated also that the main bacterial activities occurred at this level. This was experimentally shown with the segmented column in which most of the COD was

eliminated in the first 25 cm (1st segment). These results were consistent with observations by Fabiano and Danovaro (1994) who found that the vertical distribution of bacteria was characterized by a regular decline in deeper sediments. The content of total organic matter in surface sediments varied in a range between 52.4 and 93.1 mg/g dry sediment with the high concentration at the top 10 cm. The bacterial cell density varied between 1.7 - 4.5 x 10^8 cells/g dry soil with a mean of 7.3 x 10^8 cells/g dry soil, determined by direct counting or 8.6 - 50.1 x 10^5 CFU/g dry soil, determined by the plate-count method. The difference in the order of 10 magnitudes show the problems of either many dead cells or unculturable cells, which still will be counted under microscope.

It was reported by Schlieker et al. (2001) that a sequence of different reactions appeared in the first 7.5 cm of the studied soil column. The microbial reactions were those requiring oxygen (0 – 1.5 cm), followed by a zone of predominantly nitrate reduction (0-3cm), sulfide oxidation by manganeseoxiders (0 - 3 cm) and sulfate reduction (3 - 7.5 cm). Iron reduction occurred at 0 - 6 cm and between 4.5 - 6 cm found iron minerals were oxidizing sulfide. Iron sulfide was formed in depth 0 - 7.5 cm.

The bacterial numbers declined along the vertical flow path/ depth similar as reported by others (Paul and Clark, 1989; Fabiano and Danovaro, 1994; Richter and Markowitz, 1995). Kieft (1999) found that the bacterial number decreased with the depth and age of starvation both in the laboratory and in the field. It was suggested that the bacteria might die during long-term sequestration and/or retardation during transportation from the surface. This was shown by the ratio of diglyceride fatty acid (indiactive for dead cells) to phospholipid fatty acid (PLFA, living cells), which increased exponentially with depth.

The correlation between bacterial numbers and chemical parameters was calculated (Fig. 4.3.4, 4.3.10 and 4.3.14). The numbers of bacteria have a positive correlation with organic matter in soil (total carbon, COD). This supports the observation of Fabiano and Danovaro (1994) that the bacterial biomass was correlated with organic matter (total sediment protein). Contrary Alongi (1990) reported that there was no relation of the deep-sea sediment of the Solomon and Coral sea between bacterial biomass and organic matter. Zacheus and Pertti (1995) concluded from their results that the numbers of microbes were not correlated with the amount of total carbon.

The occurrence of coliforms in a cold-water distribution system has been attributed to the level of organic carbon of greater than 2.4 mg/L (LeChevallier and Norton, 1995). In this research the concentration of total organic carbon varied from 2.8 to 10.1 mg/L. The average TOC was 5.4 mg/L in processed groundwater and 7.6 mg/L in processed surface water, respectively. Recent studies have shown that the level of assimilable organic carbon (AOC) mainly effected the growth of heterotrophic bacteria. Microbial growth of heterotrophs depends on the availability of organic carbon and inorganic nutrients. A higher amount of metabolizable organic carbon may lead to a greater volume of each cell (Miettinen et al., 1997). The positive correlation between the number of bacteria and the chemical parameters suggests an association between some ions and microbes in water distribution systems.

The correlation between chemical parameter and the number of bacteria in our soil columns were calculated in 3 ways. The representative data were chosen from a high relation (R^2) type. In linear regression form, it shows the parallel or anti-parallel respond of two parameters, which depend on the correlation coefficient (R^2,+/-). In exponential form it shows that the correlation of x-y is not directly parallel, but one parameter is increasing or decreasing with power of another parameter. Both exponential and parabolic dependences showed that the correlation of the number of bacteria and a chemical parameter was related

The bacterial activity in the vadose zone was studied. Most experiments that were reported in the literatures were done under aerobic condition (Severson et al., 1991**;** Kieft et al., 1997, 1998; Kieft and Phelps, 1997) but a few also under strictly anaerobic or facultative conditions. Balkwill and Ghiorse (1985) investigated the number of aerobic and anaerobic bacteria of shallow vadose zone samples. The results showed that the anaerobic bacteria were 2 - 3 fold less numerous than aerobic bacteria. The predominate population might be facultative bacteria. Lehman *et al.* (2001) determined the aerobic and anaerobic population of core samples from an acidic and crystalline rock aquifer at different profiles depth until 120 meters. They found a higher number of anaerobic than of aerobic bacteria in most depths.

According to the results in Part 4.3.1, the ratio of aerobic and anaerobic bacteria allows to predict the redox potenital inside the soil columns. It varied with depth and the age of the respective column.

A comparison of the ratio of aerobic to anaerobic bacteria in columns IBA1 and IBA2 showed clearly that the "*older column*" was much more adapted to anaerobic

conditions inside the sand than the "*younger column*". However the conditions seemed not to be strictly anaerobic (e.g. data of IBA2, NRT1, NRT2), although the sewage was continuously purged with nitrogen gas before flowing into the columns. In the soil column NRT1, the numbers of aerobic bacteria was higher than that of anaerobic bacteria in the sludge profile on top (0 - 5 cm) had higher number of bacterial cells than in the sewage. Hence, in these depths aerobic- or anoxic conditions might have been prevalent. By diffusion of oxygen into sewage on top of the column or through the outlet hole at the bottom of the column aerobic conditions were maintained, that allowed the aerobic population of sewage to survive. When the sewage trickled into the soil profile of the column it became anaerobic rapidly because of the high activities of the aerobic bacteria (total number 10^9 CFU/g dry soil), which consumed the few mg O_2 per liter. Degrading only a few mg COD/L during oxygen respiration was observed. At 0 - 5 cm depth the numbers of anaerobic and aerobic bacteria were almost equal. For the rest of the column (after 5 cm to 90 cm) anaerobic conditions were prevalent, according to the higher number of anaerobic bacteria in comparison to aerobic bacteria. The population of anaerobic bacteria was even higher than in the sewage, indicating growth either in the biofilm or in suspension. Since aerobic bacteria were present as a major population in sewage and since these bacteria survived anaerobiosis for quite some time, it was not expected to eliminate aerobic bacteria totally at a hydraulic retention time of sewage in the soil column of around 2 liter per day.

A comparison of the population (total cells, anaerobic cells, aerobic cells, DN and SRB) of the 3 columns (IBA1, IBA2 and NRT1) was made. It was found that the numbers of bacteria in all groups were increasing with time at the same depth. The filtration effect of the sand as filling material in the column caused a decrease of all bacteria with depth in the water phase and growth of bacteria at the sand surface as a biofilm increased the population density at every depth with time. In the sand column IBA1, after 4 months observation time, lower numbers of bacteria were detected as compared to IBA2 (18 months) or NRT1 (10 months). In contrast, the numbers of aerobic on the biofilm bacteria decreased with the age of the columns. In the column IBA1 higher numbers of anaerobic bacteria were found compared to IBA 2 or NRT1. This indicated that at the beginning the main population of bacteria in the column was aerobic and with time was replaced by an anaerobic population.

The numbers of bacteria were significantly higher in the sludge sheath and dropped dramatically in the first 5 cm. The population density slightly declined

responding with the depths. The percentage of elimination of bacteria after 20 - 30 cm was stable. At this depth, the maximum supply of microorganisms with substrate was passed already. The residual carbon might be unsuitable or inhibitory for bacterial growth. A similar elimination of bacteria in aqueous samples from different depth of the segmented column (unattached bacteria Part 4.3.2) was found.

The correlation of the numbers of aerobic or anaerobic bacteria with total organic carbon or total carbon or total COD showed different results. IBA1 and IBA2 correlated highly with a linear regression type but the numbers of bacteria in NRT1 showed an exponential or parabolic relation with carbon or COD (Table 4.3.2). The different accumulation behaviour of bacteria might be due to the diameter of the column or the flow rates that have an influence on the correlation, the sewage flow rate within the column, and the filtration effect. In NRT1 the total bacteria were not related to total carbon, total COD or total organic carbon.

The statistic test (T-test) proved the significance of total anaerobic bacteria, total aerobic or SRB in comparison between NRT1- IBA1, or IBA1-IBA2 or NRT1-IBA2, at low confidence ≤ 0.2 (80 %). Due to the use of only one variable for statistic analysis it was not suitable for the complex systems. The analysis with multiple variables would be an alternative.

The amounts of total carbon in soil samples were related with time. The concentration of carbon increased in column IBA1, NRT1 and IBA2, respectively.

The SAW column was supplied with artificial wastewater, containing soluble substrates like yeast extract and peptone. This media could be easily degraded by the sewage bacteria. In both columns higher numbers of anaerobic bacteria than aerobic bacteria were found. The different running time of the two reactors showed no effect on the population of bacteria, indicating that a steady state was obtained in both colums already.

Table 5.3.3 Number of bacteria in soil of different environments.

Type of bacteria	Source	Bacterial density (cell/g dry soil)	Chemical parameter	Remark	Reference
Total bacteria	Soil-control low metal -high metal	260 x 10^7 240 x 10^7 200 x 10^7	0.9 % org. C 1.1 % 1.5 %	DAPI straining	Sandaa et al. (1999)
Neutrophilic Acidophilic	Soil -attached	ca 40 10^2-10^4		R2A medium solid medium	Lehman et al. (2001)
Anaerobic C1 utilizer Denitrification SRB Total bacteria	soil groundwater	ca 10^8 ca 10^4 ca 10^7 ca 10^3 10^6-10^7		R2A medium	Kaempfer et al. (1991)
Total cell	soil	ca 2 – 4 x 10^5 ca 5 x 10^6 – 7 x 10^7 ca 1 - 9 x 10^7		normal medium rich medium mineral medium	Dott and Kaempfer (1989)
Heterotrophic	soil	1 x 10^3 - 1.8 x 10^5 8 x 10^8 - 2.5 x 10^9 4 x 10^8 - 1.6 x 10^9		agar plates dierect cell count active cell count	Bhupathiraju et al. (1999)
	organic soil mineral soil	4.2 x 10^7 - 2.3 x 10^8 6.1x 10^6 - 7.9 x 10^7			Priha et al. (1999)
Total cell	channel sediment	3.9 - 7.0			Lemke et al. (1997)
Total cell CTC reducing bact. Micro-CFU	barley field soil	10^9 2 x 10^7 – 5 x 10^7 9 x1 0^7			Winding et al. (1994)

- Suspended bacteria

The population density in the trickling sewage declined with soil depth. In the segmented column, the highest bacterial numbers were observed after passing the 1st segment (25 cm). The removal of bacteria after trickling through the sand column occurred by interaction with soil and by adsorption at the gas-water interface. The factors and processes, which controlled bacterial transport, were studied. Results of the distribution of SRB are summarized in Table 5.3.5.

Filtration or straining is the main process that controlled the distribution of bacteria in soil. Especially the suspended particles of sewage that accumulated in the columns at the soil surface acted as a filter layer and retained the fine particles

including bacteria. Krone et al. (1958) reported that after trickling of distilled water that was contaminated with *E. coli* into a sand column, the numbers of bacteria in the effluent increased at the beginning reached a maximum and after that were constant. Herzig et al. (1970) reported that straining is a very important parameter if the ratio between the cell diameter and the grain diameter is > 0.05. According to the results by Lindqvist and Bengtsson (1991), the width of bacteria (13 strains) varied in a range of 0.8 - 1.5 μm, so any particle which had a diameter < 20 μm can trap a bacterial cell. Gannon et al. (1991) found that if a bacterial cell is shorter than 0.1 μm, it can be transported well through the soil.

Surfactants such as sodium dodecylbenzene sulfonate increased the bacterial transport through soil (Powelson and Mills, 1998). Thus, bacteria reacted with hydrophobic binding sites, which reduced the adsorption to gas-water interfaces. It was explained that the increasing removal of bacteria was due to increasing interaction between suspended cells and the sand grains and to bacterial adsorption to the gas-water interface. In contrast, Jackson et al. (1994) reported that decreasing of *ionic strength* or addition of surfactants declined the bacterial number. Increasing of pH had less effect than changes of ionic strength (Scholl and Harvey, 1992; Jewett et al., 1995).

Bacterial surface charge, hydrophobicity or extracellular polymers effects were studied by Gannon et al. (1991) in varies organisms: *Arthrobacter* sp., *Enterobacter* sp., *Pseudomonas* sp., *Bacillus* sp, *Archromobacter* sp. and *Flavobacterium* sp.. No relationship of these parameters was found with the bacterial transport through porous media. Rångeby et al. (1996) reported the removal of faecal coliforms in a wastewater stabilisation pond system in Mindelo, Cape Verde. It was found that the *retention time* was the most important factor for the reduction of bacteria in the wastewater treatment plant. A high die-off rate was observed when the pH increased. The conductivity increased at anaerobic conditions.

Cations, such as Fe^{2+}, Cu^{2+}, Zn^{2+} could influence bacterial adsorption to soil. A low ionic concentration increased the infiltration rates. A small size of bacteria favoured adsorption to soil and reduce the filtration effect.

The presence of iron oxide in sediments increased bacterial retention (Mills et al., 1994; Johnson and Logan, 1996). In contrast, the presence of sediment

organic matter increased the transport of bacteria and viruses in groundwater (Powelson, 1991).

Table 5.3.4 Numbers of bacteria found in different sources.

Type of bacteria	Source	Number (cell/ml)	chemical parameter	Remark	Reference
Total cell	Groundwater	Ca. 5 – 7 x 10^6 ca. 8 x 10^3 –1 x 10^4 ca. 4 x 10^3 – 1 x 10^4		Normal medium Rich medium Mineral medium	Dott and Kaempfer (1989)
Total cell Neutrophilic Acidophilic	Groundwater Water in pit Aerobic/anaerobic	6.6 x 10^5 2.7 x 10^5 < 10 - 10^4 10 - 10^4		acridine orange R2A Solid medium	Lehman et al. (2001)
Total cell	Atlantik Waldbach Elbe river Wastewater	1.2 x 10^6 7.0 x 10^5 9.5 x 10^6 2.5 x 10^8	0.131* 0.120* 0.149* 0.357*	* = biovolume (μm^3)	Rheinheimer (1991)
Total cell	Stream site	1.63 x 10^6		aver. of three streams	Lemke et al.(1997)
Meso. heterotrophic Total cell		8 x 10^3 -3.7 x 10^5 FU/L 1.1 x 10^8 - 1.4 x 10^8			Zacheus and Pertti (1995)

The removal of bacteria from sewage after passing of sewage through the segmented sand column was investigated. Most of the bacteria were removed in the first 25 cm of soil. Previous filtration experiments with sandy soil came to the conclusion that the main portion of bacteria was retained after a few millimetres (2 - 6 mm) at the surface of soil. Result of bacterial enumeration in the soil confirmed this assumption. The first 2 - 5 cm of soil or the sludge sheath contained higher number of bacteria than were found in further depth within a big spring (Bitton and Gerba, 1984).

A great portion of DOC or COD was eliminated at the first segmented column and the residual DOC or COD slightly declined in the following four segments. This might have been due to mainly two reasons; 1) the rest of carbon in sewage wastewater was of complex structure and 2) the requirement of a much longer retention time. The retention time was one of the major parameters that effected the degradation of organic matter (Rångeby et al., 1996). If glucose was added to sewage and the sewage was then trickled through the segmented sand column the first segment was overloaded.

More of the organic matter was degraded in the 2^{nd} – 5^{th} segment of the column (Hau et al., unpublished).

The conditions inside the column were apparently not completely anaerobic and the ratio of aerobic and anaerobic bacteria (Fig. 4.3.29) varied with depth. In the 4^{th} and 5^{th} segment, aerobic conditions must have been prevalent, due to high numbers of aerobic bacteria that were found. The appearance of nitrate was an indication for nitrifying bacteria in these areas.

Carter et al. (2000) reported that pH and conductivity had a positive correlation with numbers of bacteria on R2A plates. The temperature had also a positive correlation during cultivation on R2A agar (Spearman regression). AOC, TOC and the level of nutrients had a significant impact on bacterial numbers. In this study, there was no significant correlation between AOC and the bulk fluid bacterial number. This may be because of the very low amount of AOC detected throughout the pipe network. A significant positive correlation was detected between the amounts of bulk fluid bacterial number with TOC (> 0.8 mg/L). However, if TOC was low, there was possible to be no significant correlation (0.7 mg/L).

Ranjard and Agnes (2001) reported relationship between bacteria and organic matter distribution. A correlation higher than 0.9 was reported.

Adeline (2002, Master thesis) reported the correlation of β-D-galactosidase activity and β-D-glucuronidase activity with the number of *E. coli* by linear regressions: $Y = 0.9465\ X - 1.3639$ ($R^2 = 0.94$) and $Y = 0.4874\ X + 1.1928$ ($R^2 = 0.94$), when X = log number of cell/mL and Y = log substrate degradation $time^{-1}$ ($pmol/mL \cdot h^{-1}$), respectively. Tryland et al. (2002) measured the correlation curve of total coliforms and β-D-galactosidase activity as $Y = 1.17\ X + 2.7$ ($R^2 = 0.76$) when Y = log number of cell/100mL and X = log B-galactosidase $time^{-1}$ ($ppb\ MU\ h^{-1}$). The lower correlation (R^2) was explained by reasons such as the technique of detection of culturable bacteria or some of non coliforms bacteria. *Aeromonas* sp. and *Vibrio* sp. produced β-D-galactosidase in the samples or the sunlight killed some the bacteria. However, recent reports indicated that in samples with higher contaminated faecal sewage *E. coli* seemed to be the main producer of β-D-galactosidase (Tryland and Fikdal, 1998; Davies and Apte, 2000).

C. Sulfate reducing bacteria:

In the last 25 years scientists have focused on SRB in subsurface environments especially in the USA and in West Europe (Chapelle, 1993). The data of SRB in different ecosystems are shown in Table 5.3.5.

Within the bacteria that formed a biofilm on the sand particles of the laboratory columns, the numbers of SRB were low in all columns, especially in IBA1, which run only 4 months. The SRB contributed < 1 % to the total bacteria. This was similarly observed in reports of Ramsing et al. (1996), who found a number of sulfate-reducing bacteria in Mariager Fjord (marine environment, Denmark) in the range of 2 x 10^2 to 3 x 10^2 cells/mL by using the lactate-MPN method. The SRB contributed only 0.2 - 0.3 % to a total population of 10^5 cell/mL, as shown by in-situ hybridisation. The maximum sulfate reduction rate was between 0.05 and 0.25 µMS/d.

A low number of SRB could be caused by the following reasons: The conditions inside the column might not have been realy anaerobic. This was indicated by the appearance of nitrate in the 4^{th} and 5^{th} segment of the column. Some reports mentioned that nitrifiers were found in the intermediate zone of oxygen consumption and a zone of sulfide oxidation in natural environment (Ramsing et al., 1996) and/or they could survive under oxic conditions. The sulfate concentration in the domestic wastewater was low, approximately 1 - 2 mM, which was a limiting factor for the bacterial growth. The numbers of SRB in the biofilm on the sand or in the water between the sand declined with depth.

D. Vertical sulfate reduction rates in soil

The highest sulfate reduction rate was found in the sludge layer on top of the sand column declining with depth of the soil profile (Fig. 4.3.20). This was reported by Li et al. (1991) who investigated the sulfate reduction rate of freshwater lake sediments in Kizaki (Japan). The SRR was lower than 0.5 nM/day in the layer below 6 cm. The highest SRR was only 13 nM/day. Seasonal changes or temperature had a significant effect on the sulfate reduction rate. The SRR was high in spring and summer and decreased from autumn to winter.

Jørgensen (1982) reported that 75 - 90 % of sulfate reduction activity was found at 0-15 cm. This coincided with previous research which found the main activity of SRB at the surface. Visscher et al. (1999) found the maximum SRR at a depth of 3 - 5 mm (layer 3) of Stromatolite mats.

Table 5.3.5 Distribution and numbers of SRB at different environment.

Sources	Cells/g dry soil	Remark	References
Atlantik coastal plain sediment (<300m)	<1 - >10^5	Sediment	Jones et al. (1989)
Texas upland coastal plain	10^5 - 10^6	Sand (27 - 31 m depth)	Martino et al. (1998)
Groundwater in Sweden	10^5 cells/mL	Granitic rock(129 - 680 m)	Pederson and Ekendahl (1990)
-Mixing zone between injection and formation -oil field, Talinskoe and Mykhpay	10^5 cells/mL 10^4 - 10^5 cells/mL	Thermophilic SRB	Nazina et al. (1995)
SRB, Petroleum hydrocarbon transformation	6.6 x 10^4-1.51 x 10^5 cells/mL	Media with lactate and acetate	Kliekemper et al. (2002)
Cultivated oxic soil Forest Savanna (well aerated soil) Dessert Termite	2840 - 96000 4 - 144 28 - 840 4 - 144 60 - 520	Mineral medium	Peter and Conrad (1995)
Maringer Fjord Water column	1300 cells/mL 2 x 10^1 – 3 x 10^2 2 x 10^6 – 4 x 10^6 1 x 10^6 – 2 x 10^6	MPN MPN Ethidium bromide straining Fluorescent straining	Teske et al. (1996)

Thamdrup et al. (2000) reported that SRR increased from the surface to a maximum at a depth of 1 to 2 cm (Station I-III).

A scheme for the contribution of sulfate reduction to the degradation of COD from sewage is shown in Fig.5.1 Sewage contain sulfate and organic acids, which both are essential for sulfate reduction. Depending on the sulfate reducers present in the anaerobic bloom COD is either completely oxidized to CO_2 and hydrogrn equivalents, which are required for sulfate reduction or the COD is incompletely oxidized to acetate, liberating less hydrogen equivalents for sulfate reduction (Fig.5.1). The sulfide reacts chemically with heavy metals ions of the sewage or of the soil and leads to heavy metal precipitation under anaerobic conditions. From the unsaturated vadose zone oxygen is diffusion into the sewage bloom and may slowly oxidize the metalsulfides to metalsulfates which the sulfur react with carbonate to metalcabornates. The sulfate reduction rates are highly depending on the soil

temperature. Overall sulfate reduction contributed significantly to COD degradation in sewage below leaking sewers.

Table 5.3.6 The sulfate reduction rate in different environment

Sources	Sulfate reduction rate	Remark	References
Mesophilic SRB (sulfide production)	1.2 $\mu gS^{2-}/L\ day^{-1}$ 1.8 x 10^{-3} $\mu gS^{2-}/L\ day^{-1}$ 0.001 - 0.48 $\mu gS^{2-}/L\ day^{-1}$	Mykhpay Talinskoe Injection zone	Nazina et al. (1995)
Sediment, Denmark	0.5 x 10^{-1} - 4.5 $mMSO_4^{2-}/yr$	Radiotracer	Jakobsen and Postma (1949)
Unamended Ferrihydrite	24 ± 23 $nmol/cm^3 \cdot day$ 109 ± 42 $nmol/cm^3 \cdot day$	Depth 0.5 - 1.5 cm Depth 8 - 10 cm	Thamdrup et al. (2000)

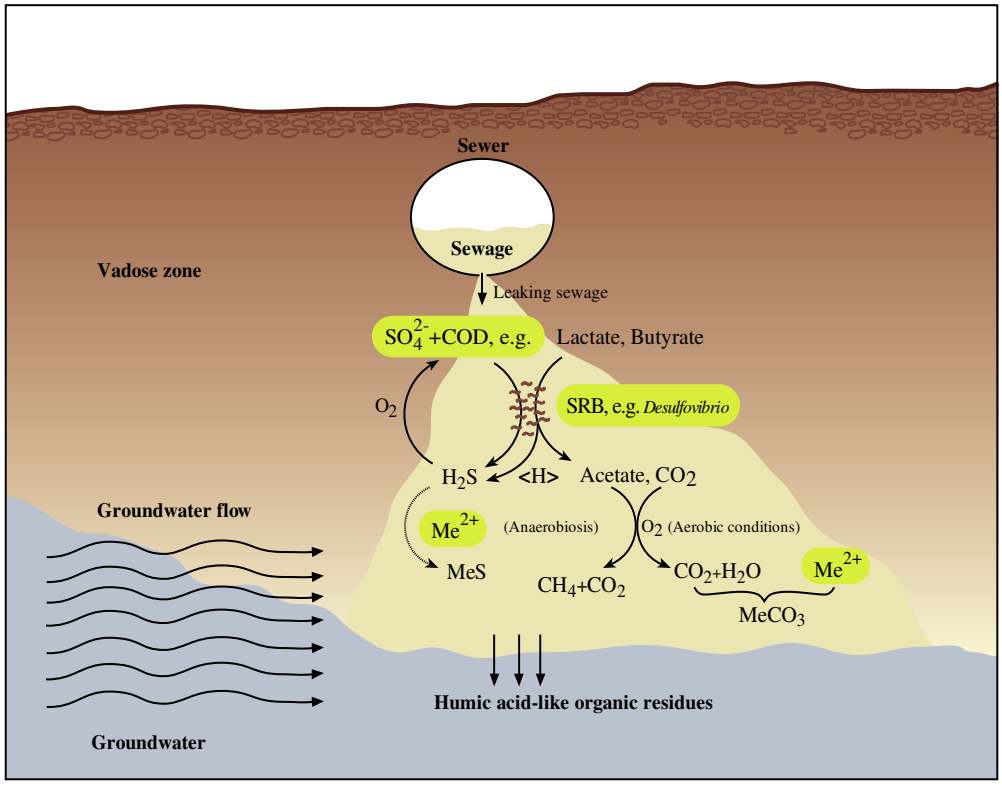


Figure 5.1 Schema of activity of sulfate-reducing bacteria in vadose zone beneath leaking sewer.

6. Conclusion

The following results were obtained:

*** Batch experiment:**

■ Enrichment of sulfate reducers culture from sewage sludge or contaminated soil, contained almost exclusively incompletely mesophilic bacteria. Lactate was the most suitable carbon source for them and resulted in high sulfate teduction rates.

■ Sulfate reducing bacteria required 36 - 63 mg/L of COD to reduce 1 mM of sulfate in the presence of BESA and 31 – 74 mg/L of COD in the absence of BESA.

■ The addition of BESA as an inhibitor of methane bacteria had no effect on SRR or % sulfate reduction in the cultures. It did not influence the proportion sulfate reduced: COD utilized.

■ Temperature had affected the velocity of sulfate reduction. If the temperature increased from 15 to 37 ^{o}C , it accelerated the sulfate reduction rate until 2.8 fold on average.

■ Addition of 0.1 mM of molybdate is a recommended concentration for studying the activity of *D. desulfuricans* under stress conditions. The velocity of the sulfate reduction rate at 37 ^{o}C in assay with Mo was decreased around 1.3 fold in comparison to controls, but no effect on the % sulfate reduction was seen. In enrichment cultures from contaminated soil of Rastatt, Mo was more effectively inhibiting growth of sulfate reducer. SRB competed with other bacterial groups for the carbon source.The V_{max} of sulfate reduction was 400 µM/hr (without Mo) and 120.5 µM/hr (with Mo).

■ The fractionation value of sulfur isotope (S^{32}/S^{34}) by sulfate reducers was affected by several factors such as SRR, temperature, sulfate concentration and/or type of electron donors (Canfield, 2001; Detmers et al., 2001). According to our results (Stoegbauer, A., S. Koydon, Z. Berner, J. Winter and D. Stueben, in press), in batch assays of *D. desulfuricans* the fractionation faktor α was 1.0147 ‰ at 37 °C and 1.116 ‰ at 15 °C. In the assays in the presence of 0.1 mM Mo the fractionation factor α was 1.021 ‰ at 37 ^{o}C, due to Mo inhibition (Stoegbauer et al. in press).

■ Natural SRB can effectively utilize sulfate and carbon sources in sewage for growth under strictly anaerobic conditions. With suitable electron donors and/or an

unlimited electron acceptor availability threshold concentration of sulfate > 3 mM accelerates sulfate reduction process.

*** Fed-batch culture and continuous culture:**

■ In fed batch culture ca 45 % of sulfate was reduced in assays with addition of electron donor (lactate) and electron acceptor (sulfate).

■ In continuous culture of *D. desulfuricans* the SRR was 67.9 µM/hr. For comparison, in contaminated soil samples the SRR was 19.2 µM/hr in assays of filtrated wastewater plus lactate and sulfate, 32.5 µM/hr in freshwater media and 5.8 µM/hr in filtrated wastewater assay without addition of sulfate and a carbon source. The SRR of contaminated soil were low compared to pure cultures due to the fact that sulfate reducers in mixed cultures had to compete with another bacteria for the carbon source.

*** Columns study:**

Distribution of suspended bacteria in the water phase of the non-segmented and segmented soil column during trickling of sewage:

■ Sulfate reducers utilized organic substrates in sewage as electron donors for growth. 40 - 55 % of the initial concentration was degraded in the first 25 cm of segmented column by sewage bacteria. From the total COD or DOC 60 – 80 % of the initial concentration were degraded in the first 25 cm and 8 – 15 % after trickling through the column until the outlet .

■ The DOC value has a positive correlation with the bacterial number in raw sewage by exponential equation: $Y = 52149^{e0.0505x}$ ($R^2 = 0.8$), when X = DOC-mg/L and Y = number of bacteria - CFU/mL. The correlation of the bacterial number in raw sewage with the amount of COD follows the exponential equation: $Y = 24260^{e0.0079x}$ ($R^2 = 0.9$), when X = COD - mg/L and Y = number of bacteria - CFU/mL.

After trickling of sewage through 125 cm sand in a non-segmented column ca 90 % of bacterial aerobic or anaerobic population, ca 76 % of *E. coli,* and ca 80 % of SRB were eliminated. In the effluent < 10^2 – 10^3 CFU/mL of *E. coli* were found. In the segmented column most of the bacterial population (83 – 95 %) was eliminated after the first 25 cm and only 2 – 5 % more removal efficiency was found after 125 cm trickling stretch.

Biofilm formation on sand particles after trickling of sewage:

The number of bacteria of all groups declined with depth. Biofilm formation could be found at the sludge level on the top of column and at 0 - 5 cm depth of the sand column. In higher loaded sand columns (high trickling gravels) high bacterial numbers were detected from 0 – 30 cm depth. SRB contributed a proportion of 0.05 – 1.6 % to the total bacteria in sand columns (NRT 1, NRT2, IBA 1, IBA 2) with trickling sewage. In sand column with artificial sewage (OECD wastewater) 0.6 –13.5 % of SRB were found. In a blank column SRB could only be detected at less than 100 CFU/ g TS soil.

Metallic sulfide precipitation:

The precipitation of heavy metals with sulfide (28) such as iron could be occured in both ways (26, 27). According to Schäfer et al. 1998, reported dominantly reaction seemed to be the equation 27 and 28.

$$FeOOH + 3/2HS^- + ½\ H_2O \rightarrow Fe^{2+} + HS^- + 1/2\ S^0 + 5/2OH^- \quad (27)$$

$$Me^{2+} + HS^- \rightarrow MeS + H^- \quad (28)$$

On average 55 mg/L of initial sulfate were eliminated. The sulfide reacted with heavy metals in sewage, e.g. with 35.9 mg/L Zn^{2+} or 38.5 mg/L Fe^{2+} and prevented transportation of heavy metals into groundwater with trickling water.

Zusammenfassung

Beitrag von Sulfatreduzierenden Bakterien unter Kanalleckagen zur Abbauaktivität der Schadstoffe:

Batchversuche mit Anreicherungskulturen von Klärschlamm oder aus den Bodenproben, die unter einer Kanalleckage gezogen wurden:

Die angereicherten sulfatreduzierenden Bakterien gehören zu den unvollständigen Oxidieren, weil sie kein Acetat verwerten können und nur mit anderen C-Quellen gewachsen waren. Lactat war das besten Kohlenstoffsubstrat für diese Bakterien.

Um 1 mM Sulfat zu reduzieren brauchten die sulfatreduzierenden Bakterien 36 – 63 mg/L CSB im Medium mit BESA und 31 – 74 mg/L CSB im Medium ohne BESA. Mit BESA findet eine Hemmung der Methanbakterien statt, ohne deutliche Änderung der Sulfatabbaurate oder ohne eine Änderung des prozentualen Sulfatumsatzes.

Der Sulfatabbau ist von der Temperatur abhängig. Wenn die Temperatur sich von 15 Grad auf 37 Grad ändert, erhöht sich die Abbaugeschwindigkeit bis zu 2,8 fach.

Eine Reinkultur wurde mit 0,1 mM Molybdat als Hemmstoff für sulfatreduzierende Bakterien angezogen. Molybdat hatte einen Effekt auf die Sulfatabbaurate und das Wachstum der Bakterien. Bei der Probe, die unter einer Kanalleckage in Rastatt entnommen wurde, hatte Mo anscheinend eine stärkere Wirkung auf das Wachstum, da die sulfatreduzierenden Bakterien durch andere Bakterien zusätzlich beeinflußt wurden. V_{max} des Sulfatumsatzes war 400 µM/hr (ohne Mo) und 120.5 µM/hr (mit Mo).

Faktoren, wie die Sulfatreduktionsrate, die Temperatur, die einer Sulfatkonzentration oder verschiedener C-Quellen haben einen Einfluss auf die Schwefelisotopen – Signatur (Canfield, 2001; Detmers et al., 2001). In der Reinkultur von *D. desulfuricans* war die Fraktionierung 1,0147 ‰ bei 37 °C und 1,116 ‰ bei 15 °C (Stoegbauer, A., S. Koydon, Z. Berner, J. winter and D. Stueben, in press). Die Gegenwart von 0.1 mM Mo beeinflusst den Fraktionierungsfaktor (α = 1,021 ‰) bei 37 ^{o}C (Stoegbauer et al. in press).

SRB können C-Quellen und Sulfat im Abwasser unter anaeroben Bedingungen verwenden. Ohne Begrenzung der Elektronendonatoren oder Elektronenakzeptoren (Sulfat > 3 mM) findet eine höhere Sulfatreduktionsaktivität statt.

In Fed-BatchKultur: Der Sulfatumsatz betrug 45 % in den Proben mit Sulfat und Laktat.

In kontinuierlicher Kultur von *D. desulfuricans* wird Sulfat mit einer Rate von 67,9 µM/hr abgebaut. Im Gegensatz zu Bodenproben betrug die Sulfatumsatzrate 19,2 µM/hr in filtriertem Abwasser plus Laktat und Sulfat, 32,5 µM/hr im Süßwassermedium und 5,8 µM/hr im filtrierten Abwasser. Das Bakterienwachstum war langsamer im Vergleich zu der Reinkultur. Wahrscheinlich greifen neben sulfatreduzierenden Bakterien andere Bakterien auf die C-Quelle zu.

Säulenversuch: Der Sulfatumsatz in segmentierten Sandsäule betrug 40 – 55 % in den ersten 25 cm im Abwasser mit sulfatreduzierenden Bakterien. Etwa 60 – 80 % CSB und DOC werden in der ersten segmentierten Säule abgebaut. Danach wurden nur noch 8 – 15 % bis zum Auslauf aus der Säule zusätzlich umgesetzt.

Die Korrelation zwischen DOC und Bakterienkeimzahl des Abwassers ist exponentiell ($Y = 52149\ ^{e0,0505x}$ ($R^2 = 0,8$), X = DOC-mg/L und Y = Bakterienkeimzahl – KEB/mL. Die Korrelation zwischen Bakterienkeimzahl des Abwassers und CSB ist exponentiell Formel ($Y = 24260^{e0,0079x}$ ($R^2 = 0,9$), X = CSB - mg/L und Y = Bakterienkeimzahl-KBE/mL.

Nach der Abwasserversickerung durch die Laborsäule (gesamte Länge 125 cm) wurden etwa 90 % aerobe und anaerobe Bakterien, ingesamt 76 % *E. coli* und etwa 80 % sulfatreduzierende Bakterien zurückgehalten. Im Ablauf finden sich aber immer noch $<10^2 – 10^3$ KBE/mL *E coli*.

Die Bakterien aus den Bodenproben nahmen mit der Tiefe exponentiell ab. Biofilmbildung findet in der Schlammschicht und den ersten 5 Zentimetern des Sandbodens statt. Bei höherer Versickerungsrate wurden in den ersten 30 Zentimetern höher Populationdichten ermittelt. SRB hatten nur einen Anteil von 0,05 – 1,6 % an der gesamten Bakteriendichte aus Sandproben der Säulen NRT1, NRT2, IBA1, IBA2.

In Bodenproben, die mit künstlichem Abwasser betrieben wurden, fanden sich 0,6 – 13,5 % SRB. Im Boden der Blanksäule (betriebt mit Leitungswasser) betrug der Anteil <100 KBE/g Boden.

Sulfid wird mit Schweremetallen wie z.B. Eisen ausgefällt. Durch 125 cm Versickerungsstrecke nahm das lösliche Sulfid um etwa 55 mg/L ab. Das gebildete Sulfid reichte aus, um 35,9 mg/L Zn^{2+} oder 38,5 mg/L Fe^{2} auszufällen. Der Rest der Schadstoffe wird direkt ins Grundwasser weiter fließen.

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