Survival of sulfate-reducing bacteria in oxic oligotrophic environments related to drinking water

vorgelegt von
Diplom-Ingenieurin
Karen Bade

Vom Fachbereich
Verfahrenstechnik, Umwelttechnik, Werkstoffwissenschaften
der Technischen Universität Berlin
zur Erlangung des akademischen Grades
Doktorin der Ingenieurswissenschaften
- Dr.Ing. –
genehmigte Dissertation

Promotionsausschuss:
Vorsitzender: Prof. Dr.-Ing. U.Stahl
Berichter: Prof. Dr. U. Szewzyk
Berichter: Prof. Dr. H. Cypionka

Tag der wissenschaftlichen Aussprache: 22.11.2000

Berlin 2000

D 83

Meiner Großmutter,
Elfriede Meyer

Danksagung

Prof. Dr. Ulrich Szewzyk gilt mein besonderer Dank für die Überlassung des Themas, die vielen wertvollen Anregungen, seine stete Diskussionsbereitschaft und das Interesse, dass er allen Problemen und Ergebnissen dieser Arbeit entgegenbrachte.

Prof. Dr. Heribert Cypionka danke ich für die Bereitschaft, als Gutachter zur Verfügung zu stehen und für die Möglichkeit, einige Versuche in seinem Labor durchzuführen.

Dr. Henrik Sass ein Danke für die Hilfe bei den Versuchen zur Sauerstoffatmung der Sulfat-Reduzierer.

Dr. Werner Manz möchte ich für die Einführung in die Technik der Hybridisierung danken.

Allen Mitarbeitern des Fachgebietes Ökologie der Mikroorganismen möchte ich meinen Dank aussprechen für das angenehme Arbeitsklima und vielfältige Formen der Unterstützung, besonders den Kolleginnen im Raum 505.

Meiner Familie und Gustav Creydt danke ich für die Ermutigung und den Rückhalt, den sie mir gaben.

Abstract

Karen Bade

Survival of sulfate-reducing bacteria in oxic oligotrophic environments related to drinking water

In this study the survival of sulfate-reducing bacteria in oxic, oligotropic habitats related to drinking water was investigated in a polyphasic approach.

Use of cultivation techniques led to the isolation of four sulfate-reducing species from the ground- and of two species from the drinking water systems of Berlin and Mülheim. Phylogenetic analysis revealed that four of the hitherto unknown species belonged to the *Desulfovibrionaceae* (strains zt3l, Mlhm, zt10e and GWE2) and two to the genus *Desulfosporosinus* (strains 5apy and Blif). Two isolates were selected for a further characterization.

The oxygen tolerance ot two of the newly isolated strains was investigated by exposition to oxygen and nutrient limitation in batch culture experiments in (i) sterile Berlin drinking water; (ii) mineral medium; and (iii) in coculture experiments with the aerobic *Aquabacterium commune*. Additionally they were compared with two strains from a culture collection, *Desulfomicrobium baculatum* (DSM 1743) and *Desulfovibrio desulfuricans* (DSM 6949), using a polyphasic approach including total cell counts, anaerobic cultivation and fluorescent in situ hybridization combined with digital image analysis. Additional experiments elucidated the influence of the culture age on the loss of culturability. Tests performed to resuscitate a ‘non-culturable’ sulfate reducer failed to succed.

The survival of sulfate-reducing bacteria in biofilm environments was then investigated in model biofilms grown in self-developed continuous culture systems. For the generation of the model system further characteristics of the in situ abundant aerobic species in the Berlin drinking water were investigated, with regard to their metabolism, the production of signalling molecules and their behaviour in biofilms. Two species, strain B3 and *Aquabacterium commune*, were selected for biofilm experiments. The ability of sulfate-reducing bacteria to establish in already developed biofilms was tested by addition of *Desulfovibrio desulfuricans* (DSM 6949) to a biofilm of *Aquabacterium commune* and exposition of this biofilm to the drinking water flow. In further experiments not only the presence but also the culturability of strain zt3l and strain zt10e was confirmed in aerobic biofilms. In situ hybridization and culturability were also used to demonstrate the ability of strain zt10e to survive and spread starting from an oligotrophic, oxic habitat. In a two stage reactor the re-attachment of detached biofilm cells in an already developed biofilm was observed.

Abstract

Karen Bade

Überleben von sulfatreduzierenden Bakterien unter oxischen, oligotrophen, dem Trinkwasser ähnlichen Bedingungen

In dieser Arbeit wurde das Überleben von sulfatreduzierenden Bakterien in oxischen, oligotrophen Habitaten mittels eines mehrstufigen Ansatzes untersucht.

Kultivierung führte zur Isolierung von vier sulfatreduzierenden Stämmen aus Grund- und von zwei Stämmen aus Trinkwasserverteilungssystemen in Berlin bzw. Mülheim. Die phylogenetische Einordnung der Stämme ergab, dass vier der bis dahin unbekannten Stämme den *Desulfovibrionaceae* (Stamm zt3l, Mlhm, zt10e und GWE2) zugerechnet werden konnten, während zwei weitere dem Genus *Desulfosporosinus* (Stamm 5apy und Blif) zugeordnet wurden.

Für eine weitergehende Charakterisierung der Sauerstofftoleranz der Sulfat-Reduzierer wurden zwei der neu isolierten Stämme (zt3l und zt10e) in Batch-Kulturen Sauerstoffstress und Nährstoffarmut ausgesetzt, und zwar in (i) sterilem Berliner Trinkwasser, (ii) Mineralmedium und (iii) Co-Kultivierungsansätzen mit dem aeroben *Aquabacterium commune*. Zwei Stämme aus Stammsammlungen, *Desulfomicrobium baculatum* (DSM 1743) und *Desulfovibrio desulfuricans* (DSM 6949), wurden in den Vergleich mit einbezogen, der mittels der direkten Bestimmung der Gesamtzellzahl, anaerober Kultivierung und in situ Hybridisierung kombiniert mit digitaler Bildanalyse durchgeführt wurde. Weitere Experimente belegten den Einfluß des Kulturalters auf den Verlust der Kultivierbarkeit. Der Versuch einer Reaktivierung von ‚unkultivierbaren' Sulfatreduzierern erwies sich als erfolglos.

Das Überleben von sulfatreduzierenden Bakterien unter natur-ähnlichen Bedingungen wurde anschließend in Modell-Biofilmen untersucht, die in selbst-entwickelten kontinuierlichen Systemen gezüchtet wurden. Dazu wurde der Stoffwechsel, die Produktion von Signalmolekülen und das Verhalten in Biofilmen von häufig im Berliner Trinkwasser vorkommenden aeroben Bakterien untersucht. Zwei Arten, der Stamm B3 und *Aquabacterium commune*, wurden für die nachfolgenden Biofilmversuche ausgewählt. Die Fähigkeit von Sulfatreduzierern in einem aeroben Biofilm zu Überleben wurde durch die Zugabe von *Desulfovibrio desulfuricans* (DSM 6949) zu einem aus *Aquabacterium commune* bestehenden Biofilm getestet, der anschließend dem Trinkwasser ausgesetzt wurde. In weiteren Versuchen wurde nicht nur die Anwesenheit sondern auch die Kultivierbarkeit der Stämme zt3l und zt10e in aeroben Biofilmen nachgewiesen. In situ Hybridisierung und Kultivierung zeigten auch die Fähigkeit von Stamm zt10e in einem oxischen, oligotrophen Habitat zu überleben und sich aus diesem Habitat heraus zu verbreiten. In einem zweistufigen Reaktor konnte die Wiederansiedlung von abgelösten Zellen beobachtet werden.

Table of contents

Results

Discussion

Abbreviations

A	adenine
AHL	*N*-acyl homoserine lactones
C	cytosine
CTC	5-cyano-2,3-ditolyltetrazoliumchloride
Cy3	5,5'-disulfo-1,1'di(γ-carbopentynyl-)3,3,3',3'-tetramethyl-indolocarbocyanine (cyanine Cy3.18
DAPI	4',6-diamidino-2-phenylindole
dH_2O	deionized water
DOC	dissolved organic carbon
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
DNA	desoxynucleic acid
DVC	direct viable count
EPS	exopolymeric substances
FISH	Fluorescent in situ hybridization
FLUOS	5(6)-carboxyfluorescein-*N*-hydroxysuccinimide-ester
G	guanine
HLA	homoserine lactone analog
HSL	mixture of different *N*-acyl-homoserine lactone analogs
MALDI-TOF	Matrix assisted laser desorption ionization time of flight
N	cell counts
NTA	nitrilo-triacetic acid
OD	optical density
PAC	probe active counts
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE-HD	polyethylene high-density
PE-LD	polyethylene low-density
RNA	ribonucleic acid
rDNA	ribosomal desoxynucleic acid
rRNA	ribosomal ribonucleic acid
SDS	sodium dodecyl sulfate
SE	standard error

SRB	sulfate-reducing bacteria
T	thymine
Tris	tris-(hydoxymethyl)-aminomethane
Tween 40	polyoxyethylenesorbitan monopalmitate
Tween 80	polyoxyethylenesorbitan monooleate
U	unit
μ	growth rate
ν	division rate
v/v	volume/volume
VBNC	viable but non culturable
X-Gal	5-bromo-4-chloro-3-indolyl β–D-galactopyranoside
w/v	weight/volume

Introduction

Sulfate-reducing bacteria (SRB), a heterogenous group of anaerobic heterotrophs, are known to be implicated in cases of microbially influenced corrosion arising in a wide range of natural and industrial systems. In accordance with the universal strategy of microorganisms to attach to substrates and to grow in consortia with a variety of species, SRB are able to adhere to inert surfaces and develop biofilms, also together with aerobic microorganisms (Characklis and Cooksey, 1983; Costerton et al., 1987). Especially the accumulation of SRB in such mixed population biofilms has been correlated with corrosion (Bryant et al., 1991) in industrial water supplies (Tatnall, 1990; Coutinho et al., 1994) and drinking water distribution systems (Tuovinen et al., 1980; Tuovinen and Hsu 1982), regardless of potentially high indigenous oxygen concentrations.

The first pure culture of SRB was isolated in 1895 by Beijerinck, who also proved the process of sulfate reduction to be catalysed by bacteria. *Spirillum desulfuricans*, as the organism was called, was highly sensitive towards oxygen and was described as strictly anaerobic. However, in the last years, the presence and metabolic activity of SRB could be demonstrated in many oxic and periodically oxic habitats. In first studies about SRB in oxic sediment their presence was explained by anoxic microenvironments, enhancing the survival of SRB (Jørgensen, 1977), although the natural occurrence of these microenvironments has never been directly demonstrated. In the following years the number of studies reporting the presence of SRB in habitats with different degrees of oxygen exposure increased. Apart from marine sediments (Laanbroek and Pfennig, 1981; Jørgensen and Bak, 1991) numerous studies reported the occurrence of SRB or their metabolic activity in oxic regions of microbial mats (Canfield and Des Marais, 1991; Fründ and Cohen, 1992; Minz et al., 1999) and in aerobic waste water treatment plants (Kühl and Jørgensen, 1992; Ramsing et al., 1993; Lens et al., 1995; Santegoeds et al., 1998; Manz et al., 1998), although their activity could not be confirmed in aerated sludge flocs (Schramm et al., 1999). In freshwater habitats the sulfate-reducing activity of SRB was shown (Smith and Klug, 1981) as well as their presence (Bak and Pfennig, 1991) even under oligotrophic conditions and with a peak of the number of SRB at the oxic/anoxic sediment interlayer (Sass et al., 1997) and with a high diversity (Sass

et al., 1998). Common features of this habitats were high numbers of aerobic organisms in microbial mats, biofilms or sediments or the presence of surfaces in form of particles in sediments or even in the freewater phase (Teske et al., 1996). The positive influence of particle association on the survival of SRB in oxic habitats was previously proven by Fukui and Takii (1990).

The effect of particle association or adhesion to surfaces seems also to be important for the presence of SRB in drinking water distribution systems. In contrast to most aerobic habitats supporting the presence of SRB investigated so far, drinking water is characterized not only by highly oligotrophic conditions, but also by low numbers of bacteria and particles, which might result in highly unfavorable growth or even survival conditions for SRB. Nevertheless, Tuovinen and Hsu (1982) isolated SRB from ‘tubercles’, a biological mound which covers pits in steel (Tatnall, 1990), on pipe surfaces of the Columbus, Ohio, drinking water system.

But SRB were not only detectable in oxic habitats, additionally several studies proved their physiological tolerance towards oxygen as well. Hardy and Hamilton (1981) detected culturable cells of *Desulfovibrio vulgaris* after incubation in oxygenated seawater for 72 hours. In another investigation few percent of SRB remained culturable for more than fifty days after exposure to air on the surface of solidified mineral medium (Wall et al, 1990). Cypionka et al. (1985) exposed several taxonomic groups of SRB to oxygen and determined differences in the survival rates. Oxygen sensitivity was increased considerably by the presence of sulfide or sulfhydryl group-containing agents. In chemostat experiments low oxygen concentrations resulted in an increase of the NADH oxidase, of the superoxide dismutase and of the respiration activity of *Desulfovibrio desulfuricans*, suggesting a defence mechanism in this organism (Abdollahi and Wimpenny 1990).

Finally the strict anaerobiosis of SRB was challenged by results obtained by Dilling and Cypionka (1990), who proved the ability of different SRB to respire with oxygen and gain ATP by this process. In the presence of different electron acceptors oxygen was even used prior to sulfate or other sulfur compounds (Krekeler and Cypionka, 1995), as substrates different organic and reduced sulfur compounds (Dannenberg et al., 1992; Kuhnigk et al., 1996) could be used. Nevertheless, pure cultures of SRB showed only weak growth in the presence of oxygen (Marschall et al., 1993).

All these findings make the presence of SRB in oxic habitats less unusual, although their behaviour in natural environment may differ signifcantly from results obtained in pure culture experiments.

In industrial systems the presence of SRB may have negative effects caused by the production of H_2S, which is also a possible precursor of odorants. Additionally SRB are able to enhance microbially mediated corrosion of materials. The microbial influence on corrosion is already well established (Hamilton, 1985; Ford and Mitchell, 1990), although many of the mechanisms are still not fully understood. Severe corrosion problems are caused by SRB in different technical systems as sewer systems, sewage treatment systems, in the pulp and paper industry (Odom, 1993), but especially in the oil industry (Coutinho et al., 1994a and 1994b). The corrosive effect of SRB seems to be amplified by the proximity to oxic/anoxic interfaces, which appears to stimulate maximal corrosion weight loss with associated deep pitting (McKenzie and Hamilton, 1992). In tubercles, the presence of SRB in aerobic drinking water systems could be determined (Tuovinen et al.,1980; Tuovinen and Hsu 1982). Tubercles present a unique ecological habitat. They are initially formed by microbiological deposition of iron and manganes oxides, which yields a large, localized mass of cells and minerals. The bacteria produce exopolymers that bind the mineral deposits together and limit diffusion of oxygen in and out of the developing tubercle. Steep chemical gradients are formed, which lead to an outer layer with a near neutral pH, while inner regions are highly reduced and acidic. A diverse microbial community can therefore coexist within the tubercle ecosystem (Ford and Mitchell, 1990). The tuberculation of drinking water systems may have a number of undesirable features as the possible release of opportunistic pathogens, toxins and slugs of toxic metals to the water, the imaginable esthetic degradation in form of odour or taste problems and the loss of hydraulic capacity (Tuovinen et al., 1980).

In many natural and artificial systems more metabolically active bacteria are found to be associated with interfaces than in the free water phase. The differences between attached and planktonic cells led to an increase in research focussed on surface colonization and the formation of biofilms. But although there is a consensus that surfaces do influence bacterial metabolism, the experimental observations are not always consistent, with studies reporting metabolic activities at interfaces being enhanced, inhibited, or unaffected (van Loosdrecht et al., 1990) in comparison to

planktonic cells. The lack of experimental consistency is probably due to the great variations in the tested substrata, the kind and concentration of the nutrients and the condition of the investigated microorganisms. In oligotrophic habitats, most investigators reported increased metabolic activities of the adhered microbial population (ZoBell, 1943; Kjelleberg et al., 1982; Marshall, 1988; Manz et al., 1993; Kalmbach et al., 1997a).

A typical oligotrophic habitat is drinking water and the related distribution network, where low contents of assimilable organic carbon (LeChevallier et al., 1987; van der Kooij, 1992) or the insufficient availability of phosphorus (Miettinen et al., 1996; Miettinen et al., 1997) may lead to limited bacterial growth. Nevertheless, the formation and presence of biofilms in drinking water systems has been frequently proven, both with microscopical techniques (McCoy et al., 1981; Ridgway and Olson, 1981; Costerton et al., 1986; Pedersen, 1990; Kalmbach et al., 1997a and b) and with cultivation methods (LeChevallier et al., 1987; Donlan et al., 1994; Rogers et al., 1994).

An important point for the survival of SRB in oxic biofilm systems as demonstrated in waste water systems (Ramsing et al., 1993; Santegoeds et al., 1998), is the coexistence with the aerobic autochthonous microbial population. Kalmbach and coworkers (1997b) investigated biofilms in the Berlin drinking water system by fluorescent in situ hybridization (FISH) and demonstrated the in situ dominance of the beta-*Proteobacteria*. As a molecular tool, FISH not only provides phylogenetic and therefore ecological information, but can also be correlated with the metabolic activity of bacteria by microfluorimetry (Schaechter et al., 1958; DeLong et al., 1989). The development of automated digital image analysis (Poulsen et al., 1993; Møller et al., 1995) enabled the quantification of the fluorescence intensities emitted from hybridized single cells on a reproducible and unbiased basis. Nevertheless, no general statement on the metabolic activity of SRB can be made on the basis of the detection by FISH. Several investigators found no sulfate-reducing activity of SRB although their ribosome content made them detectable with specific oligonucleotide probes in oxic habitats (Santegoeds et al., 1998; Schramm et al., 1999).

Other methods used to elucidate the metabolic state of bacteria in their natural environment are for example based on the reduction of fluorochromes as 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) (Rodriguez et al., 1992) or on the division repressing effect of gyrase inhibiting antibiotics (Kogure et al., 1979). The method

was modified by Kalmbach et al. (1997a) to the probe active count (PAC) method, by combining the effect of antibiotics with the detection of metabolically active cells with FISH. Investigations performed with these methods in drinking water revealed that the amount of metabolically active cells was much higher than heterotrophic plate counts (Schaule et al., 1993; Coallier et al., 1994; Kalmbach et al., 1997a). In oligotrophic systems such as drinking water, usually less then 1% of the bacterial population can be cultivated (Staley and Konopka, 1985), a number also found for the percentage of culturable SRB related to the number of total SRB from estuarine sediment (Gibson et al., 1987), although Bak and Pfennig (1991) claimed higher cultivation rates calculated on basis of the sulfate respiration rate. One reason for this inability of bacteria from oligotrophic ecosystems to form colonies might be due to individual cells having entered a viable but nonculturable (VBNC) state (Roszak and Colwell, 1987; Oliver, 1993).

Thus, for investigations of natural and laboratory scale systems, a combination of molecular and cultivation based methods should be used. FISH provides the appropiate tool for the in situ identification and enumeration of bacteria in the environment, while pure cultures provide data about the physiological capacities and abilities of isolated organisms.

In the last years several investigations proved the presence of SRB in oxic habitats, their oxygen tolerance and their ability to cause biocorrosion. Environmental conditions as high numbers of aerobic organisms or the possible adhesion to surfaces enhanced their ability to survive in aerobic systems. In oligotrophic drinking water systems, characterized by a low bacterial density and few particles, the presence of SRB in biofilms was demonstrated, but no data are available on the distribution and proliferation of SRB in this habitat.

As an oligotrophic, oxic habitat the Berlin ground- and drinking water system, which has been extensively described by molecular and cultivation based methods, was chosen. In a first step to demonstrate the presence of SRB in this system in situ hybridization with oligonucleotide probes and cultivation based methods for the isolation of typical SRB were to be used. Physiological and taxonomical features of the isolated SRB were to be scrutinized, particularly with regard to their oxygen tolerance. The development of specific 16S rDNA probes should be performed to facilitate further investigations of the metabolic potential and the presence of SRB in

different systems. In a model system resembling conditions relevant during their distribution in drinking water the survival and metabolic activity of SRB should be characterized. For the investigation of their survival in biofilms methods to allow the cultivation of defined mixed species biofilms were to be developed. The ability of SRB to colonize surfaces and biofilms under oxic conditions and their possible detachment under different nutrient conditions should be investigated. Their spatial distribution within the biofilms should be characterized in order to achieve a more comprehensive picture of the survival of SRB in this habitat.

Material and methods

1. Generation of biofilms in different devices

1.2 Robbins Device

Biofilms from the Berlin drinking water distribution system were obtained on glass and various plastic materials exposed in modified Robbins devices (McCoy et al., 1981; Manz et al., 1993; Kalmbach, 1998), which were installed in the Berlin drinking water distribution system. They consisted of stainless steel cylinders (180 by 150 mm) with 10 threaded holes (diameter 30 mm), each fitted with a stainless steel screw. Glass and plastic slides were mounted on the front of stainless steel screws by insertion into a 3 mm cleft on top of the srews, fixed by a stainless steel plate and aligned parallel to the water flow. Plastic materials used were polyethylene high-density (PE-HD, Hoechst, Frankfurt, Germany) and polyethylene low-density (PE-LD, neolab, Heidelberg, Germany). PE-LD slides were mounted together with a glass support slide in order to confer stability to the material.

The modified Robbins devices were installed at two different locations of the Berlin drinking water distribution system: in well 3 of the waterworks Jungfernheide, located immediately after the pumping station, and in Berlin-Lichterfelde, parallel to a main distribution pipe at a distance of about 5 km from the waterworks.

The raw water source at the water works Jungfernheide, Berlin, Germany, is mainly artificially recharged groundwater. The groundwater resources are replenished by infiltration of pre-treated water (coagulation/flocculation – sedimentation – filtration) from the river Spree (Heinzmann and Wascher, 1997). The hydrogeological situation is characterized by sand, gravel and tills of glacigenic origin. Further treatment of the groundwater involves aeration followed by rapid sand filtration without final chlorination, ozonation or UV treatment. The physical and chemical parameters of the raw water from well 3 of the waterworks Jungfernheide and of the Berlin drinking water were analyzed at the waterworks Jungfernheide according to standard procedures (Anonymous, 1990) and are summarized in Table 1.

Table 1: Chemical parameters of the Berlin raw and drinking water obtained from the waterworks Jungfernheide

Parameter (unit)	Raw water (well 3)	Drinking water	Tap water at the TU Berlin
Temperature °C	12.2	12.5	13.0
pH	7.3	7.4	7.5
O_2 (mg/l)	2.8	8.6	n.d.[b]
Total hardness °dH	20.2	20.2	n.d.
$KMnO_4$-O_2 (mg/l)	2.8	2.3	n.d.
DOC (mg/l)	3.9	3.5	5.3
Ca^{2+} (mg/l)	123	124	n.d.
Mg^{2+} (mg/l)	12.3	12.6	n.d.
Na^{2+} (mg/l)	45	50	n.d.
K^{+} (mg/l)	8.5	8.2	n.d.
Total Fe (mg/l)	0.78	0.03	n.d.
Total Mn (mg/l)	0.42	0.02	n.d.
F^{-} (mg/l)	0.21	0.19	n.d.
Cl^{-} (mg/l)	70	81	n.d.
NH_4^{+}-N	0.35	0.0	<0.05
NO_2^{-} (mg/l)	0.01	0.00	<0.05
NO_3^{-} (mg/l)	0.62	0.84	2.96
SO_4^{2-} (mg/l)	184	188	100
PO_4^{3-} (mg/l)	0.08	0.0	0.1

[a] DOC, dissolved organic carbon
[b] n.d. not determined

1.3 Centrifuge tube bioreactor

Artificial biofilms were generated in 15 ml polypropylene centrifuge tubes (Renner, Dannstadt, Germany). A silicon tube was connected to the lid of the centrifuge tube by melting a hole into the plastic material followed by direct insertion of the tube, which was fixed with silicone rubber. Into the bottom of the centrifuge tube a hypodermic needle (Ø 0,9 mm) was inserted as medium inlet, providing further coupling of gas permeable silicon tubes by Luer-connectors. As substratum glass slides, cut to a size of 8 x 13 mm, were positioned into the centrifuge tubes parallel to the medium flow. The centrifuge tube reactor was operated as an up-flow reactor with a total surface of 64.31 cm^2. The whole system was sterilized by autoclaving. The same principle was applied to 50 ml centrifuge tubes. Sterile medium was pumped (Ismatec, Wertheim-Mondfeld, Germany) from reservoir bottles to the biofilm

reactors, the effluent was collected in separate sterile containers. Bacterial colonization of the tubes into the reservoir bottles was prevented by heating the silicon tubes between reservoir bottle and pump to 80°C in a water bath. Afterwards the medium was cooled to room temperature. The biofilm reactors were inoculated by pumping the respective inoculum into the biofilm reactor until it was filled. Pumping was stopped for 24 hours, followed by a complete exchange of the medium by pumping through at least the fivefold of the reactor volume. Afterwards a constant minimum flow through of two reactor volumes per hour was adjusted. For hybridization the glass slides were aseptically removed and immediately fixed in 3.7% (v/v) formaldehyde solution as described below. For examination of culturable cell counts the slides were scraped off into 2 ml mineral medium and subjected to dilution series, followed by aerobic and anaerobic cultivation.

1.4 Continuous flow microchamber

One type of continuous flow microchambers used in the experiments was described by Szewzyk and Schink (1988). The microchamber comprised a central elliptical well of 2 ml volume which was milled into a plexiglass slide (80 x 35 x 5 mm). Two holes of 1 mm diameter drilled through the side of the plexiglass slide of the central chamber served as medium inlet and outlet. The other type was a modified construction of the flow cell described by Wolfaardt et al. (1994), which was used together with a bubble trap. The microchambers of this system had a size of 15 x 4 x 4 mm. The chambers of each system were covered with two glass cover slips (0.15 mm thick), which allowed direct microscopic investigation of biofilm development. All parts of the continuous-flow equipment except the chambers and the bubble trap were sterilized by autoclaving. Before starting an experiment, the tubes were connected to the plexiglass slides. In a first step 2% (v/v) H_2O_2 was pumped through the system and washed out with sterile dH_2O. Afterwards the system was filled with 70% (v/v) Ethanol and left for at least 2 h. The ethanol was washed out by pumping 100 ml of growth medium through the chamber. The chambers were inoculated via a side port septum adjacent to the inlet pipe. Pumping, collecting of effluent and the prevention of bacterial colonization of the tubes into the reservoir bottles was handled as described for the centrifuge tube bioreactor.

1.5 Combination of biofilm reactor and continuous flow microchamber

For the production of thicker biofilms a combination of different biofilm reactors was used (Fig. 1).

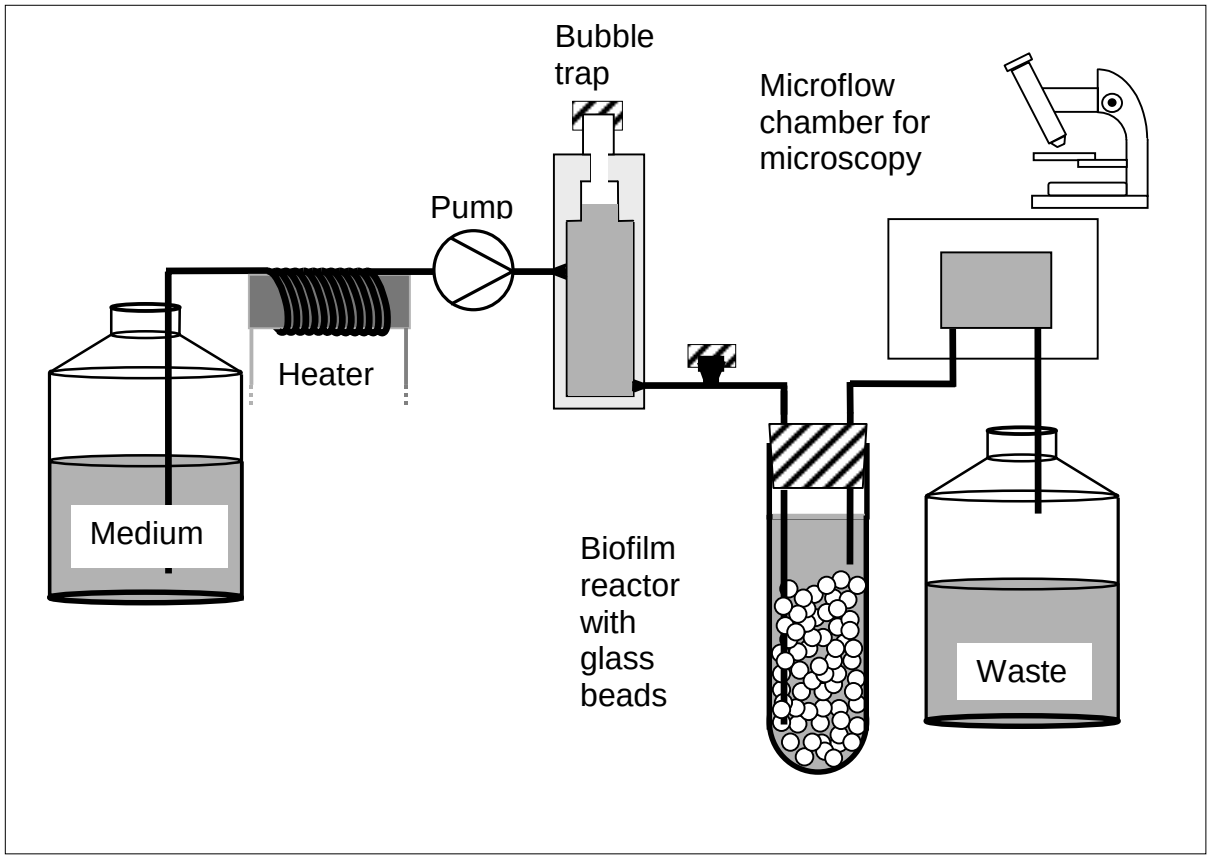


Figure 1: Schematic diagram of the combination of different continuous flow biofilm reactors.

The combination consisted of a glass test tube (Ø 16 mm x 98 mm), closed with a silicon stopper and filled with 15 g glass beads (Ø 3mm). The test tube was operated as an up-flow fixed bed reactor by means of hypodermic needles (Ø 0,9 mm) of different length. The total growth surface comprised 130 cm^2. A bubble trap was located between pump and inlet of the column. Between the outlet of the column and the waste container a continuous flow microchamber as described above was placed. All parts of the continuous-flow equipment were connected by gas permeable silicon tubes and were sterilized by autoclaving except the microflow chambers and the bubble trap. The handling of the system was performed as described above. For examination of cell counts 10 ml effluent was collected in intervals of 3 to 4 days in sterile containers on ice and subjected to dilution series. Samples were taken for the detection of total cell counts by DAPI-staining and for aerobic and anaerobic

cultivation. Cell counts and composition of the biofilm were examined after detaching the biofilm from the immobilisation material in a total volume of 10 ml of freshwater buffer. After dilution aerobic and anaerobic cultivation was performed, another fraction was immediately fixed in 3.7% (v/v) formaldehyde solution for hybridization. Spatial distribution of specific organisms in the biofilm was examined by fixation and hybridization of whole microchambers at the end of the experiment.

2. Media and culture conditions

2.1 Reduced media

Reduced media were prepared in special pressure proof glass vessels (Ochs, Bovenden, Germany), according to Widdel (1980). The vessel was autoclaved at 121 °C with loosened screw caps. The salt composition of the reduced freshwater medium consisted of 1 g NaCl, 0.2 g KH_2PO_4, 0.27 g NH_4Cl, 0.41 g $MgCl_2 \cdot 6H_2O$, 0.52 g KCl and 0.15 g $CaCl_2 \cdot 2H_2O$ per liter dH_2O (Widdel and Bak, 1992), to the sulfate reducer medium 2.81 g Na_2SO_4 per liter (final concentration: 20 mM) were added. After autoclaving the reduced media were cooled and stirred under an N_2/CO_2 (80/20, v/v) atmosphere. The cold media were completed by adding from stock solutions (per liter): 30 ml $NaHCO_3$-solution (final concentration 2.5 g/l), 1 ml selenite/tungstate solution, 1 ml trace element solution SL 9, 1 ml vitamin-B_{12}-solution, 0.5 ml vitamin mixture, 0.1 ml resazurin as redox indicator, and 3 ml $Na_2S \cdot 9H_2O$-solution (final concentration 0.27 g/l) as reducing agent. Supplements were added aseptically, while escaping gas prevented the entry of air. The pH was adjusted to 7.0 - 7.2 by addition of sterile 2 M HCl-solution. The media were dispensed and stored in screw cap bottles.

$NaHCO_3$-solution

The suitable quantity of bicarbonate solution was prepared immediately before use from 84 g $NaHCO_3$ per liter dH_2O. The head space of the bottle and the solution were saturated with CO_2 by repeated flushing and shaking. The bottle was tightly closed with rubber-fitted screw caps and autoclaved.

Trace element and vitamin solutions

All solutions were prepared with dH_2O. The trace element solution SL9 and the selenite-tungstate solution were sterilized by autoclaving.

Trace element solution SL9 (modified from Tschech und Pfennig, 1984)	
Stock solution (1000 x):	
NTA	12.8 g/l
$FeCl_2 \cdot 4H_2O$	2 g/l
$ZnCl_2$	70 mg/l
$MnCl_2 \cdot 2H_2O$	80 mg/l
H_3BO_3	6 mg/l
$CoCl_2 \cdot 6H_2O$	190 mg/l
$CuCl_2 \cdot 2H_2O$	2 mg/l
$NiCl_2 \cdot 6H_2O$	24 mg/l
$Na_2MoO_4 \cdot 2H_2O$	36 mg/l
NaOH	ad pH 6.0
Dilution: 1 ml per liter medium	

Selenite-tungstate solution (Widdel, 1980)	
Stock solution (1000 x):	
NaOH	200 mg/l
$Na_2SeO_3 \cdot 5H_2O$	6 mg/l
$Na_2WO_4 \cdot 2H_2O$	8 mg/l
Dilution: 1 ml per liter medium	

Vitamin mixture (modified from Widdel (1980))	
Stock solution (2000 x):	
4-Aminobenzoic acid	40 mg/l
D (+)-biotin	10 mg/l
Nicotinic acid	100 mg/l
Ca-D (+) pantothenic acid	50 mg/l
Pyridoxine hydrochloride	150 mg/l
Thiaminchloride dihydrochloride	100 mg/l
Dilution: 0.5 ml per liter medium	

Vitamin-B_{12}-solution	
Stock solution (1000 x):	
Cyanocobalamine	50 mg/l
Dilution: 1 ml per liter medium	

Resazurin solution	
Stock solution (10000 x):	
Resazurin	5 mg/ml
Dilution: 0.1 ml per liter medium	

All vitamin solutions and the resazurin solution were filter sterilized and stored at 4 °C in the dark.

Sulfide solution

The sulfide solution was also prepared immediately before use. 0.9 g $Na_2S \cdot 9H_2O$ was anoxically dissolved in 10 ml boiled dH_2O, gassed with N_2, tightly closed with rubber-fitted screw caps and autoclaved.

Preparation of organic substrates and further additions

All stock solutions were prepared with MilliQ-water. The pH was adjusted to 7.0 ± 0.1 with HCl or NaOH. Heat-sensitive solutions were sterilized by filtration (pyruvate, fumarate, sulfite, Fe(III)-NTA, $FeSO_4$), all others were autoclaved. All solutions were stored at 4 °C in the dark.

ethanol, 1,2 propane-diol,	1 M
Fe(III)-NTA, $FeSO_4$	1 M

Prepared from sodium salts:

acetate, propionate, butyrate, fumarate, pyruvate, malate and succinate	
sulfide, sulfite, thiosulfate and nitrate	1 M
benzoate	0.5 M

Cultivation of *Desulfomicrobium baculatum* (DSM 1743) and *Desulfovibrio desulfuricans* (DSM 6949) was performed in sulfate reducer medium supplemented with lactate (10 mM) at the recommended temperatures of 30°C and 37 °C, respectively (DSMZ-catalogue).

2.2 Anoxic media

Anoxic media were prepared as described for the reduced freshwater medium, but without the addition of any reducing agents. The phosphate-buffered, anoxic medium was based on the salt solution described for the reduced freshwater medium. The medium was cooled and stirred under an N_2-atmosphere after autoclaving. The cold medium was completed by adding from stock solutions (per liter): 2 ml 0.5 M phosphate buffer and 1 ml trace element solution SL9. The pH was adjusted to 7.0 - 7.2 by addition of sterile 1 M NaOH-solution. The medium was dispensed and stored in screw cap bottles.

For the detection of iron oxidizing, nitrate reducing bacteria a bicarbonate buffered medium containing (per liter dH_2O) 0.3 g NH_4Cl, 0.05 g $MgSO_4 \cdot 7H_2O$, 0.4 g $MgCl_2 \cdot 6H_2O$, 0.6 g KH_2PO_4 and 0.1 g $CaCl_2 \cdot 2H_2O$ was prepared (Straub et al., 1998). After cooling under an N_2/CO_2 (80/20, v/v) atmosphere the medium was completed by adding 30 ml $NaHCO_3$-solution, 1 ml selenite/tungstate solution, 1 ml

trace element solution SL 9, 1 ml vitamin B_{12} solution and 0.5 ml vitamin mixture per liter medium. $FeSO_4$, nitrate and acetate were added to a final concentration of 10 mM, 4 mM and 0.5 mM, respectively. Upon the addition of $FeSO_4$ a white precipitate, most likely consisting of ferrous carbonate and phosphate, formed. The medium was dispensed under stirring and stored in screw cap bottles.

Phosphate buffer

0.5 M phosphate-buffer was prepared from 4 parts 0.5 M KH_2PO_4 mixed with 6 parts 0.5 M K_2HPO_4. After adjustment of the pH to 7.0 the buffer was autoclaved.

2.3 Aerobic media

Modified R2A medium (Kalmbach et al., 1999) was used for the cultivation of the drinking water bacteria strains B1, B2, B3, B5, as well as *Aquabacterium citratiphilum* (B4), *A. parvum* (B6) and *A. commune* (B8). The medium contained per liter dH_2O: 0.5 g yeast extract, 0.5 g Difco proteose peptone no. 3, 0.5 g casamino acids, 0.5 g glucose, 0.3 g sodium pyruvate, 0.3 g K_2HPO_4, 0.05 g $MgSO_4 \cdot 7H_2O$, the pH was adjusted to pH 7.2 by addition of K_2HPO_4 or KH_2PO_4. Soluble starch was replaced by 0.1% (v/v) Tween 80 (Sigma, Deisenhofen, Germany). For solid medium, 15 g agar (Difco, Augsburg, Germany) was added per liter modified R2A medium.

Agrobacterium tumefaciens was cultured in nutrient broth (Difco, Augsburg, Germany) or on nutrient agar, containing per liter dH_2O: 3 g beef extract, 5 g peptone and 15 g agar, respectively. Agar plates for *Agrobacterium tumefaciens* NTL4(pZLR4) were additionally supplemented with 30 µg gentamicin per ml. Tests for AHL-production were performed on AB-medium (Chilton et al., 1974), containing per liter dH_2O: 3 g K_2HPO_4, 1 g NaH_2PO_4, 1 g NH_4Cl, 0.3 g $MgSO_4 \cdot 7H_2O$, 0.15 g KCl, 0.01 g $CaCl_2$, 0.0025 g $FeSO_4$. The agar concentration was 7 g or 15 g per liter medium.

The mineral medium for stress experiments and for the generation of biofilms was based on the salt composition described for the reduced freshwater medium. For the generation of biofilms different quantities of R2A and 0.014 g Na_2SO_4 per liter were added and the medium was autoclaved. The pH was adjusted to pH 7.2 and 1.0 ml of 0.5 M phosphate-buffer per liter was added. Without the addition of R2A and Na_2SO_4 the medium was used as freshwater buffer and for stress experiments.

3. Sources and isolation of SRB

3.1 Sources

3.1.1 Berlin raw water

For isolation of SRB from Berlin raw water, 10 l water samples of raw water were taken from the distribution line connecting the pumping station of well 3 of the waterworks Jungfernheide with the aeration tower. Pressure drops led to the detachment of biofilm material from the pipe wall, characterized by the brownish colour of ferric oxides. 10 l samples were also taken from an anaerobic well (gallery R, well 3) at the waterworks Friedrichshagen. Cells were concentrated by centrifugation (8000 x g, 25 min, Sorvall RC-5B centrifuge, Sorvall, Bad Homburg, Germany) and subsequently enriched in sulfate reducer medium with different electron donors.

3.1.2 Berlin drinking water

A modified Robbins device was installed in Berlin Lichterfelde, parallel to a main distribution pipe at a distance of about 5 km from the waterworks. The flow through was adjusted to 180 l/h. Glass slides were removed after an exposure time of two months and immediately transferred into sulfate reducer medium with a mixture of electron donors.

3.1.3 Other water systems

For the isolation of SRB from the Duisburg drinking water system, biofilm was detached from a 28 year old PVC-tube by use of sterile glass-beads (size 0.5 mm) in June 1999. The biofilm sample was directly inoculated into the sulfate reducer medium, supplemented with the electron donor mixture.

3.2 Isolation of SRB

The enrichment medium for the SRB consisted of the sulfate reducer medium supplemented with lactate, (20 mM), ethanol, (10 mM), benzoate, (5 mM), acetate, (20 mM), propionate (10 mM) or a mixture of these electron donors. Growth of SRB in the enrichment cultures was determined by turbidity and by detection of sulfide according to Widdel (1980). 0.5 ml culture liquid was mixed with 2 ml of the reagent, consisting of 50 mM HCl and 5 mM $CuSO_4$. In the presence of sulfide a brownish

precipitate, CuS, was formed. The intensity of the brown colour gave a hint on the quantity of sulfide. Isolation of SRB was performed by use of deep agar dilution series (Widdel and Bak, 1992). For this purpose 30 g agar (Difco, Augsburg, Germany) per liter dH_2O was washed four times. After melting the suspension was dispensed in portions of 3 ml to thick walled test tubes (Ochs, Bovenden, Germany), which were closed with culture plugs, autoclaved and stored in plastic bags. For the preparation of dilution series 8 agar tubes were heated in boiling water to remelt the agar. The culture plugs were replaced by rubber stoppers (Reichelt Chemietechnik, Heidelberg, Germany) and the tubes were placed in a bath at about 45 °C. 6 ml prewarmed medium of 45 °C was added to each tube to produce an agar medium of 0.9%. After the addition of some dithionite crystals a suitable amount from the enrichment culture was added to the first tube, which was mixed by turning. An estimated amount was pipetted from this tube into the next and mixed again, which was repeated till the last tube. After each transfer the previous tube was placed in a cold water bath and subsequently gassed with N_2/CO_2 (80/20, v/v). For a second dilution series colonies were picked by means of a drawn Pasteur pipette. From this series pure cultures were picked and transferred into liquid medium. After isolation strains zt3l, Mlhm and Blif were cultivated with lactate as electron donor at a temperature of 30°C, strains zt10e and GWE2 with ethanol at 30°C and strain 5apy with pyruvate (20 mM) at 26°C.

4. Phenotypic characterization and physiological parameters

4.1 Cell morphology

4.1.1 Phase contrast

Wet mounts of cells grown to exponential phase (approximately 10^8 cells/ml) in sulfate reducer medium were observed on agarslides (Pfennig and Wagener, 1986) by phase-contrast microscopy (Axioplan, Zeiss, Oberkochen, Germany).

4.1.2 Gram staining

Gram-staining was performed using the Gram-color kit from Merck (Darmstadt, Germany) according to the recommended protocol.

4.1.3 Flagella staining

Staining of flagella was performed according to the protocol of Heimbrook et al. (1989). The flagella stain consisted of two solutions. Solution 1 contained 10 ml of 5% aqueous solution of phenol, 2 g of tannic acid and 10 ml of saturated aqueous solution of $AlK(SO_4)_2 \cdot 12H_2O$. 10 parts of this solution were mixed with 1 part of solution 2, containing 1.2 g crystal violet in 10 ml 95% ethanol. For the staining a wet mount was prepared on a glass slide and covered with a cover slip. After 5 to 10 min two drops of the stain were applied to the edge of the cover slip. The cells were examined for flagella after 5 to 15 min at room temperature. In modification of the original protocol the microscopic investigation was performed by phase-contrast with a Zeiss Axioplan (Oberkochen, Germany).

4.2 Catalase activity

For measuring catalase activity bacterial species were grown in liquid media. After centrifugation (13000 x g) pellets were resuspended on glass slides with a drop of 3% hydrogen peroxide. Bubbles indicated the presence of catalase.

4.3 Presence of desulfoviridin

Detection of desulfoviridin was performed according to Postgate (1959). 10 ml of a fully grown culture were centrifuged, the cells resuspended in 1 ml water. One drop of 20% NaOH was added and the suspension inspected immediately with light at 365 nm. A brilliant red fluorescence implied the presence of desulfoviridin.

4.4 Utilization of electron donors

Utilization of various electron donors was studied in sulfate reducer medium. The substrates were supplemented to final concentrations of 10 mM with ethanol, 1,2 propane-diol, formate, acetate, benzoate, fumarate, malate, succinate, lactate, pyruvate or fructose. Tween 80 and glycerol were used at final concentrations of 0.1% (v/v). H_2 was directly injected into the headspace of a N_2/CO_2 (80/20, v/v) gassed, septum closed bottle. Bacterial growth was determined in completely filled test tubes with screw caps by measurement of the optical density at λ = 588 nm with a Dr. Lange (Berlin, Germany) spectrophotometer and by determination of sulfide production as described above.

4.5 Fermentation and utilization of electron acceptors

Utilization of different electron acceptores was tested in reduced freshwater medium at final concentrations of 20 mM. Electron donors were 10 mM formate for strain zt3l and 10 mM ethanol for strain zt10e. Fermentation of 20 mM pyruvate or lactate was tested in reduced freshwater medium. Bacterial growth was determined by measurement of the optical density at λ = 588 nm as described above.

4.6 Aerobic respiration

The ability of pure cultures of SRB to use oxygen as electron acceptor was tested in a multi-electrode device based on an oxygen-electrode (HansaTech Bachofer, Reutlingen, Germany) according to Cypionka (1994). In the device a concomitant measurement of the sulfide concentration, the oxygen concentration, the pH and the redox potential was possible. Measurements were performed with washed cells (0.5 to 4 mg protein per ml) in 150 mM KCl. The obtained data were analysed by use of a Turbo Pascal program kindly provided by Prof. Dr. H. Cypionka (Institut für Chemie und Biologie des Meeres, Universität Oldenburg).

Measurements were performed at 20°C, the substrates were added from 1 M N_2-saturated stock solutions by use of Hamilton syringes. O_2 and H_2 were added from a 150 mM KCl-solution saturated with O_2 and H_2, respectively.

5. Genotypic characterization

5.1 Extraction of genomic DNA

For the extraction of genomic DNA, bacterial species were grown in liquid media. After centrifugation (13000 x g) the DNA was isolated by using the QIAamp Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

5.2 Amplification of the 16S rRNA genes

16S rRNA genes were amplified with the universal primers 616V and 1492R or 63f and 1387r (sequences and target sites given in Table 2). PCR mixtures contained 200 µM of each deoxynucleotide, 3 mM magnesium chloride, PCR buffer (10 mM Tris/HCl, 50 mM KCl, pH 8.0), 20 pM of each primer, 100 to 125 ng of genomic DNA, and 2.5 U of *Taq* polymerase (Boehringer, Mannheim, Germany). The PCR was performed in a Personal cycler (Biometra, Göttingen, Germany); the samples were

subjected to an initial denaturing step of 90 s at 96 °C, followed by addition of 2.5 U of *Taq* polymerase to each sample. The thermal profile consisted of 35 cycles of 30 s at 96 °C, 90 s at 52 °C, and 90 s at 72 °C, with an increment of 5 s. The amplification products were checked for the correct size by electrophoresis in 1% (wt/vol) agarose gels, the DNA-fragments were stained with ethidium bromide. The PCR products were purified with the QIAquick PCR purification kit (Qiagen).

Table 2: Oligonucleotides used as PCR-primers

Oligonucleotide[a)]	Common name	Sequence (5'-3')	Target organisms
S-D-Bact-0008-a-S-20	616V	AGA GTT TGA TYM TGG CTC AG	*Bacteria*
S-D-Bact-1492-a-A-18	1492R	CGG YTA CCT TGT TAC GAC	*Bacteria*
S-D-Bact-0063-a-S-21	63f	CAG GCC TAA CAC ATG CAA GTC	*Bacteria*
S-D-Bact-1387-a-A-18	1387r	GGG CGG WGT GTA CAA GGC	*Bacteria*
S-D-Bact-1114-a-A-15	699RII	RGG GTT GCG CTC GTT	*Bacteria*
S-D-Bact-0531-a-A-17	610RII	ACC GCG GCT GCT GGC AC	*Bacteria*
S-D-Bact-0515-a-S-17	610VII	GTG CCA GCA GCC GCG GT	*Bacteria*
S-D-Bact-0338-a-S-18	nonEUB	ACT CCT ACG GGA GGC AGC	*Bacteria*

a) Nomenclature according to Alm et al., (1996)

5.3 16S ribosomal DNA sequencing

Cycle sequencing of 16S ribosomal DNA was done with the BigDye Terminator Ready Reaction Kit (Perkin-Elmer Applied Biosystems, Weiterstadt, Germany) according to the manufacturer's protocol. The reaction mixture contained 5 µl DNA, 4 µl BigDye Kit and 1 µl primer (1 pM). Primers used in this study are summarized in Table 2. DNA amplification was performed in a Personal cycler (Biometra, Göttingen, Germany); the samples were subjected to an initial denaturing step of 2 min at 96 °C. The thermal profile consisted of 35 cycles of 10 s at 96 °C, 5 s at 50 °C, and 4 min at 60 °C. Samples were held at 4 °C until precipitation with ethanol/sodium acetate. 10 µl of sample was mixed with 90 µl of ultrapure water, 10 µl of 3 M sodium acetate, pH 4.6 and 275 µl of 96% ethanol, left at room temperature for 15 min to precipitate the extension products and centrifuged for 20 min at 13000 rpm. The DNA-pellet was washed with 250 µl of 70% ethanol, centrifuged again and dried at room temperature. Sequences were generated with an ABI373-sequencer (Perkin-Elmer Applied Biosystems, Foster City, USA) and analyzed with the sequence analysis software version 3.3 at Sequenzierservice Meixner (Berlin, Germany).

5.4 Reconstruction of phylogenetic trees

The phylogenetic analysis of the sequences was performed with the ARB software package (Strunk et al.,1995). The 16S rDNA sequences were aligned by using the Aligner tool of the ARB software package and manually corrected according to primary and secondary structure similarities. Distance matrices were constructed from the aligned sequences and corrected for multiple base changes at single positions by the method of Jukes and Cantor (1969). Phylogenetic trees were constructed by the neighbor joining method of Saitou and Nei (1987).

6. Fluorescent in situ hybridization

6.1 Cell fixation

6.1.1 Biofilms

PE-LD, PE-HD and glass slides were removed from the Robbins device or the centrifuge tubes, immediately placed in 3.7% (v/v) formaldehyde solution and incubated for 1 h at 4 °C. Slides were washed in phosphate buffered saline (PBS, containing per liter: 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, pH 7.2), air dried and stored at room temperature. Light microscopic examinations of randomly chosen biofilms on PE-LD, PE-HD and glass were performed prior to fixation in order to control eventual loss of cells or biofilm fragments. The detachment of parts of the thick layer of ferric oxides from 3 month old raw water biofilms grown on PE-HD during fixation and dehydration could usually not be prevented. It was assumed, that the composition of the detached parts corresponded with the still adhering biofilm. Glass slides from centrifuge tubes showed usually no cell loss.

6.1.2 Liquid cultures

Liquid cultures of bacterial strains were centrifuged at 10000 x g for 10 - 15 min. The pellet was resuspended in 1 ml 3.7% (v/v) formaldehyde solution and incubated for 1 h at 4 °C. Cells were washed with PBS, resuspended in a 1:1 mixture of PBS with ethanol and stored at -20 °C.

6.2 Oligonucleotides

The oligonucleotides used in this study and their specificity are listed in Table 3. Oligonucleotides labeled with 5(6)-carboxy-fluorescein-*N*-hydroxysuccinimide-ester (FLUOS) were purchased from TIB MOLBIOL (Berlin, Germany), labeled with the indocarbocyanine dye Cy3 from Metabion (München, Germany).

Table 3: Oligonucleotide probes, target-organisms and stringencies used in this study (FA = percentage formamide in hybridization buffer).

Probe [a, b]	Common name	Target-organisms	FA (%)	[NaCl] (mM)	Reference [c]
S-D-Bact-0338-a-A-18	EUB338	Domain *Bacteria*	20	250	A
S-D-Bact-0338-a-S-18	non-EUB338	serves as negative-control	20	250	
S-Sc-aProt-0019-a-A-17	ALF1b	α-subclass of *Proteobacteria*	35	88	B
S-Sc-bProt-1027-a-A-17	BET42a	β-subclass of *Proteobacteria*	35	88	B
S-St-IsoB3-0474-a-A-17	beta3	strain B3	40	62.4	C
S-St-IsoB8-0633-a-A-19	beta8a	*Aquabacterium commune*	40	62.4	C
S-St-IsoB8-0069-a-A-19	beta8b	*Aquabacterium commune*	50	31.2	C
S-Sc-gProt-1027-a-A-17	GAM42a	γ-subclass of *Proteobacteria*	35	88	B
S-F-Srb-0385-b-A-18	SRB385Db	Most members of delta subclass of *Proteobacteria* including *Desulfobacteriaceae*	35	88	D
S-G-Dsv-0214-a-A-17	DSV214	Members of the *Desulfovibrionaceae*, *Desulfomicrobium baculatum* (DSM 1743)	10	500	E
S-G-Dsv-0221-a-A-20	221	Members of the *Desulfobacteriaceae*	35	88	F
S-St-Dsbo-0224-a-A-19	DSBO224	*Desulfobotulus sapovorans*	60	15.6	E
S-G-Dsv-0407-a-A-18	DSV407	Members of the *Desulfovibrionaceae*	50	31.2	E
S-G-Dsb-0488-a-A-20	DSMA488	Members of the *Desulfobacteriaceae*	60	15.6	E
S-G-Dsb-0651-a-A-18	DSR651	Members of the *Desulfobacteriaceae*	35	88	E
S-G-Dsb-0658-a-A-18	DSS658	Members of the *Desulfobacteriaceae*	60	15.6	E
S-G-Dsb-0660-a-A-20	660	Members of the *Desulfobacteriaceae*	60	15.6	F
S-G-Dsv-0698-a-A-20	DSV698	Members of the *Desulfovibrionaceae*	35	88	E
S-G-Dsb-0985-a-A-19	DSB985	Members of the *Desulfobacteriaceae*	20	250	E
S-G-Dsv-1292-a-A-18	DSV1292	Members of the *Desulfovibrionaceae*, *Desulfovibrio desulfuricans* (DSM 6949)	35	88	E
S-St-zt3L-0468-a-A-21	3L468	strain zt3l	20	250	
S-St-zt10E-0458-a-A-21	10E458	strain zt10e	35	88	

[a] *Escherichia coli* numbering (Brosius et al.,1981)

[b] probe nomenclature as described by Alm et al., (1996)

[c] References:

A: Amann et al., (1990)
B: Manz et al., (1992)
C: Kalmbach et al., (1997b)
D: Rabus et al., (1996)
E: Manz et al., (1998)
F: Devereux et al., (1992)

6.3 Hybridization procedure

All oligonucleotide probes were diluted in sterile dH_2O to a concentration of 50 ng/µl and stored at -20 °C. Hybridizations were performed in a humid chamber at 46 °C for 1.5 to 4 h as described by Manz et al. (1993). The hybridization solution consisted of 5 ng/µl oligonucleotide probe, 0.9 M Nacl, 20 mM Tris/HCl (pH 8), 0.01% SDS, and the appropriate amount of formamide as given in Table 3. The slides were washed by immersion of the slides for 15 min in 46 °C washing solution containing 20 mM Tris/HCl, 0.01% SDS. The NaCl-concentration of the washing solution corresponded to the formamide concentration of the hybridization solution and is given in Table 3.

6.3.1 Biofilms

After fixation of the attached cells on PE and glass slides, the slides were dehydrated with increasing concentrations of ethanol (50, 80 and 96%, 3 min each). The slides were cut into several smaller pieces, which were mounted on glass slides with 15 µl of hybridization solution and overlaid with additional 50 µl of hybridization solution. Incubation and washing of the pieces was performed as described above. For total cell counts, biofilms were stained with 4',6-diamidino-2-phenylindole (DAPI) at a final concentration of 1 µg/ml according to Kalmbach et al. (1997a). Attached cells were stained by placing 50 to 100 µl of DAPI solution onto pieces of PE or glass slides and gentle rinsing after 5 min of incubation with dH_2O. For microscopic examination the pieces were mounted on glass slides, using the antifading reagent Citifluor AF2 (Citifluor Ltd., London, UK).

6.3.2 Liquid cultures

10 µl fixed cell suspension was placed on each cavity of a teflon-coated microscopic slide (Marienfeld, Bad Mergentheim, Germany), dried at 46°C and subjected to a heat-fixation. The slides were dehydrated with ethanol as described for biofilms. 10 µl of hybridization solution was placed on each cavity, incubated, washed, stained with DAPI and mounted with Citifluor.

6.4 Design and evaluation of specific oligonucleotide probes

Strain specific oligonucleotides for the isolated strains zt3l and zt10e were designed by using the Probe_Design tool of the ARB software package (Strunk et al., 1995). The ARB data set of 13807 complete and partial 16S rDNA sequences (http://isar.mpi-bremen.de, December 1998) was complemented with sequences of δ-subclass sulfate reducers and with sequences of sulfate-reducing bacteria from the *Heliobacterium* group from the National Center for Biotechnology Information GenBank (http://www.ncbi.nlm.nih.gov/Taxonomy). The database was scanned for probes as specific as possible, i.e. other bacteria should be discriminated by at least one mismatch in the target region. Potential candidates for analytical in situ probes were compared to the ARB database by using the Probe_Match tool of the ARB software package to search for organisms with complete homologies within the target sequences. The newly developed probes 3L468 and 10E458 were labeled with Cy3 and purchased from Metabion (München, Germany).

6.4.1 Determination of hybridization stringencies

The optimization of the hybridization stringency was performed as described by Manz et al. (1998). In situ hybridizations were performed as described above using target and non-target organisms displaying one to two mismatches within the target region. The hybridization stringency was adjusted by the stepwise addition of formamide to the hybridzation buffer in concentration steps of 5%. The sodium chloride concentration of the washing buffer was adjusted to the formamide concentration used in the hybridization buffer.

7. Microscopy and image processing

7.1 Epifluorescence microscopy

Epifluorescence microscopy was performed with a Zeiss Axioplan (Oberkochen, Germany) fitted with a 100 W high-pressure bulb and Zeiss light filter set no. 01 for DAPI (excitation 365 nm, dichroic mirror 395 nm, suppression 397 nm), no. 09 for Fluos (excitation 450-490 nm, dichroic mirror 510 nm, suppression 520 nm), and HQ light filter 41007 (AF Analysentechnik, Tübingen, Germany) for Cy-3-labelled probes (excitation 535-550 nm, dichroic mirror 565 nm, suppression 610-675 nm). Color micrographs were taken on Kodak EES 1600 color reversal film, black-and-white

micrographs were taken on Agfapan APX 25 ASA film, exposure times were 8-30 s. For statistical evaluation at least 10 microscopic fields (100 by 100 µm) and a minimum of 1000 cells were chosen randomly and enumerated. Statistical analysis (standard error (SE)) were done with SigmaPlot 4.0 for Windows (Jandel Scientific, Erkrath, Germany) software programs.

7.2 Acquisition of digital images

Digital images were captured with a 12-bit cooled slow scan charge coupled device (CCD) camera (Visicam 1280, Visitron systems, Puchheim, Germany), controlled by the MetaMorph Imaging Software (Universal Imaging Corp., USA) which was also used for the analysis of the acquired digital images. The exposure time was 100 ms. A shading correction for the removal of uneven illumination was performed, followed by a background subtraction. For the analysis of the signal intensity, all cells in the acquired digital image were evaluated. Before measurement, images were subjected to a noise reduction and to a sharpening process for the increase of contrast in order to facilitate edge detection. The light intensity was measured by determination of the average gray value, defined as the sum of gray values divided by the number of pixels. For each analysis, the average intensity of three different images was used. Statistical analysis was performed as mentioned above. The average fluorescence intensity at the beginning of the assay was normalized to 100, with all other values calculated depending on the initial value.

7.3 Image optimization

Photos were scanned with a Agfa Snapscan 310, slides with a Nikon Coolscan II (LS20) (Nikon, Düsseldorf, Germany). All images were optimized with Corel Photo Paint (Corel Inc., Ottawa, Canada).

8. Determination of total cell counts (DAPI staining)

Bacteria from liquid cultures were sampled by microfiltration of the appropriate dilution through polycarbonate membranes (25 mm diameter, 0.2 µm pore size, Millipore, Eschborn, Germany) on regenerated cellulose support membranes (0.45 µm pore size, Sartorius, Göttingen) by using a vacuum filtration unit (Sartorius). The cells were stained according to Kalmbach et al. (1997a) by placing 20 µl of DAPI

solution onto the polycarbonate membranes, followed by immediate mounting on glass slides using Citifluor. Microscopic enumeration was performed as described above.

9. Lectin staining

Lectin staining was used for the staining of extracellular polymeric substances (EPS) in raw water biofilms. It was performed on dried hybridized biofilms prior to the DAPI-staining. 50 to 100 µl of the staining solution at a concentration of 50 ng/µl was placed onto pieces of PE or glass slides and gently rinsed after 5 min of incubation with dH_2O, followed by drying and DAPI-staining as described above. All lectins were labeled with the fluorochrome FLUOS and purchased from Sigma (Deisenhofen, Germany). The lectins used in the experiment are listed in Table 4.

Table 4: Lectins used in this investigation and their specificities

Lectin from	Specificity (given by supplier)
Triticum vulgaris	N-acetyl-β-D-glucosaminyl residues
	N-acetyl-β-D-glucosamine oligomers
Tetragonolobus purpureus	α-L-fucose, α-L-fucosyl residues
Arachis hypogaea	β-D-galactose(1→3)glucuronic acid
Lens culinaris	α-D-mannosyl and α-D-glucosyl residues
Ulex europaeus	α-L-fucose
Bandeiraea simplicifolia	terminal α-D-galactosyl and N-acetyl-α-D-galactosaminyl residues
Canavalia ensiformis (Concanavalin A)	terminal α-D-mannosyl and α-D-glucosyl residues
Limulus polyphemus	N-acetylneuraminic acid, glucuronic acid, phosphorylcholine analogs
Pseudomonas aeruginosa	D-galactose and derivatives

10. Stressing of SRB

10.1 Preparation of cultures subsequently exposed to stress

Nutrient and oxygen stress conditions were exerted on cultures of SRB in Berlin drinking water, in mineral medium and in mineral medium in coculture with *Aquabacterium commune* (DSM 11901). Precultures of *Desulfomicrobium baculatum*

(DSM 1743), *Desulfovibrio desulfuricans* (DSM 6949), strain zt3l, strain zt10e were cultivated in sulfate reducer medium with the appropriate electron donor at the suitable temperature as described above. *Aquabacterium commune* was cultivated in modified R2A. The organisms were harvested in the end-log phase by centrifugation and washed two times in anoxic mineral medium or 0.2 µm-filtered Berlin drinking water, respectively. Washed cells were added to mineral medium or Berlin drinking water in Erlenmeyer vessels closed with silicon-caps (Ochs, Bovenden, Germany), which provide high diffusion rates of oxygen. Cell numbers were adjusted to approximately 10^8 cells/ml for each strain by the determination of the OD 580 based on calibration curves. One coculture experiment was performed with cell numbers adjusted to approximately 10^5 cells/ml for each strain. For the maintenance of the required aeration, Erlenmeyer vessels were rotated at 120 rpm on a rotary shaker at 25 °C.

10.2 Measurement of O_2

For the detection of oxygen in liquid cultures, samples were taken randomly. O_2 was measured with the CellOx 325 microelectrode (WTW, Weilheim, Germany).

10.3 Cell counts of culturable aerobic and anaerobic bacteria

Dilution series were prepared in anoxic mineral medium. For aerobic bacteria, suitable dilutions were spread on modified R2A-agar plates. For anaerobic bacteria 1 ml of each dilution was pipetted into 9 ml of the described agar medium, supplemented with the appropriate electron donors and 20 mM sulfate.

11. Determination of growth characteristics

Batch cultures of *Desulfomicrobium baculatum* (DSM 1743), *Desulfovibrio desulfuricans* (DSM 6949), strain zt10e and zt3l were cultivated in sulfate reducer medium with the appropriate electron donor. *Desulfovibrio desulfuricans* was cultivated at 37°C, all other organisms at 30°C. *Aquabacterium commune* and B3 were cultivated in modified R2A at room temperature (21°C). All media were inoculated with bacteria collected in the late exponential growth phase. The correlation between optical density and cell number was examined by measurement at λ = 588 nm with a spectrophotometer.

Growth of strain B3 and *Aquabacterium commune* was followed by measuring the optical density as described above and by determination of the total cell counts. The growth rate μ of exponentially growing cultures of *Aquabacterium commune* and B3 was calculated from the equation

$$OD(t) = OD_0 e^{\mu t} \qquad \text{(Equation 1)}$$

where OD(t) is the optical density at time *t* and OD_0 is the optical density at the start of the measurement.

Additionally the division rate ν for strain B3 and *Aquabacterium commune* was determined according to the equation:

$$\nu = \frac{\lg N - \lg N_0}{\lg 2\ (t - t_0)} \qquad \text{(Equation 2)}$$

where *N* is the cell count at time *t* and N_0 is the cell count at the start of the measurement, the time t_0.

The division rate of the strains zt3l and zt10e was determined for growth at room temperature (21°C) in the above described media.

12. Activation of SRB

12.1 Biofilms and liquid cultures

The probe active count method (PAC) described by Kalmbach et al. (1997a) was modified using the antibiotic cocktail (ABC) proposed by Joux and LeBaron (1997). Biofilms on glass slides were incubated for 48 h, 96 h and 144 h in sulfate reducer medium supplemented with a mixture of electron donors (lactate, (20 mM), ethanol, (10 mM), benzoate, (5 mM), acetate, (20 mM), propionate (10 mM)). After incubation, biofilms were fixed in 3.7% (v/v) formaldehyde.

Bacteria from liquid cultures were incubated by mixing equal volumes of liquid culture with reduced mineral medium supplemented with the appropriate electron donors and a cocktail of different antibiotics (ABC). Final concentrations of antibiotics were: nalidixic acid 20 µg/ml, piromidic acid 10 µg/ml, pipemidic acid 10 µg/ml, cephalexin 10 µg/ml and ciprofloxacin 0.5 µg/ml. Stock solutions of antibiotics were prepared at concentrations of 10 mg/ml in 0.05 M NaOH and stored at -20 °C. The assay was performed in injection bottles, the headspace was flushed with N_2/CO_2 (80/20, v/v). Samples were taken under anoxic conditions with sterile syringes. Cells

were harvested onto polycarbonate membranes. Total cell counts and the cell lengths of 100 randomly chosen SRB were measured microscopically after DAPI-staining.

The same method was used for the examination of the effect of different *N*-acyl homoserine lactones (AHL) on the activation of *Desulfovibrio desulfuricans* (DSM 6949) in liquid cultures. Addition of medium and antibiotics was followed by admixture of different AHL's. The assays were supplemented with homoserine lactone (HLA) (0.5 pmol) or a mixture of different homoserine lactones (HSL) (1 pmol).

12.2 Synthesis of AHL

A homoserine lactone analog (HLA) and a mixture of various homoserine lactone analogs (HSL) was kindly provided by R. Kalnowski, Department of Environmental Microbiology and Hygiene, Technische Universität Berlin. HLA and HSL were synthesized according to the following protocol, which describes the synthesis of HSL.

11 mM of various acyl acid chlorides (Merck, Darmstadt, Germany) were used and mixed with cylic isopropylidene malanoate (Fluka, Deisenhofen, Germany) at a molar ratio of 10 to 11. The molar ratio of cylic isopropylidene malanoate to pyridine (Merck, Darmstadt, Germany) was 1 to 2. 60 mM (8.64 g) cylic isopropylidene malanoate was dissolved in 150 ml dichloromethane (Merck, Darmstadt, Germany). 11 mM of the following acyl acid chlorides (butyric chloride, 1.15 ml, hexanoic chloride, 1.51 ml, octanoic chloride, 1.83 ml, decanoic chloride, 2.21 ml, dodecanoic chloride, 2.62 ml, tetradecanoic chloride, 2.98 ml) and 120 mM (9.68 ml) pyridine were added. After gassing with N_2, the mixture was stored overnight at 4 °C in the dark. The mixture was washed with 60 ml 1 M hydrochloric acid (Merck, Darmstadt, Germany) and with 60 ml dH_2O. Evaporation of the washed solution resulted in a waxy substance. A part of the waxy substance was mixed with homoserinelactone hydrochloride (Sigma, Deisenhofen, Germany) at a molar ratio of 1.5 to 1, in this case 387.4 mg of the waxy substance was mixed with 137 mg homoserinelactone (1 mM) hydrochloride in 10 ml pyridine. In a second evaporation step superfluous pyridine was removed. The mixture was dissolved in methanol and stored at 4 °C in the dark. Based on a yield of 50% of *N*-acyl homoserine lactones 0.5 mM of the autoinductors were synthesized.

The molecular weight of HLA and HSL was examined with MALDI-TOF, which revealed the presence of several AHL-analogs with different molecular weights, given in Table 5, in HLA and HSL.

Table 5: Molecular weights and corresponding formulas of AHL-analogs from HLA and HSL

		Present in	
Molecular weight	Formula	HLA	HSL
358.2565	$C_{20}H_{40}NO_4$		x
386.2998	$C_{22}H_{44}NO_4$	x	x
414.3431	$C_{24}H_{48}NO_4$		x
442.3861	$C_{26}H_{52}NO_4$		x

13. Characterization of additional parameters influencing the biofilm community

13.1 Determination of *N*-acyl homoserine lactone production

The drinking water bacteria strains B1, B2, B3, B5, *Aquabacterium citratiphilum*, *A. parvum* and *A. commune* and the sulfate-reducing bacteria *Desulfomicrobium baculatum* (DSM 1743), *Desulfovibrio desulfuricans* (DSM 6949), strain zt3l and strain zt10e were tested for their ability to produce *N*-acyl homoserine lactones according to Shaw et al. (1997). By the same method the efficiency of the synthesized AHL was qualitively measured. The *Agrobacterium tumefaciens* test system was kindly provided by S.K. Farrand, Department of Microbiology, University of Illinois, and included *Agrobacterium tumefaciens* NT1(pTiC58Δ*accR*), constitutively synthesizing *N*-(3-oxo-octanoyl)-L-homoserine lactone, *Agrobacterium tumefaciens* NT as negative control and *Agrobacterium tumefaciens* NTL4(pZLR4) as reporter strain indicating the presence of different homoserine lactones. *Agrobacterium tumefaciens* NTL4(pZLR4) is a clone containing inserts form pTiC58 that encode a) a *traG*::*lacZ* fusion and b) *traR*. *Agrobacterium tumefaciens* NTL4(pZLR4) does not express its own AHL; consequently, the lacZ reporter fusion is not expressed unless an exogenous active AHL is added (Cha et al., 1998), only then a colour reaction is possible.

For the detection of AHL produced by SRB culture supernatants as well as extracts were tested. For the preparation of extracts, bacteria were removed by centrifugation. The supernatant was extracted twice with equal volumes of ethyl-acetate, then combined and dried over anhydrous magnesium sulfate, filtered and evaporated to dryness.

For the assay, soft agar suspensions of the reporter strain in AB minimal medium with 0.7% agar and containing 5-bromo-4-chloro-3-indolyl β–D-galactopyranoside (X-Gal) at 40 µg/ml were prepared. The suspension was overlayed onto a plate containing a base of AB minimal medium (1.5% agar) with mannitol (0.5 g/l) as carbon source and X-Gal. After the overlay had solidified a colony of the bacterium to be tested or 10 µl of supernatant or of the extracted supernatant was patched onto the surface. The plates were incubated overnight at 28 °C. A diffuse blue zone spreading out from the test colony or supernatant spot indicated a positive reaction. Agar plates without reporter strain were treated as described above to determine whether the samples were able to cause hydrolysis of the X-Gal. The ability of *Agrobacterium tumefaciens* NTL4(pZLR4) to detect AHL was controlled by testing a colony and culture supernatant of *Agrobacterium tumefaciens* NT1(pTiC58Δ*accR*).

13.2 Determination of iron-oxidation

The ability of the strains B1, B2, B3, B5, *Aquabacterium citratiphilum*, *A. parvum* and *A. commune* to reduce nitrate coupled with the oxidation of ferrous iron was tested in the bicarbonte-buffered anoxic medium as described above. Immediately before withdrawing samples, cultures were agitated to disperse iron precipitates homogeneously. Samples were taken with anoxic syringes. For the measurement of ferrous iron the sample was immediately acidified with HCl to a final concentration of 1 M. The sample was mixed with 0.1% (w/v) ferrozine in 50% (wt/v) ammonium acetate solution. Spectrophotometric detection of the ferrozine-iron complex was performed at λ = 562 nm (Stookey, 1970). The concentration of total iron was measured the same way after reduction of all iron in the sample to the ferrous state with 10% (w/v) hydroxylamine hydrochloride. Ferric iron concentration was calculated as the difference between measured total iron and ferrous iron.

Results

1. Investigation of the natural habitat

1.1. Detection of SRB in raw and drinking water systems

Berlin raw and drinking water represent oligotrophic habitats with different degrees of oxygen saturation. The raw water contained approximately 2.8 mg/l oxygen, while the drinking water is saturated with 8.6 mg/l. The presence of SRB in biofilms in this habitat was studied on different substrata exposed to the water flow in modified Robbins devices installed in the waterworks Berlin-Jungfernheide and in the main pipe in Berlin-Lichterfelde.

1.1.1 In situ hybridization of raw water biofilms with probes specific for Gram-negative SRB

In situ hybridization was performed with twelve 16S rRNA targeted oligonucleotide probes specific for mesophilic SRB within the δ-subclass of *Proteobacteria* developed in earlier studies. The probes and their specificities are given in Table 3. Hybridization was performed with young (14 days) as well as with old (up to 90 days) biofilms. Regardless of the age, the number of hybridization-positive cells was very low in all biofilms and their detection was rather incidentally. The best signals were obtained with the probe SRB385Db as shown in Fig. 2A. The low number and the random distribution of the hybridization-positive cells made a statistical evaluation of the cell number unreasonable. One reason for this result might be the fact that out of six newly isolated SRB from this habitat only two had target-sequences responding to the previously developed probes. Nevertheless, the newly developed probes 3L468 and 10E458 did not yield a particular different result. An additional reason might be the low metabolic activity of SRB in this unfavorable habitat, making the detection of SRB by in situ hybridization very difficult.

1.1.2 In situ hybridization combined with activation

For the improvement of this low rate of detectability the PAC-method developed by Kalmbach et al. (1997a) was modified and was performed with biofilms from Berlin-Jungfernheide (14 days old) and from Berlin-Lichterfelde (30 days old). An

improvement of the detectability of cells by in situ hybridization with probes specific for SRB was not detectable in any of the biofilms after any incubation period.

1.2 Description of a raw water biofilm

1.2.1 Interference microscopy of raw water biofilms

Young biofilms from Berlin raw water have already been described (Kalmbach, 1998). However, the small number of SRB detectable by in situ hybridization in the natural system was usually found in older biofilms. Therefore the microbial population of 90 days old biofilms from Berlin-Jungfernheide was described. A general characteristic of these biofilms were iron precipitates, which appeared frequently in combination with *Gallionella*-like structures (Fig. 2B). Light microscopic identification was also possible for other typical inhabitants, which belonged to the *Sphaerotilus-Leptothrix* group (Fig. 2B).

1.2.2 Determination of the population composition by in situ hybridization

The cell density and the phylogenetic affilitation of the adherent microbial population on different substrata was investigated by in situ hybridization with oligonucleotide probes targeting the domain *Bacteria* and the alpha-, beta- and gamma-subclasses of *Proteobacteria*. All oligonucleotides were labeled with the fluorochrome Cy3. For the investigation of substrata induced variations in the species composition three different materials were used: glass, PE-LD and PE-HD. The population composition obtained on the different substrata is shown in Table 6.

Figure 2: Raw water biofilm on glass surfaces exposed in a Robbins device connected to a tap at well 3 of the waterworks Berlin Jungfernheide. (A) In situ hybridization with Cy3-labeled probe SRB385Db of a 90 day old biofilm obtained in winter 1997 (left), and corresponding phase-contrast image (right), demonstrating the presence of Gram-negative SRB in the habitat. (B) interference contrast micrograph of a two weeks old biofilm obtained in summer 1996. Note the *Gallionella*-like structures, stained orange by ferric oxides, and the empty and filled sheathes of members of the *Sphaerotilus-Leptothrix*-group. (C) Lectin staining of 90 day old biofilm acquired in spring 1999 (left) with lectin from *Ulex europaeus* and corresponding phase contrast image (right), showing the presence of EPS within the filamentous structures which cover the biofilm. Scale bar equals 10 µm.

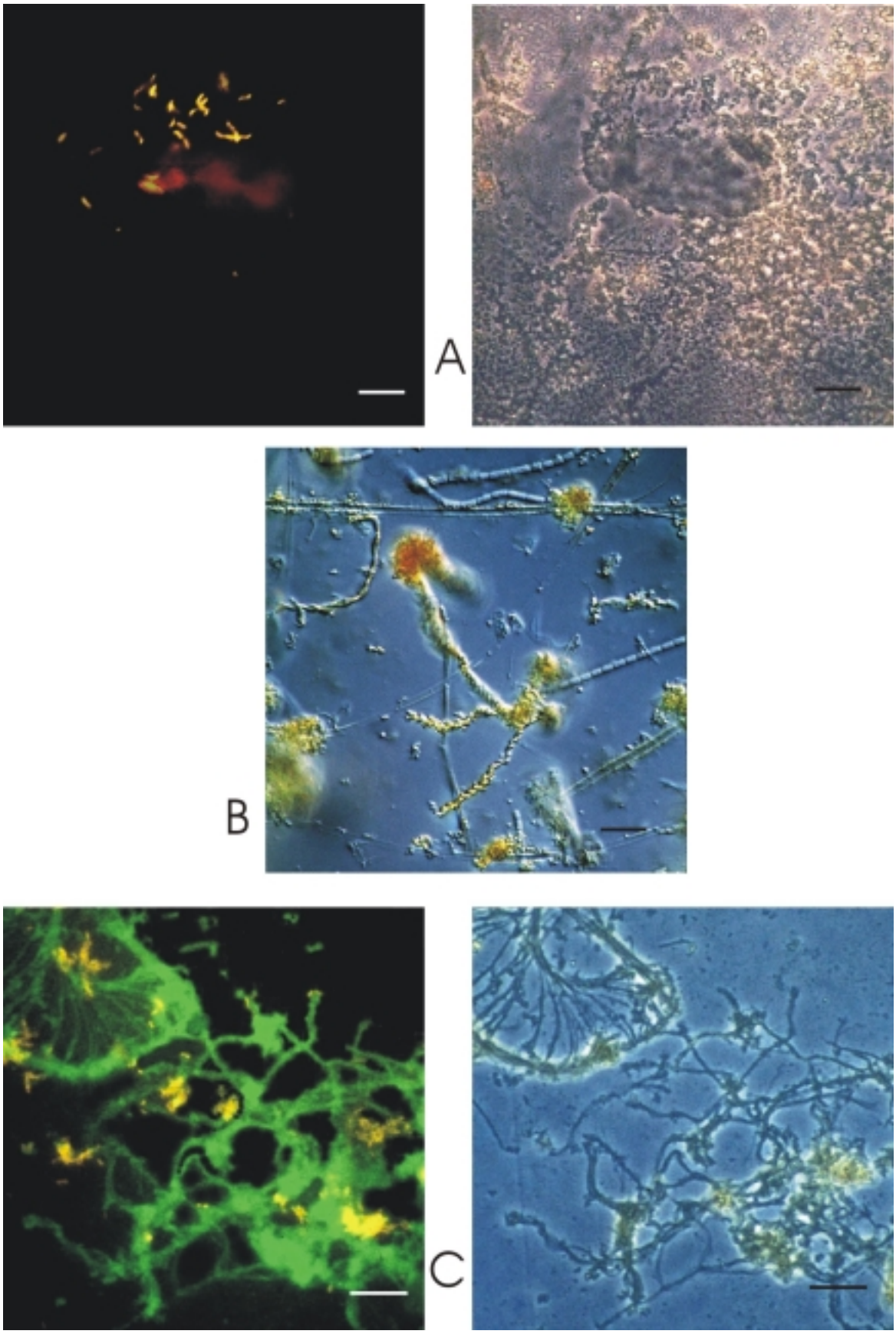

A
B
C

Table 6: Population composition of 90 day old biofilms obtained from raw water in Berlin-Jungfernheide in spring 1999.

	PE-LD	Glass	PE-HD
Cell counts by DAPI [per cm^2][a]	1.9×10^6 ($\pm 7 \times 10^5$)	7.4×10^5 ($\pm 1.2 \times 10^5$)	4.7×10^6 ($\pm 1.6 \times 10^5$)
Probe: ALF1b[b]	18.6 (± 5.7)	11.2 (± 4.7)	0.1 (± 0.1)
Probe: BET42a[b]	45.0 (± 6.1)	25.4 (± 4.5)	0.2 (± 0.1)
Probe: GAM42a[b]	5.6 (± 1.3)	8.5 (± 1.3)	0.1 (± 0.1)
Probe: EUB338[b]	64.4 (± 5.6)	45.0 (± 8.0)	1.7 (± 0.4)

a: Mean of all samples (SE of mean value)

b: Mean % of total bacteria (SE)

Remarkable was the low percentage of hybridization positive cells detectable on PE-HD, although the number of total cells exceeded those detected on the two other substrata. One reason might be the detachment of upper layers of the very thick biofilm grown on PE-HD during fixation and dehydration.

1.2.3 Lectin staining of raw water biofilms

Lectin staining of the EPS in raw water biofilms was possible with a variety of lectins with different specificities. A signal was observable with lectins from *Canavalia ensiformis* (Concanavalin A), *Ulex europaeus*, *Pseudomonas aeruginosa, Lens culinaris* and *Triticum vulgaris*. For the specificities of these lectins see Table 5. The fluorescence signal revealed the presence of stainable substances predominantly within the *Gallionella*-like structures, which traverse the whole biofilm. Probably these structures, together with the high amount of oxygen present in this habitat, are jointly responsible for the high amount of iron precipitates within the biofilms (Fig. 2C).

2. Isolation and characterization of SRB from raw and drinking water systems

2.1 Isolation

For isolation of SRB, material from different locations was sampled. Water samples from the waterworks Jungfernheide were taken in March and April 1996. The strains zt3l and zt10e were isolated after enrichment from cultures with the electron donors lactate and ethanol, respectively. Strain 5apy was isolated from the enrichment culture growing on acetate, but was subsequently cultivated on pyruvate,

resulting in faster growth and in higher cell numbers. Strain Blif was enriched from biofilms sampled in Berlin-Lichterfelde in November 1997 and was first cultivated in the described mixture of electron donors, followed by cultivation on lactate. Strain GWE2 was isolated after enrichment on ethanol from water samples taken at the waterworks Friedrichshagen in July 1998. Detachment of biofilm from a PVC-pipe in the drinking water system of Duisburg-Rheinhausen in June 1999 resulted in the isolation of strain Mlhm, which was subsequently cultivated with lactate as electron donor.

2.2 Phylogenetic characterization

2.2.1 16s rDNA based phylogeny

Comparative 16S rDNA sequence analysis identified the newly isolated strains zt3l, Mlhm, zt10e and GWE2 as members of the genus *Desulfovibrio*. The phylogenetic tree shown in Fig. 3 reflects the phylogenetic relationships of strain zt3l, Mlhm, zt10e and GWE2 within the *Desulfovibrionaceae*. The strains zt10e and GWE2 clustered with *Desulfovibrio alcoholovorans* (GenBank acc. no. AF053751), *Desulfovibrio fructosovorans* (GenBank acc. no. AF050101), *Desulfovibrio burkinensis* (GenBank acc. no. AF053752) and the sulfate-reducing bacterium R-SucA1 (GenBank acc. no. AJ012592). The strains zt3l and Mlhm clustered with *Desulfovibrio sp.* STL10 (GenBank acc. no. X99501) and the sulfate-reducing bacterium R-LacA1 (GenBank acc. no. AJ012593). The 16S rDNA sequence similarity matrix for the strains zt10e, GWE2, zt3l, and Mlhm and their next known relatives is given in Table 7.

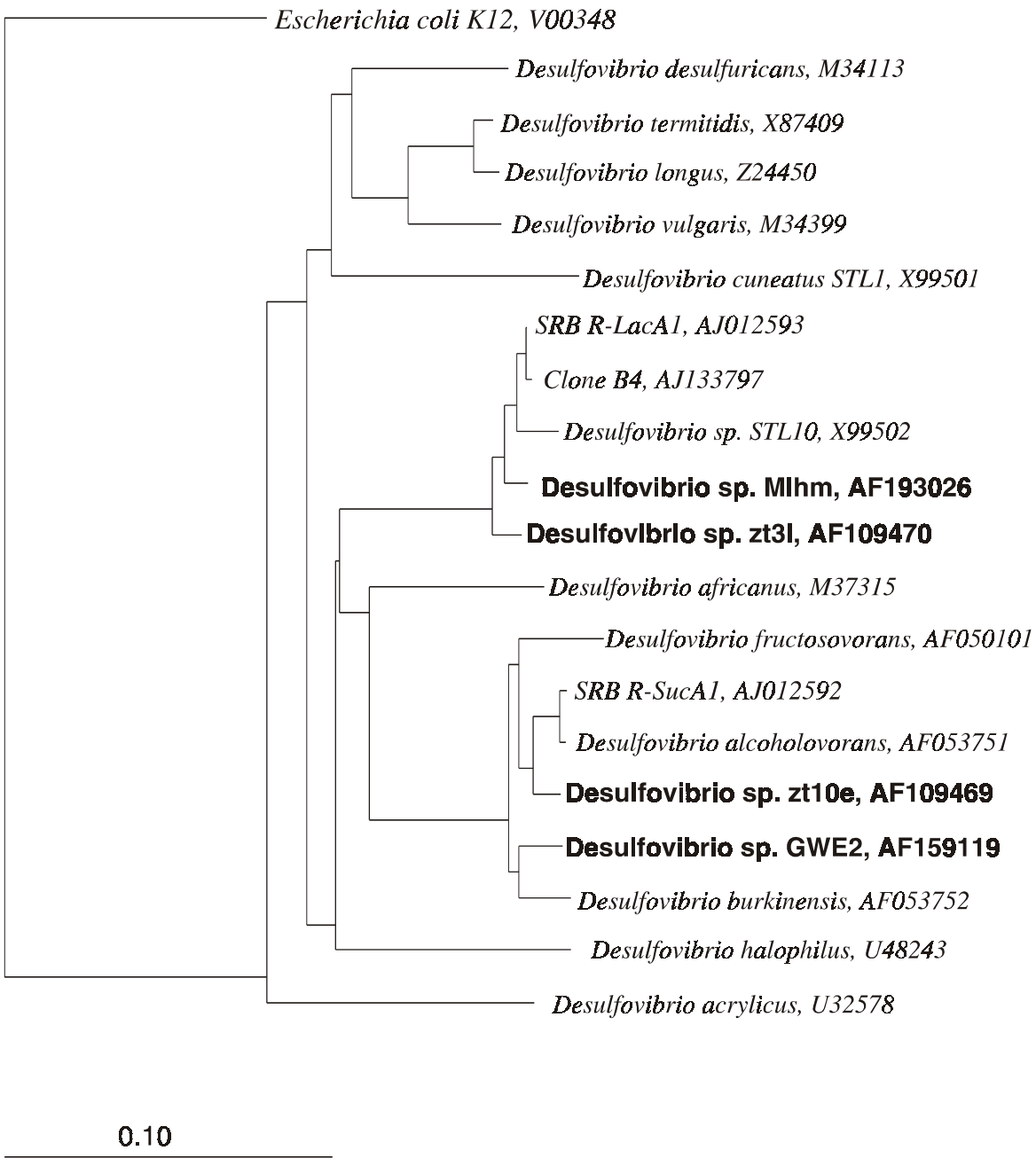


Figure 3: Phylogenetic tree inferred from 16S rDNA sequence data, showing the relationships of the four isolated strains belonging to the genus *Desulfovibrio*, zt3l, Mlhm, zt10e and GWE2, to their closest relatives. The scale bar represents 10 nucleotide substitutions per 100 nucleotides.

Table 7: 16S rDNA sequence similarity matrix for the strains zt3l, zt10e and GWE2 and their next known relatives

	% rDNA sequence similarity			
Taxon	zt10e	GWE2	zt3l	Mlhm
strain zt10e				
strain GWE2	97.5			
strain zt3l	87.3	88.3		
strain Mlhm	86.5	87.2	98.1	
Desulfovibrio sp. STL10	85.2	85.8	97.0	97.4
Clone B4	86.1	86.8	97.7	98.6
sulfate-reducing bacterium R-LacA1	86.3	87.0	97.6	98.7
sulfate-reducing bacterium R-SucA1	98.2	96.8	88.3	87.1
Desulfovibrio alcoholovorans	98.1	96.5	88.0	87.0
Desulfovibrio fructosovorans	96.3	95.6	86.0	84.5
Desulfovibrio burkinensis	96.9	97.1	87.3	86.8

The comparative 16S rDNA sequence analysis of the strains 5apy and Blif revealed their close relatedness with members of the genus *Desulfosporosinus*. The phylogenetic relationships of 5apy and Blif within this genus is shown in Fig. 4. The closest relatives of 5apy and Blif included *Desulfotomaculum auripigmentum* (GenBank acc. no. U85624), *Desulfosporosinus orientis* DSM 8344 (GenBank acc. no. Y11571), *Desulfosporosinus orientis* DSM 765 (GenBank acc. no. Y11570), and the *Desulfosporosinus sp.* T2 and S8 (GenBank acc. nos. AF076526 and AF076247), the similarity matrix is shown in Table 8.

Table 8: 16S rDNA sequence similarity matrix for the strains 5apy and Blif and their next known relatives

	% rDNA sequence similarity	
Taxon	5apy	Blif
5apy		
Blif	96.8	
Desulfosporosinus sp. T2	96.2	96.8
Desulfosporosinus sp. S8	96.4	96.9
Desulfotomaculum auripigmentum	96.0	97.0
Desulfosporosinus orientis DSM 765	96.4	97.2
Desulfosporosinus orientis DSM 8344	96.1	96.8

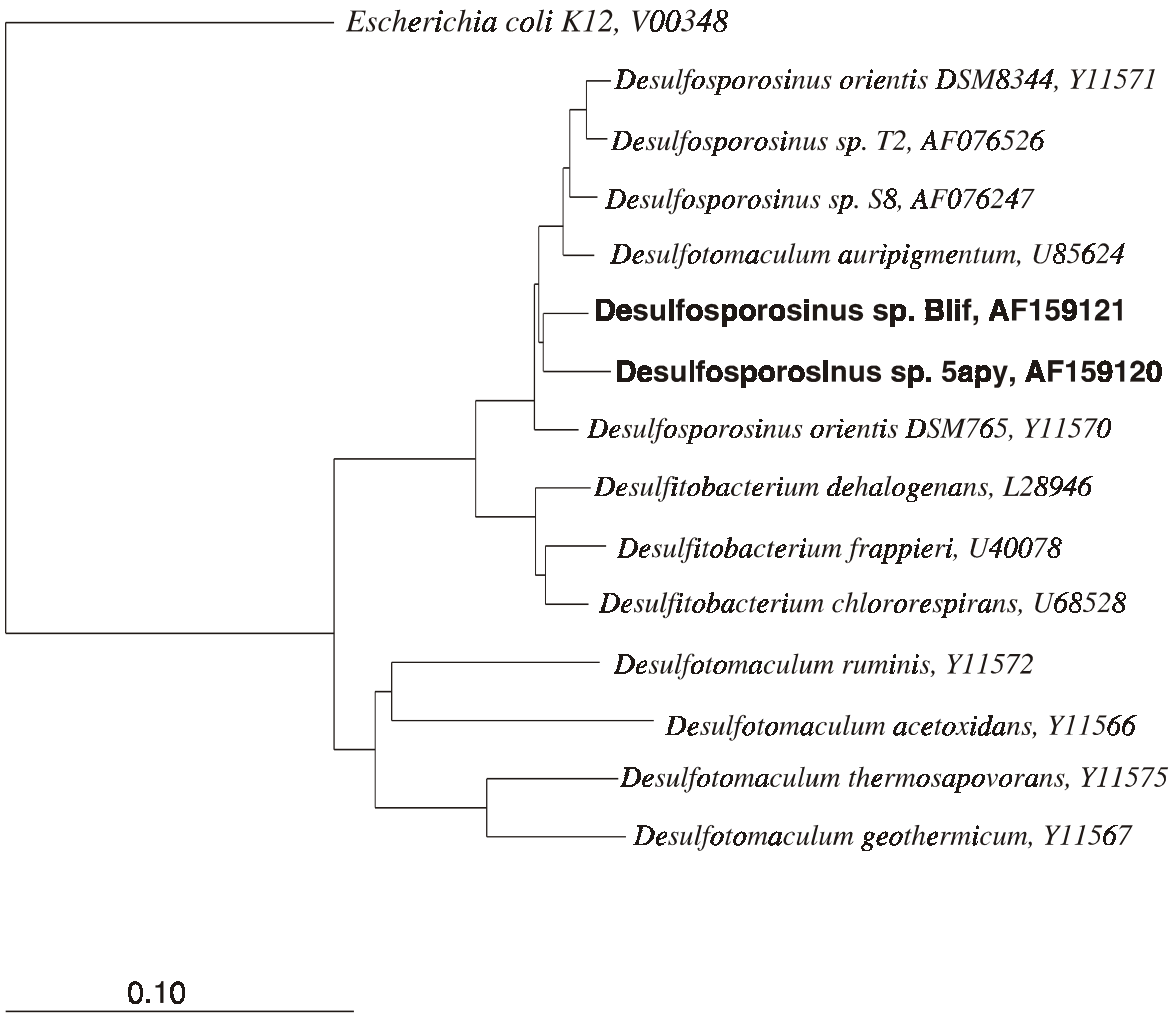


Figure 4: Phylogenetic tree inferred from 16S rDNA sequence data, showing the relationships of the two isolated strains belonging to the *Heliobacterium* group, 5apy and Blif, to their closest relatives. The scale bar represents 10 nucleotide substitutions per 100 nucleotides

2.2.2 Nucleotide sequence accession numbers

The 16S rDNA sequences of the newly isolated strains were submitted to the GenBank database, the accession numbers are: AF109469 (strain zt10e), AF109470 (strain zt3l), AF159119 (strain GWE2), AF159120 (strain 5apy), AF159121 (strain Blif), and AF193026 (strain Mlhm).

2.2.3 Design and evaluation of specific oligonucleotide probes

Strain specific probes 3L468 (strain zt3l) and 10E458 (strain zt10e) were designed complementary to diagnostic regions of the 16S rDNA sequence by using the ARB software package (Strunk et al., 1995). Computer aided sequence comparison

applying the Probe_Match tool of ARB revealed at least one mismatch to all other accessible 16S rDNA sequences for both probes. The sequences and positions of the oligonucleotides are summarized in Table 9.

The probe specificities (labeled with Cy3) were evaluated against selected reference strains. The reference strain for zt3l was *Desulfovibrio gigas* (DSM 5433), revealing two mismatches within the target region, for strain zt10e it was *Desulfovibrio alcoholovorans* (DSM 1382), revealing one mismatch. The hybridization stringencies necessary to discriminate between the target and the nontarget organisms were adjusted by the stepwise addition of formamide to a concentration of 35% for probe 3L468 and to 20% for probe 10E458. The sodium chloride concentration in the washing buffer was adjusted to 88 mM for probe 3L468 and 250 mM for probe 10E458.

Table 9: Sequences of 16S rDNA oligonucleotide probes specific for the sulfate-reducing strains zt3l and zt10e

Oligonucleotide[a]	Common name	Sequence (5'-3')	Target organism
S-St-3L-0468-a-A-21	3L468	GGTACCGTCAGCCTCAAGCCC	strain zt3l
S-St-10E-0458-a-A-21	10E458	GTCCCAAGACTTATTCAGCCT	strain zt10e

[a] Nomenclature according to Alm et al. (1996)

2.3 Morphological characterization

Size and cell form of the four isolates affiliated to the *Desulfovibrionaceae* varied considerably. Strain zt3l showed a morphology typical for this genus. The vibrioid rods were 2 to 4 µm long and had a diameter of 0.5 µm with a monopolar, single flagellum. Its closest relative, strain Mlhm, revealed an atypical morphology of straight, uncurved rods with a length of 3 to 8 µm and a diameter of only 0.3 µm (Fig. 5A). The flagellum differed as well, as it was subterminal and appeared sometimes in pairs.

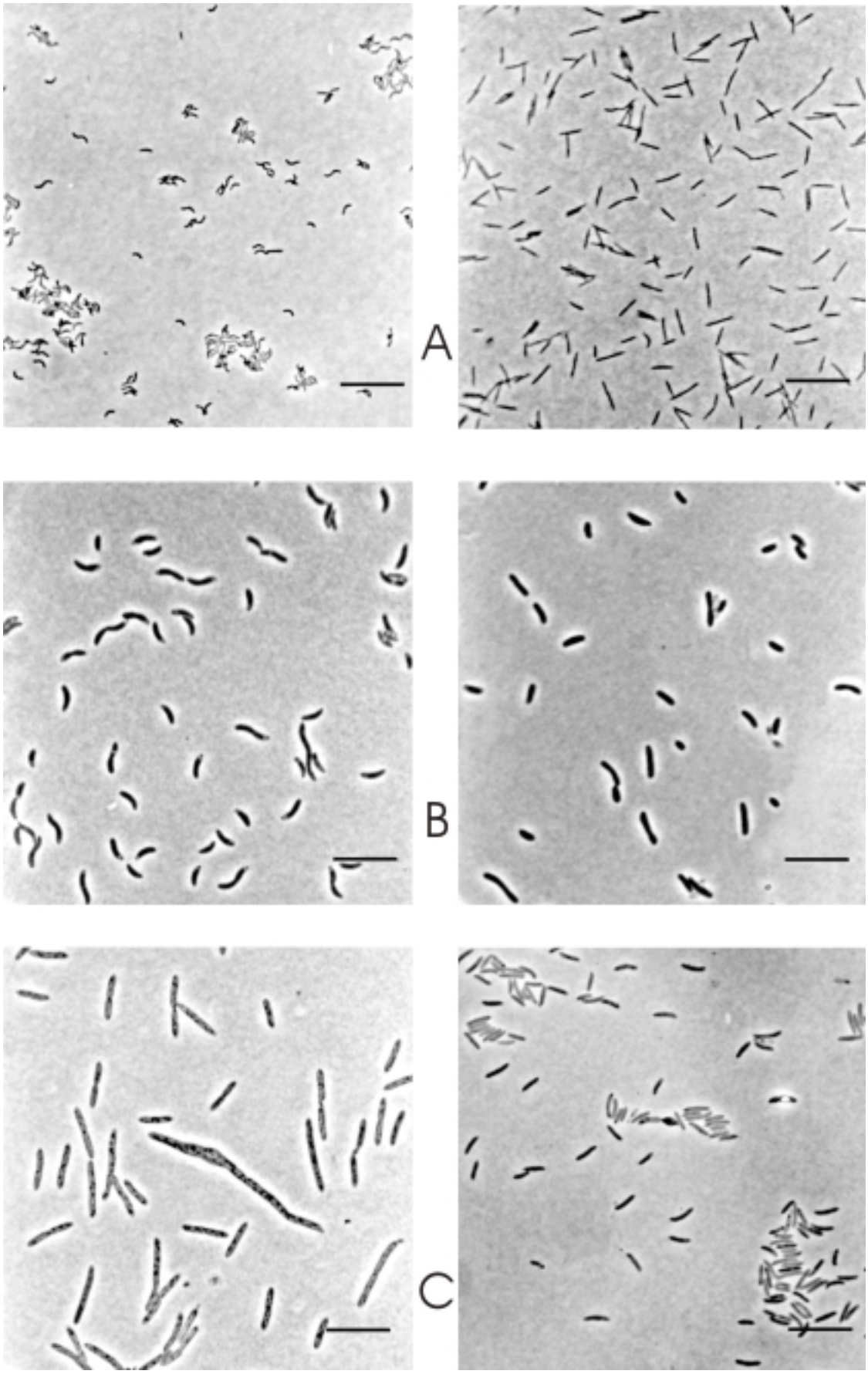


Figure 5: Phase contrast micrographs of (A) strain zt3l (left) and strain Mlhm (right) (B) strain zt10e (left) and strain GWE2 (right) and (C) strain 5apy (left) and strain Blif (right) on agar slides. The scale bar corresponds to 10 µm.

Cells of strain zt10e also showed the typical vibrioid morphology, their length varying between 4 to 6 µm with a diameter of 1.3 µm. A more heterogeneous morphology was shown by the closely related strain GWE2, the rods were less curved and somewhat irregular. The cell length was more variable, the cells were 3 to 8 µm long and had a diameter of 1.3 µm (Fig. 5B). Both had monopolar, single flagella, those of strain zt10e were sometimes in pairs.

The strains affiliated to the genus *Desulfosporosinus* were both rod-shaped, motile and were both able to form spores. Strain 5apy formed spores predominantly when cultivated with acetate or acetate/butyrate as electron donor. When cultivated on pyruvate, the cells had a length of 5 to 10 µm and a diameter of 1.5 µm. The cells of strain Blif were smaller, with a length of 4 to 6 µm and a diameter of 1 µm (Fig. 5C). The organisms had similar monopolar located single flagella.

2.4 Biochemical characteristics

Desulfoviridin could be detected in all strains affiliated to the *Desulfovibrionaceae*, and as expected none of the strains belonging to the genus *Desulfosporosinus* showed any reaction when tested for this enzyme. Catalase reaction was detectable in three of the strains, all belonging to the *Desulfovibrionaceae* as shown in Table 10.

Table 10: Detection of catalase and desulfoviridin in the newly isolated SRB

Strain	Catalase reaction	Desulfoviridin
zt3l	++	+
Mlhm	-	+
zt10e	+	+
GWE2	+	+
5apy	-	-
Blif	-	-

2.5 Physiological characterization of Gram-negative strains from Jungfernheide raw water

The substrate utilization patterns of the strains zt3l and zt10e were determined in sulfate reducer medium. Bacterial growth was determined by use of a spectrophotometer by measuring changes in the optical density at λ = 588 nm for periods of up to two months and additionally by the detection of sulfide.

2.5.1 Electron donor utilization

Both strains belonged to the physiological group of incompletely oxidizing sulfate-reducing bacteria. Both were able to use lactate, pyruvate, formate and H_2 as electron donors. Their substrate utilization patterns are summarized in Table 11.

Table 11: Electron donor utilization of strain zt3l and strain zt10e

Electrondonors	zt3l	zt10e
Lactate	+	+
Pyruvate	+	+
Ethanol	-	+
Formate	+	+
H_2/CO_2	+	+
Acetate	-	-
Benzoate	-	-
Fumarate	-	+
Malate	-	+
Succinate	-	+
Glycerol	+/-	+
Propanediol	-	+
Tween 80	-	-
Fructose	-	-

2.5.2 Electron acceptors and fermentation

Both isolates were able to grow with thiosulfate, sulfite and nitrate as electron acceptors. Weak iron reduction could be shown for strain zt3l. Both strains were able to ferment pyruvate. The complete results are summarized in Table 12.

Table 12: Electron acceptors and fermentation of strain zt3l and strain zt10e

Electron acceptors	zt3l	zt10e
SO_4^{2-}	+	+
$S_2O_3^{2-}$	+	+
SO_3^{2-}	+	+
S^o	+	-
NO_3^-	+	+
Fe(III)	+/-	-
Fermentation of		
Pyruvate	+	+
Lactate	+	-

2.5.3 Growth characteristics

For the determination of the growth behaviour of strain zt3l and strain zt10e both were cultivated in sulfate reducer medium at 30°C with lactate and ethanol, respectively, used as electron donors. The optical density was measured at λ = 588 nm. Strain zt3l had a μ of 0.042 per hour and a doubling time of 16.5 hours. Strain zt10e had a μ of 0.119 per hour and a doubling time of 5.8 hours.

The division rate ν of the strains zt3l and zt10e was determined at room temperature (22°C) with lactate (10 mM) or ethanol (10 mM) as electron donors. Strain zt3l had a division rate of 0.1049, corresponding to a generation time of 9.53 hours, strain zt10e had a ν of 0.0989, which means a generation time of 10.11 hours.

2.5.4 Aerobic respiration

Both isolates were able to use oxygen as electron acceptor. Strain zt3l was able to use H_2 and formate, weak respiration could be shown with lactate. Strain zt10e respired with formate, H_2 was only metabolized when the oxygen concentration was below 20 mM. After termination of the respiration experiment strain zt3l was again able to form sulfide, indicating the still present ability to reduce sulfur-compounds, a feature which was not observed in strain zt10e, implying a higher sensitivity towards oxygen.

3. Characteristics of in situ dominant drinking water bacteria possibly influencing the biofilm community

The in situ dominant bacteria in young biofilms of the Berlin drinking water system belonged to the beta-subgroup of the *Proteobacteria* and were recently isolated (Kalmbach, 1997b). Several biochemical features of these bacteria, like the oxidation of ferrous iron and the production of AHL, were assumed to play a role in the biofilm community of drinking water bacteria and SRB. After their examination, strain B3, which showed a high abundancy within the distribution system, and the in situ dominant *Aquabacterium commune* were chosen for further experiments.

3.1 Oxidation of ferrous iron coupled to the reduction of nitrate

Different Berlin drinking water bacteria from the beta-subgroup of the proteobacteria are closely related to species, which are able to oxidize ferrous iron coupled to the reduction of nitrate. Based on this relationship the Berlin drinking water bacteria were tested for their ability to perform the same metabolism, making an iron cycle in the iron rich parts of the Berlin drinking water system possible. Nevertheless, only strain B2 was able to oxidize more than 45% of the offered ferrous iron, all other tested bacteria were able to oxidize only a maximum of 11.5%. The amounts of ferrous iron oxidized by the Berlin drinking water bacteria are summarized in Figure 6.

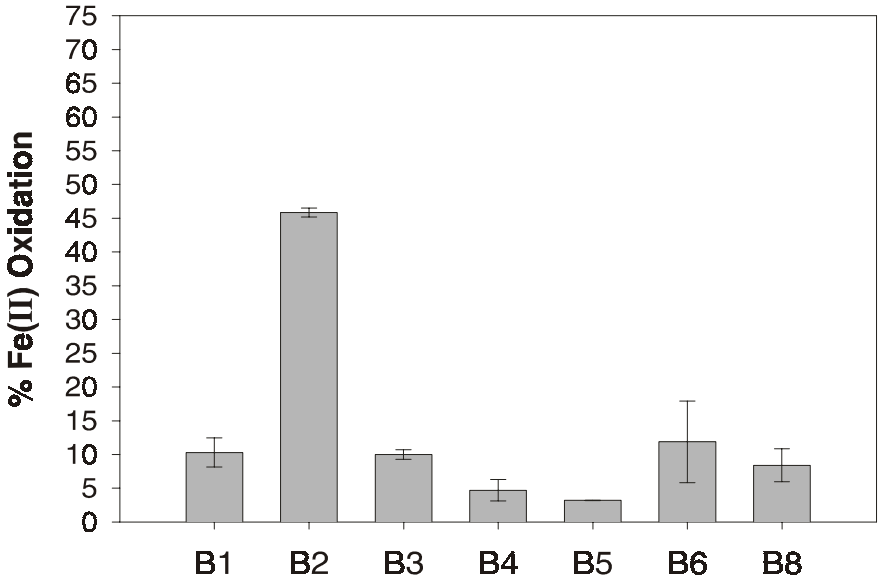


Figure 6: Oxidation of ferrous iron coupled to the reduction of nitrate. The obtained values were normalized to the initial concentration of ferrous iron. Error bars represent standard error. *Aquabacterium citratiphilum*, *Aquabacterium parvum* and *Aquabacterium commune* are identified as strains B4, B6 and B8, respectively.

3.2 Production of *N*-acyl homoserine lactones

N-acyl homoserine lactones (AHL) are supposed to play an important role in the physiology of biofilms. Therefore the ability of the beta-*proteobacteria* isolated from the Berlin drinking water to form this molecule was tested by means of a biological test system. *Agrobacterium tumefaciens* NTL4(pZLR4) is able to detect acyl homoserine lactones with 3-oxo-, 3-hydroxy- and 3-unsubstituted side chains of a length of up to 12 C-atoms with the exception of *N*-butanoyl-L-homoserine lactone. Tests performed with colonies as well as with culture supernatants revealed the ability of the strains B1 and B3 to produce detectable AHL. All other strains showed no colour reaction.

3.3 Nutritional characteristics of strain B3

After selection of *Aquabacterium commune* and strain B3 for further experiments the substrate utilization pattern of strain B3 was determined by use of the Biolog system. The strain was able to grow on a great variety of substrates as fatty acids, carboxylic acids, alcohols, proteins and several sugars.

The strain produced colour reactions with α-cyclodextrin, dextrin, glycogen, Tween 40, Tween 80, D-arabitol, D-fructose, α-D-glucose, maltose, D-mannitol, D-mannose, β-methyl D-glucoside, D-psicose, D-sorbitol, D-trehalose, turanose, methyl-pyruvate, mono-methyl-succinate, acetic acid, cis-aconitic acid, D-gluconic acid, D-glucuronic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, γ-hydroxybutyric acid, α-keto butyric acid, α-keto valeric acid, D,L-lactic acid, propionic acid, quinic acid, sebaic acid, succinic acid, bromo succinic acid, succinamic acid, alaninamide, D-alanine, L-alanine, L-alanyl glycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl-L-glutamic acid, L-histidine, hydroxy L-proline, L-leucine, L-phenylalanine, L-proline, L-pyroglutamic acid, D-serine, L-serine, L-threonine, γ-amino butyric acid, urocanic acid, inosine, 2,3-butanediol, glycerol, and D,L-α-glycerol phosphate.

3.4 Characterization of growth of strain B3 and *Aquabacterium commune*

The growth behaviour of strain B3 and *Aquabacterium commune* was tested at room temperature (20°C) in R2A by determination of the optical density and of the total cell counts with DAPI. The growth curves of both organisms are given in Figure 7. Strain B3 had a μ of 0.30 per hour and a doubling time of 2.31 hours. *Aquabacterium commune* had a μ of 0.26 per hour and a doubling time of 2.64 hours. The division rate ν was 0.59 for strain B3 and 0.57 for *Aquabacterium commune*, the generation time was 1.70 hours for strain B3 and 1.76 hours for *Aquabacterium commune*.

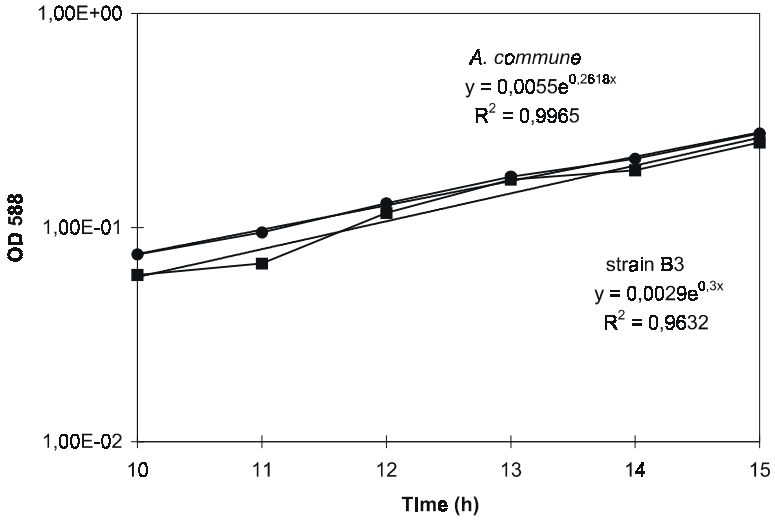


Figure 7: Growth curves of strain B3 and *Aquabacterium commune*. The growth curves were subjected to an exponential regression.

3.5 Dynamics during surface colonization and after starvation

For the characterization of the biofilm building behaviour of strain B3 and *Aquabacterium commune* monospecies biofilms were cultivated in a continuous flow microchamber, which allowed direct microscopic investigation of the biofilm development. Inoculation of each species was performed by injection of 3 ml of a late-exponential-phase culture, which was diluted to a cell number of 1 x 10^6 cells/ml. The flow was stopped for 18 hours to allow attachment and growth of the cells to a cell number of approximately 5 x 10^8 cells/ml in the bulk phase. After re-start of the medium flow, the flow rate was adjusted to 3 to 4 volume exchanges per hour (6 to 8 ml/h). As growth medium fiftyfold diluted R2A for biofilms was used.

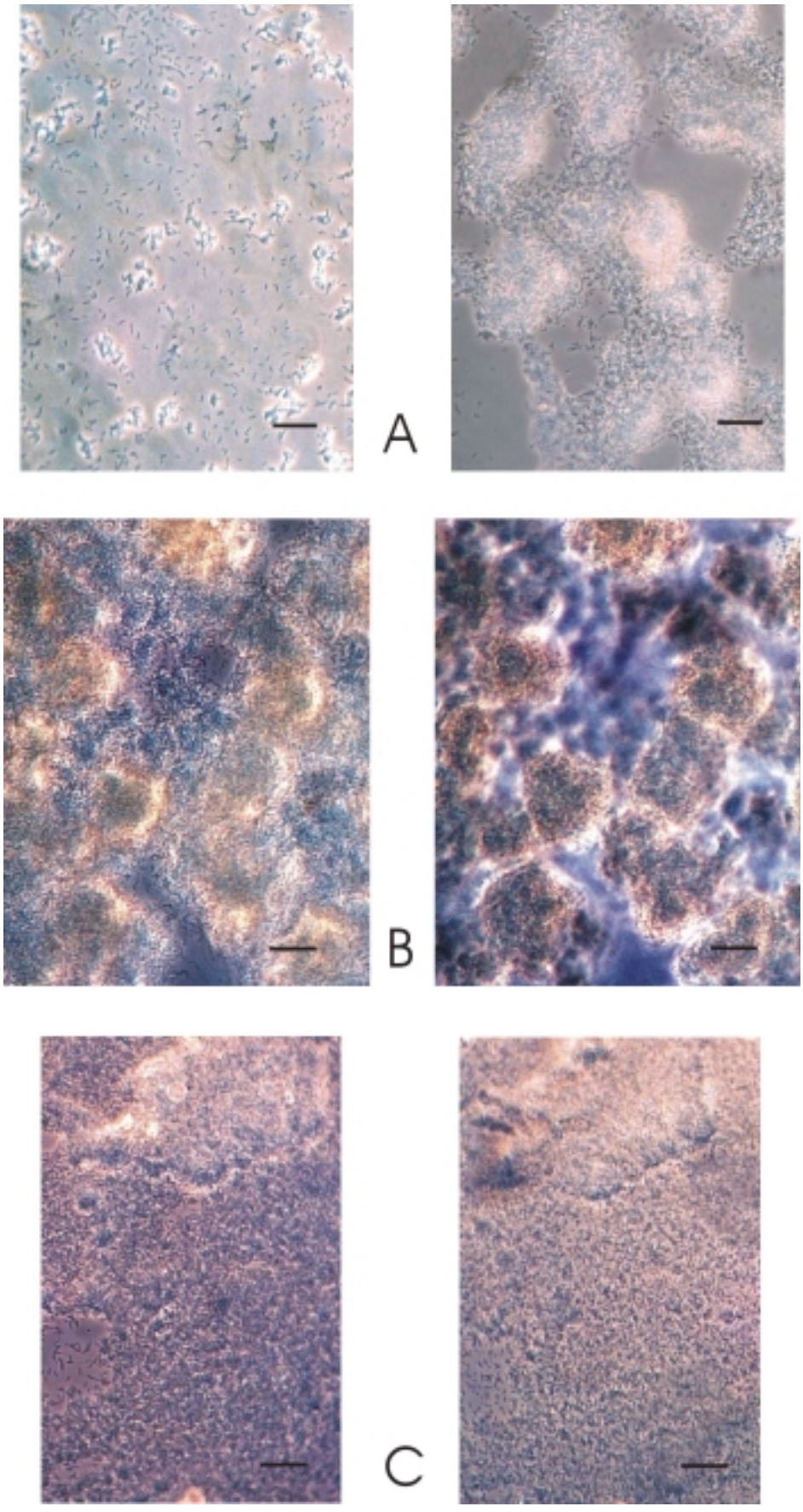

A
B
C

Figure 8: Phase contrast micrographs of monospecies biofilms of strain B3 and *Aquabacterium commune* cultivated on diluted R2A in microchambers. For flow rate see above. (A) Attachment of strain B3 to the glass substratum and beginning of microcolony formation before start of the flow (left), biofilm growth originating from the microcolonies after 7 days medium flow with 50fold diluted R2A (right), (B) micrograph of the identical area of a thick biofilm of *Aquabacterium commune* cultivated on 20fold diluted R2A with the bottom layer (left) and the top layer (right), demonstrating the mushroom structure of the thick biofilm. (C) micrograph of the identical area of a biofilm of strain B3 after two weeks growth on 50fold diluted R2A (left), followed by carbon starvation for 4 weeks (right). Note the constant biofilm structure and the decrease of cell size. Bar corresponds to 10 µm.

Both species showed a very similar biofilm building behaviour. In the first stages of biofilm building single cells attached to the substratum which subsequently started to form microcolonies (Fig. 8A). During this phase both species occasionally formed filamentous bacteria which seemed to connect the microcolonies. Further biofilm development, in both the horizontal and vertical directions, was dominated by growth originating from the microcolony clusters (Fig. 8A), which were randomly distributed throughout the biofilm. As already found in pretests, the biofilm organization remained stable after 10 to 14 days, and the films were then viewed as mature. Use of 20fold diluted R2A resulted in the growth of thicker biofilms showing mushroom-structures (Fig. 8B).

Carbon starvation of the mature biofilms was induced by replacing the diluted R2A-medium with freshwater mineral medium at the same flow rate. The observation of changes of the biofilm community was difficult as changes occured slowly and were not very obvious. The biofilm structure remained the same even after four weeks of starvation. The major indication of the altered carbon supply was the reduction of the cell size of each species during carbon starvation (Fig. 8C). The length of strain B3 was reduced from an average of 4 (± 1.6) µm to 1.3 (± 0.9) µm, the length of *Aquabacterium commune* decreased from 3.2 (± 1.2) µm to 1.2 (± 0.8) µm. Further changes such as detachment or formation of motile cells were not observed.

4. Behaviour of selected sulfate-reducing bacteria under oligotrophic conditions and oxygen stress in particle-free systems

This part of the study addressed the question how SRB are able to spread and proliferate in drinking water systems. Therefore a model system was established for the investigation of the survival and metabolic potential of SRB under conditions relevant in the above described drinking water. SRB were exposed to oxygen stress in oligotrophic and particle free batch cultures, represented either by carbon-free mineral medium or by sterile-filtered drinking water. Possible influences of autochthonous aerobic bacteria were investigated by coexposure with *Aquabacterium commune*. The behaviour of the SRB was determined in long-term experiments by a polyphasic approach with regards to total cell counts, culturability, and the assessment of metabolic potential by FISH and digital image analysis.

4.1 Effect of stress conditions on cell shape and total cell counts

The average oxygen saturation of the particle free systems was 98%. No differences in motility and cell shape of SRB exposed to oxygen in either mineral medium or sterile drinking water were detectable. However, loss of motility was observed within 48 hours after the shift to oxic conditions at the start of each assay for all SRB used in the particular experiments. An alteration of the cell shape or decrease in cell size of the tested SRB strains was not detectable during the whole experiment. Incubation in Berlin drinking water and simultaneous aeration resulted in lysis of cells of strain zt3l after 25 days, and for *Desulfomicrobium baculatum* (DSM 1743) and *Desulfovibrio desulfuricans* (DSM 6949) after 40 days, respectively. Only strain zt10e was detectable by DAPI for longer than 70 days in aerated Berlin drinking water. In mineral medium, total cell counts of all investigated SRB strains remained constant throughout the investigation period, which was 73 days for pure culture experiments and 37 days for coculture tests with *Aquabacterium commune* in mineral medium (Fig. 9).

Exposure of *Aquabacterium commune* to the same stress factors as the sulfate reducers in mineral medium resulted in changes in cell shape as well as in total cell counts in pure and coculture experiments with SRB. In pure culture tests performed in mineral medium, total cell counts of *Aquabacterium commune* decreased from initial cell numbers of 1.2×10^8 to 5×10^6 cells/ml within 10 days. When inoculated to an initial cell number of 1.2×10^5 cells/ml only slight changes of the total cell numbers

occured during carbon starvation in mineral medium, with cell counts decreasing to 2.4 x 10^4 cells/ml within 50 days. Within 24 hours after the onset of carbon starvation in mineral medium, the cells reduced their size from an average length of 3 µm to 1 µm.

In coculture assays with the sulfate-reducing bacteria, *Aquabacterium commune* could be differentiated by size and cell morphology from SRB cells after staining with DAPI. The initial concentration of *Aquabacterium commune* in the coculture assays was 1.3 x 10^8 cells/ml. *Aquabacterium commune* remained detectable by DAPI for just three days after the start of the coculture experiment. The detection limit of *Aquabacterium commune* cells in the coculture experiments was reached at a concentration of 3 x 10^6 cells/ml.

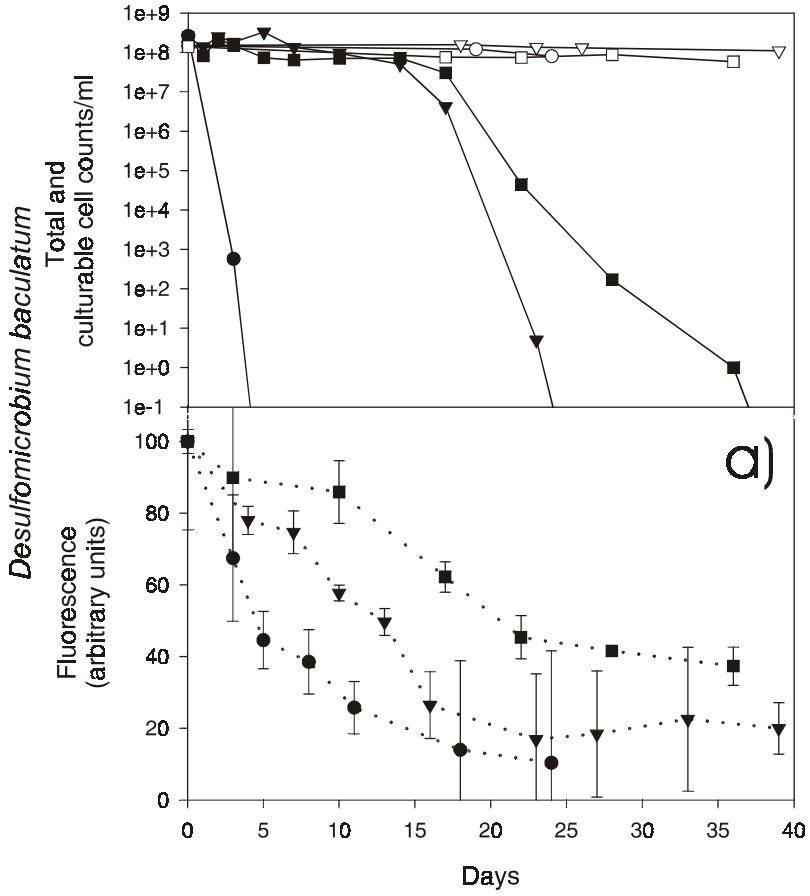
4.2 Effect of stress conditions on culturability

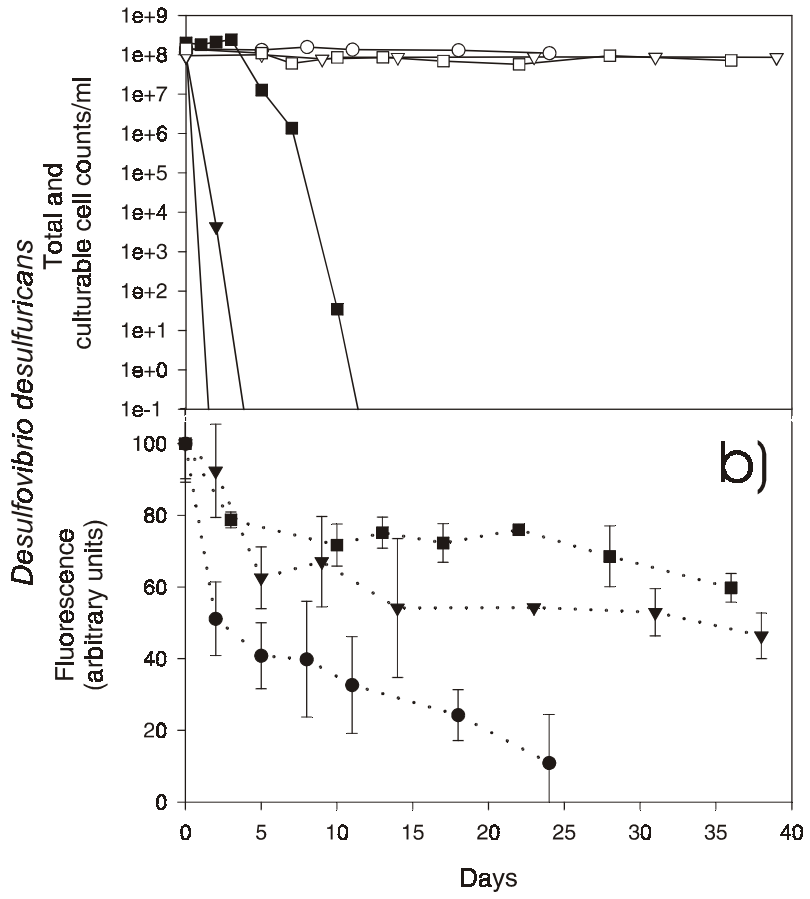
For testing the influence of the media composition on the culturability of stressed microorganisms, both drinking water and defined mineral media were applied.

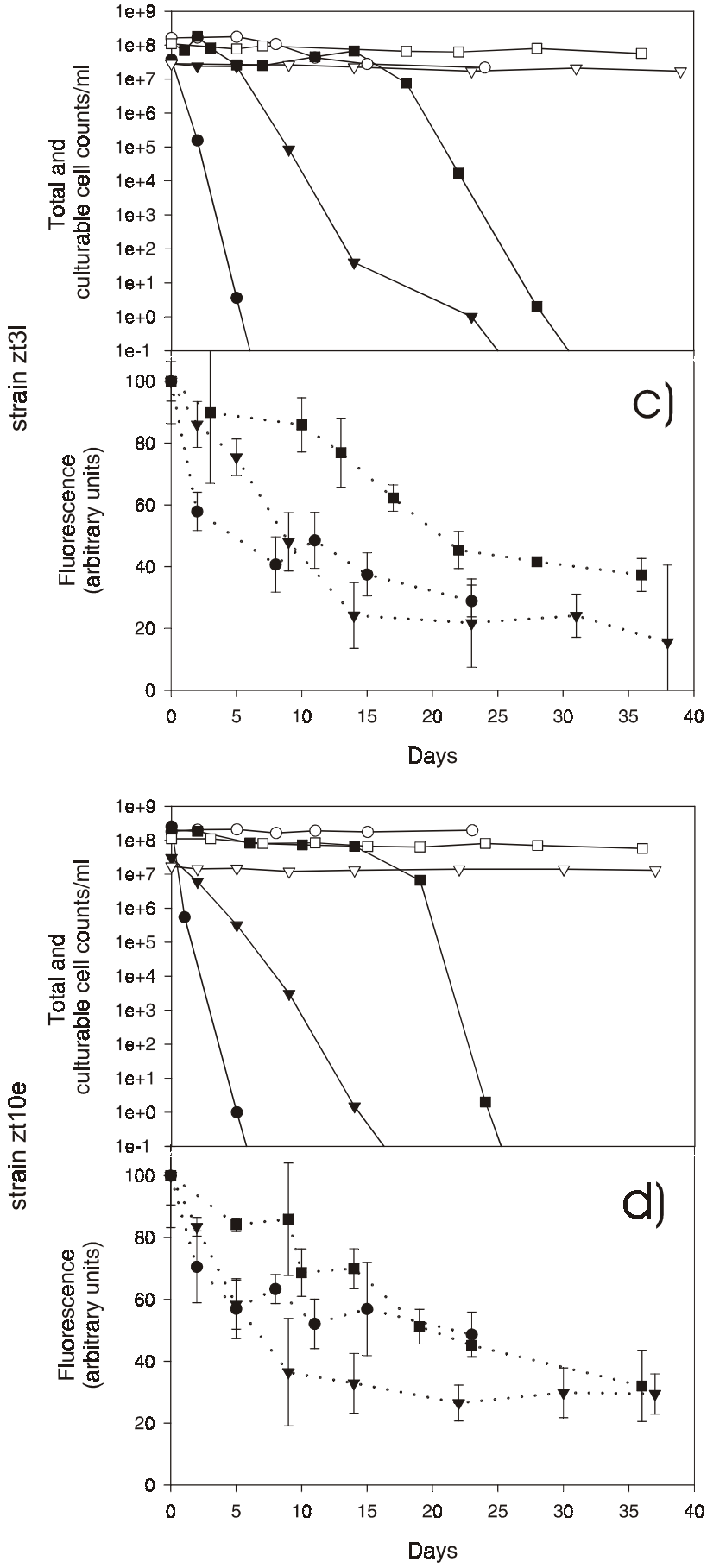
4.2.1 SRB stressed in Berlin drinking water

Desulfomicrobium baculatum remained culturable for 4 days (Fig. 9a), whilst for the environmental isolates zt3l and zt10e, one colony forming unit (cfu) per ml was still detectable after 6 days (Fig. 9c, 9d). Inoculation of *Desulfovibrio desulfuricans* in particle free aerated Berlin drinking water resulted in a total loss of culturability within two days after the start of the experiment (Fig. 9b).

Figure 9: Dynamics of total cell counts, culturability and FISH intensities of SRB under oxic conditions and carbon limitation. a) *Desulfomicrobium baculatum*, b*) Desulfovibrio desulfuricans*, c) strain zt3l and d) strain zt10e grown in anaerobic medium and subsequently starved under oxic conditions in different media. Berlin drinking water is indicated by (○,●), mineral medium by (▽,▼) and coculture with *Aquabacterium commune* by (□,■). Empty symbols with solid lines correspond to total cell counts, solid symbols with solid lines to culturable cell counts and solid symbols with dotted lines to the FISH signal intensities. Time of starvation is given in days. The standard error of total and culturable cell counts was below 5% in all experiments. Standard error of the FISH intensities is represented by error bars.


Desulfomicrobium baculatum
Total and culturable cell counts/ml
1e+9
1e+8
1e+7
1e+6
1e+5
1e+4
1e+3
1e+2
1e+1
1e+0
1e-1
a)
Fluorescence (arbitrary units)
100
80
60
40
20
0
0
5
10
15
20
25
30
35
40
Days


Desulfovibrio desulfuricans
Total and culturable cell counts/ml
1e+9
1e+8
1e+7
1e+6
1e+5
1e+4
1e+3
1e+2
1e+1
1e+0
1e-1
b)
Fluorescence (arbitrary units)
100
80
60
40
20
0
0
5
10
15
20
25
30
35
40
Days


strain zt3l
Total and culturable cell counts/ml
1e+9
1e+8
1e+7
1e+6
1e+5
1e+4
1e+3
1e+2
1e+1
1e+0
1e-1
Fluorescence (arbitrary units)
100
80
60
40
20
0
c)
0
5
10
15
20
25
30
35
40
Days
strain zt10e
Total and culturable cell counts/ml
Fluorescence (arbitrary units)
d)
Days

4.2.2 In mineral medium

In mineral medium, differences between the particular strains became more obvious. Culturability of *Desulfomicrobium baculatum*, which was inoculated at a cell density of 2 x 10^8 cells/ml, slowly decreased to 10^7 cfu/ml for 14 days, followed by a sharp decrease and the complete loss of culturability after 27 days (Fig. 9a). In contrast to this, *Desulfovibrio desulfuricans* started to loose its culturability immediately after inoculation into mineral medium, and reached the detection limit of 1 cfu/ml after 6 days (Fig. 9b). Culturability of the environmental strain zt3l remained on an almost constant level for 6 days, before the number of cfu declined rapidly. After 19 days culturable cells were below 1 per ml (Fig. 9c). Strain zt10e showed a continuous decline in culturability for 15 days (Fig. 9d).

4.2.3 SRB stressed in coculture with *Aquabacterium commune*

In coculture experiments with the aerobic bacterium *Aquabacterium commune* in aerated mineral medium, culturability of all tested SRB was significantly prolonged compared to the respective pure culture experiments. The decrease in culturable cell counts of the four investigated SRB showed a similar pattern: culturability remained on a high level for approximately 8 days for *Desulfovibrio desulfuricans* (Fig. 9b) and up to 18 days for *Desulfomicrobium baculatum* (Fig. 9a), followed by a sudden decline and complete loss of culturability. Strain zt3l showed culturability on a high level for 18, strain zt10e for 20 days before the culturability declined rapidly. The investigated strains differed in the total period of culturability, which was 37 days for *Desulfomicrobium baculatum*, while the detection limit of strain zt3l was reached after 29 days and for zt10e after 24 days (Fig. 9c and 9d). *Desulfovibrio desulfuricans* remained culturable for only 11 days (Fig. 9b).

4.2.4 Coculture experiments in mineral medium inoculated with low cell numbers

This experiment was performed with *Desulfomicrobium baculatum* (DSM 1743), *Desulfovibrio desulfuricans* (DSM 6949) and strain zt3l in mineral medium. Test conditions were identical to the experiment described above. The initially inoculated cell numbers of the SRB and *Aquabacterium commune* are given in Table 13.

Table 13: Effects of oxygen stress and carbon starvation in coculture experiments in mineral medium inoculated with low cell numbers

Strain	Inoculated cells [ml^{-1}] of *A. commune*	Inoculated cells [ml^{-1}] of SRB
Desulfomicrobium baculatum	9.7×10^4	1.8×10^5
Desulfovibrio desulfuricans	8.2×10^4	8.2×10^4
strain zt3l	7.2×10^4	2.7×10^4

The culturable cell counts of Aquabacterium commune showed only few changes throughout the 52 days of the experiment. In contrast, the similarity of the decrease in culturable cell counts of the investigated SRB compared to the experiment performed with high cell numbers was striking: culturability remained on a high level for different periods before a rapid loss of the ability to form colonies made the detection of culturable cells impossible. Nevertheless, the period of detection of culturable cells is much shorter than in assays with high cell numbers (Fig. 10).

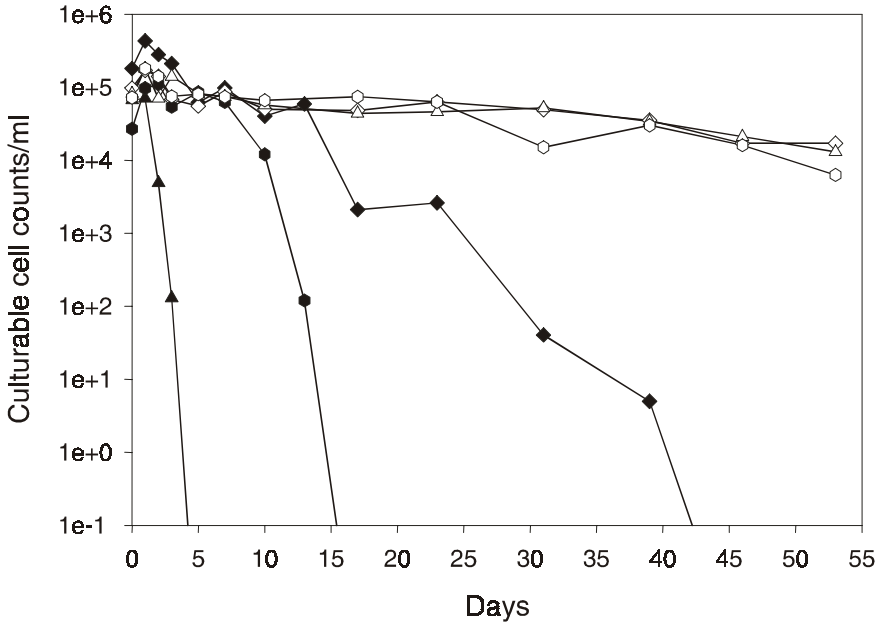


Figure 10: Dynamics of culturable cell counts of Aquabacterium commune and different SRB under oxic conditions and carbon limitation after inoculation with low cell numbers. *Aquabacterium commune* combined with *Desulfomicrobium baculatum* is indicated by (◆), combined with *Desulfovibrio desulfuricans* by (▲) and in combination with zt3l by (⬢). Filled symbols indicate culturable cell counts of SRB, empty symbols indicate culturable cell counts of *Aquabacterium commune.* Time of starvation is given in days.

4.3 Effect of stress conditions on the FISH signal intensity

Probe conferred fluorescence intensities of the SRB subjected to starvation and oxygen stress were determined by digital image analysis as an indicator for the metabolic potential. The average signal intensity of all hybridized SRB declined after stress exposure (Fig. 9). The dynamics of the decrease, however, showed considerable variations depending on the strains and on the medium used.

4.3.1 SRB stressed in Berlin drinking water

The course of signal intensity decrease was not consistent for the different SRB exposed to oxygen in Berlin drinking water. For *Desulfomicrobium baculatum* and *Desulfovibrio desulfuricans* the signal intensities consistently declined until they reached about 10% of the initial value (Fig. 9a and 9b), and culturability was lost during this decrease. *Desulfovibrio desulfuricans* was culturable for only 2 days and *Desulfomicrobium baculatum* for a maximum of 4 days. The signal intensities of the environmental strains zt3l and zt10e showed a steep decline during the first 4 days, but then remained at an almost constant level of 40% and 50%, respectively. This change in the decrease of the fluorescence intensity coincided with loss of culturability (Fig. 9c and 9d).

4.3.2 SRB stressed in mineral medium

The signal intensity of all sulfate-reducing strains exposed to stress in mineral medium decreased rapidly during the first 15 days, and subsequently remained at almost constant levels with a minimum value around 20%. For most strains a correlation between reaching a stable level of signal intensities and the rate of culturability could be determined (Fig. 9), though this relationship was not so strong for *Desulfomicrobium baculatum* (Fig. 9a).

4.3.3 SRB stressed in coculture with *Aquabacterium commune*

The relationship between signal intensity and culturability in this experiment was similar to that for SRB in mineral medium. The loss of signal intensities of the sulfate-reducing strains occured at a constant rate, but lower than that for pure culture experiments in Berlin drinking water and mineral medium. For *Desulfomicrobium baculatum, Desulfovibrio desulfuricans* and strain zt3l, culturability was no longer

detectable when the signal intensities were on an almost stable level, while the signal intensity of strain zt10e showed a slight further decrease (Fig. 9).

Strain specific differences between *Desulfovibrio desulfuricans* and strain zt3l displayed the fact that there was no tight correlation between the average fluorescence intensity and the rate of culturability. In all experiments culturability of *Desulfovibrio desulfuricans* was no longer detectable at fluorescence intensities around 60% (Fig. 9b), whilst zt3l still formed colonies when fluorescent intensity values were much lower, 20-40% (Fig. 9c). However, experiments with *Desulfomicrobium baculatum* proved that there were no consistent strain specific distinct values of signal intensity, from when culturability ceased. For example, culturable cells were obtained after stress in Berlin drinking water when signal intensities were 70%, in contrast, culturable cells in mineral medium were still present at signal intensities of 20% (Fig. 9a).

4.4 Influence of the culture age on the stress response

For the measurement of the influence of the culture age on the stress response an experiment was performed with strain zt3l in mineral medium. The growth of the original culture on reduced mineral medium supplemented with lactate was interrupted at different times. Growth was interrupted during the exponential phase, in the beginning of the stationary phase and 96 hours after reaching the stationary phase. The cells were then washed in mineral medium and inoculated into the mineral medium as described above with initial cell numbers of 1.4×10^8 ($\pm 1 \times 10^7$) cells/ml. Culturability of the cells was investigated as described above.

The best stress tolerance could be shown for cells which were cultivated until the beginning of the stationary phase. Culturable cells were detectable for up to 15 days (Fig. 11). Culturability of cells cultivated to the exponential phase and of cells in the stationary phase was only detectable for 9 and 11 days, respectively (Fig. 11).

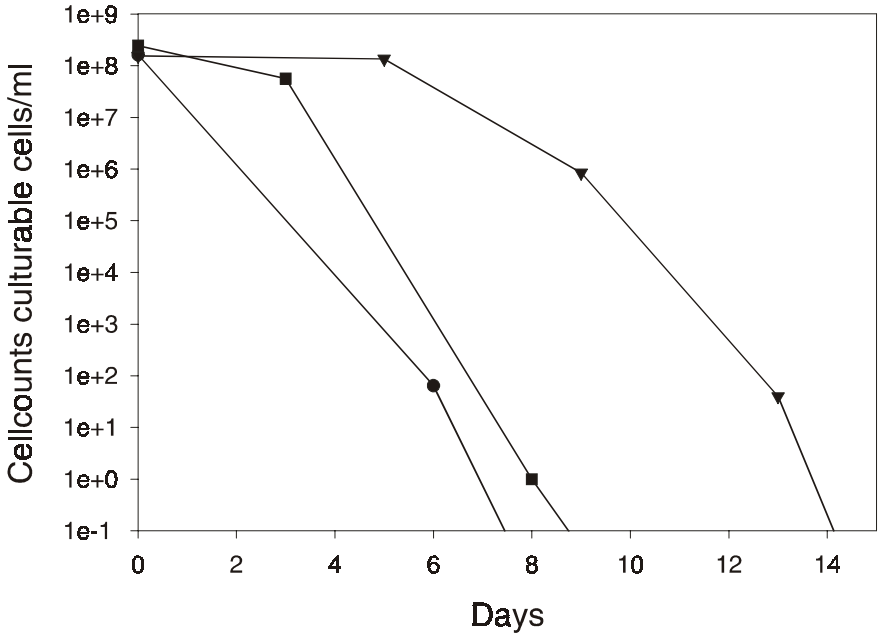


Figure 11: Dynamics of culturability of strain zt3l under oxic and carbon limited conditions exerted after different times of cultivation. Culturability of stressed cells was investigated after growth to the exponential phase (●), after growth to the beginning of the stationary phase (▼) and after cultivation 96 hours after reaching the stationary phase (■).

4.5 Physiological activity of stressed, nonculturable SRB

Physiological activity of pure cultures of strain zt3l and strain zt10e was determined in the multi-electrode device also used for the determination of aerobic respiration. Both strains were stressed in Berlin drinking water according to the method described above. Strain zt3l was inoculated at an initial concentration of 1.8×10^8 ($\pm 1 \times 10^7$), strain zt10e at 3.6×10^6 ($\pm 2 \times 10^5$) cells per ml. After 12 days aeration no culturable cells of strain zt3l could be detected in an anaerobic agarshake, while 6 cells per ml of strain zt10e were still detectable after 14 days. After this time both strains showed endogeneous oxygen respiration, which could not be influenced by substrate addition. For strain zt3l also sulfide formation could be demonstrated, which was stimulated by addition of H_2, though no other substrate had any effect. Strain zt10e showed only a very low rate of endogeneous respiration and sulfide formation could not be determined under these circumstances, indicating a loss of physiological activity.

4.6 Activiation of stressed, 'non-culturable' *Desulfovibrio desulfuricans* (DSM 6949)

Each strain of the above stressed sulfate-reducing bacteria lost its culturability after a specific period of time and presumably entered the viable-but-nonculturable state. By addition of different nutrients and other supplements to sulfate reducer medium the ability of viable but nonculturable *Desulfovibrio desulfuricans* (DSM 6949) to resuscitate and thus to regain the ability to grow was tested. This was combined with the PAC-method by Kalmbach et al. (1997), which was modified by using the antibiotic cocktail (ABC) proposed by Joux and LeBaron (1997). Addition of *N*-acyl homoserine lactones (AHL) has been shown to accelerate the recovery of nitrifying biofilms from starvation (Batchelor et al., 1997), its efficiency on the resuscitation of the oxygen-sensitve *Desulfovibrio desulfuricans* (DSM 6949) was therefore tested as well.

4.6.1 Modifications of the PAC method for *Desulfovibrio desulfuricans*

In the original PAC assays aerobic bacteria were incubated in aerobic media. For anaerobic bacteria like *Desulfovibrio desulfuricans*, however, sulfate reducer medium had to be used. Therefore the evaluation of the gyrase inhibitors was performed with stationary phase cells of *Desulfovibrio desulfuricans* incubated in sulfate reducer medium supplemented with 20 mM lactate. As gyrase inhibitors pipemidic acid with a final concentration of 30 mg/l and an antibiotic cocktail (ABC) with final concentrations of the following antibiotics: nalidixic acid 20 µg/ml, piromidic acid 10 µg/ml, pipemidic acid 10 µg/ml, cephalexin 10 µg/ml and ciprofloxacin 0.5 µg/ml were tested.

The initial cell density of 2.5×10^7 ($\pm 2.1 \times 10^6$) cells/ml remained nearly constant for both inhibitors after incubation for 24 hours. After 48 hours pipemidic acid did no longer effectively suppress cell division, cell numbers increased to 3.1×10^7 ($\pm 2.3 \times 10^6$) cells/ml (Table 14).

Table 14: Evalution of the cell division inhibiting effect of pipemidic acid and an antibiotic cocktail on *Desulfovibrio desulfuricans*

Exposure time [h]	antibiotic	cell density [cells/ml][a]	average cell length [µm][b]
0		2.5×10^7 (± 2.1×10^6)	2.9 ± 0.1
24	pipemidic acid	2.1×10^7 (± 1.6×10^6)	7.0 ± 0.8
24	ABC	2.6×10^7 (± 1.3×10^6)	5.9 ± 0.3
48	pipemidic acid	3.1×10^7 (± 2.3×10^6)	n.d.[c]
48	ABC	2.6×10^7 (± 2.0×10^6)	n.d.

[a]: Total cell counts determined by DAPI staining (± standard error)
[b]: Mean cell length of 100 randomly chosen bacteria (± standard error)
[c]: not determined

4.6.2 Influence of nutrient concentration and different *N*-acyl homoserine lactones on the behaviour of stressed *Desulfovibrio desulfuricans*

In a first test, the ability to resuscitate was investigated with the oxygen-sensitve *Desulfovibrio desulfuricans* (DSM 6949) stressed for 10 days in oxic mineral medium, whose culturability was no longer detectable in agarshakes. Lactate and sulfate, the normal growth substrates of *Desulfovibrio desulfuricans*, were chosen as nutrients for the resuscitation at low concentrations of 1 mM lactate and 2 mM sulfate and at high concentrations of 10 mM lactate and 20 mM sulfate. Furthermore the influence of the addition of one homoserine lactone analog (HLA) and of a mixture of different *N*-acyl-homoserine lactone analogs (HSL) was tested. For the inhibition of cell division the antibiotic cocktail was added.

No elongation of cells was detectable in any of the assays at any time. The average cell length of 100 randomly chosen cells at the beginning of the test was 2.35 (± 0.07) µm, after 6 days it was 2.1 (± 0.04) µm in the assays without AHL and 2.3 (± 0.04) µm in the assays supplemented with HSL. After 16 days the average cell length of *Desulfovibrio desulfuricans* in the assay with high nutrient concentration was 1.78 (± 0.06) µm while the addition of HSL led to a cell length of 2.05 (± 0.07) µm. Nevertheless, addition of HSL resulted in an increase of the DAPI-stainable particles within 7 days after the start of the experiment regardless of the nutrient concentration (Fig. 12). The particle counts were 1.5 fold higher than the initial particle counts. After 20 days an increase of the number of particles in the assays with a high nutrient concentration supplemented with HLA and without HSL supplementation (Fig. 12) was found. This was accompanied by a reduction of the

cell length into a range where accurate measurement was no longer possible (< 1 µm). In contrast, the morphological integrity of cells incubated with HSL remained stable for 35 days, although the cell size decreased as well to 1.52 (± 0.03) µm.

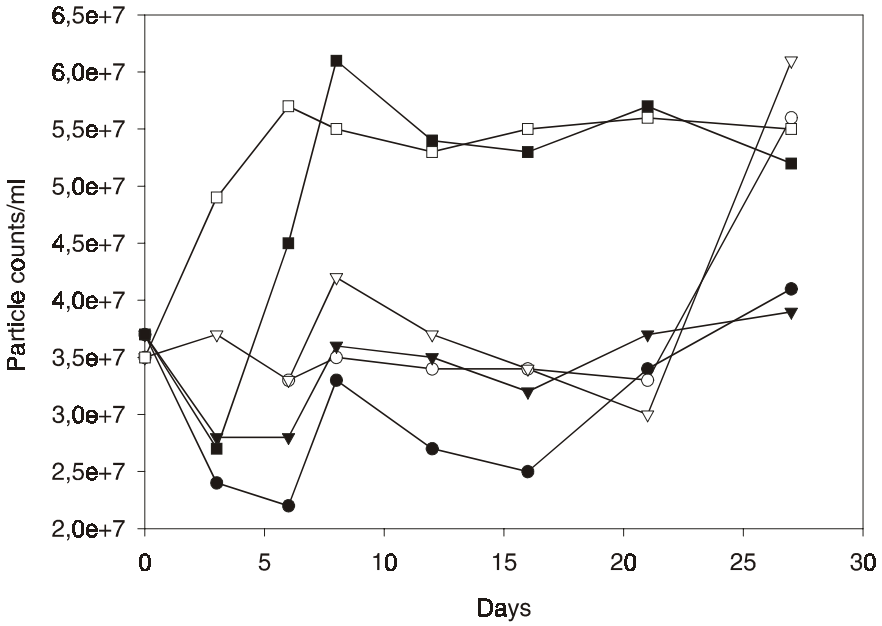


Figure: 12: Activation of *Desulfovibrio desulfuricans*: Effect of nutrient concentration and different *N*-acyl homoserine lactones. Filled symbols correspond to the low nutrient concentration of 1 mM lactate and 2 mM sulfate, unfilled symbols represent 10 mM lactate and 20 mM sulfate. (●, ❍): no further additions, (▼, ▽) addition of HLA and (■, □) addition of HSL.

As the reason for the unexpected increase of particle numbers without cell elongation after supplementation with HSL was unclear, a second test was performed. To exclude the possibility of utilization of any other supplement of the activation assay, every component was tested alone and in all possible variations. Sulfate was used in all tests at a concentration of 20 mM, lactate was used where specified at a concentration of 10 mM. HSL and ABC were used at the concentrations given above.

Again no elongation of cells was detectable, but the increase of particle counts after the addition of HSL within 7 days was found once more, and again the increase was 1.5 fold. Particle counts of the assay supplemented with lactate, HSL and the antibiotic cocktail increased from 2.5×10^7 ($\pm 1.4 \times 10^6$) particles/ml to 3.8×10^7 ($\pm 2.2 \times 10^6$) particles/ml, without the antibiotic cocktail but with lactate and HSL, the particle counts increased to 3.9×10^7 ($\pm 3.1 \times 10^6$) particles/ml (Fig. 13)

Additionally, the increase in particle counts in the assays supplemented with lactate but without HSL after 21 days due to cell lysis was detectable as well, regardless of the addition of the antibiotic cocktail. After 21 days an increase from the initial 2.5×10^7 ($\pm 1.4 \times 10^6$) particles/ml to 3.3×10^7 ($\pm 9 \times 10^5$) particles/ml was observed (Fig. 13). Assays without any carbon source, but supplemented with HSL, with ABC or with both showed no changes neither in the number of particles (Fig. 13) nor in the cell length throughout the experiment.

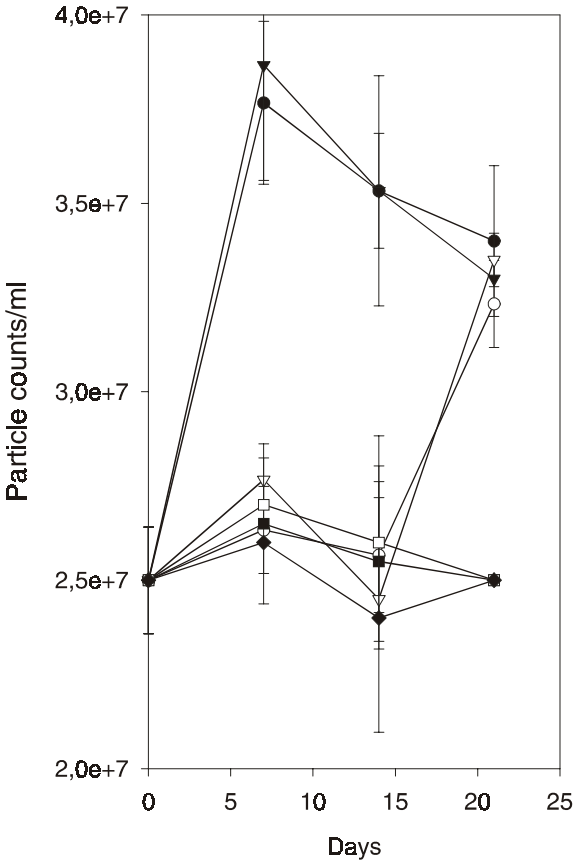


Figure 13: Activation of *Desulfovibrio desulfuricans*: Effect of HSL. Sulfate reducer medium was supplemented with: (●) 10 mM lactate, HSL and anitbiotic cocktail (ABC), with (❍)10 mM lactate and ABC, with (▼)10 mM lactate and HSL, with (▽) 10 mM lactate, with (■) HSL, with (□) HSL and ABC, with (◆) ABC. Error bars represent standard error. Note the early increase of the particle counts in the assays supplemented with HSL and 10 mM lactate regardless of the presence of ABC.

For the detection of resuscitated cells, which possibly regained the ability to grow and form colonies, samples were taken after 7 days and cultivated in agarshakes with 10 mM lactate. Formation of colonies could not be observed regardless of the supplementation of the initial assay.

4.6.3 Detection of AHL produced by sulfate-reducing bacteria

The unspecific reaction of *Desulfovibrio desulfuricans* with AHL-analogs suggested a response of SRB on this type of molecules and hence a production of individual *N*-acyl homoserine lactones by SRB. Therefore the ability of *Desulfomicrobium baculatum* (DSM 1743)*, Desulfovibrio desulfuricans* (DSM 6949), strain zt3l and strain zt10e to form this type of molecules was tested with the biological test system described above. Tests were performed with culture supernatants as well as with ethyl-acetate extracts. However, no colour reaction could be observed, neither with culture supernatants nor with ethyl-acetate extracts.

5. Behaviour of SRB in artificial aerobic biofilms

5.1 Exposition of sulfate-reducing bacteria in aerobic biofilms to drinking water

To determine the ability to establish themselves in biofilms under aerobic conditions, SRB were added to a biofilm of the in situ dominant drinking water bacterium *Aquabacterium commune*. Afterwards the biofilm was exposed to the drinking water flow. As growth substratum glass slides placed in 50 ml centrifuge tubes were used. In situ hybridization with probes directed against the 16S rRNA was used to detect the SRB in the biofilm.

Biofilms were grown in R2A by inoculation of the centrifuge tube reactor to an initial cell number of 1×10^6 cells/ml with late-exponential phase cells of *Aquabacterium commune*. Biofilms were allowed to develop for 2 days to a DAPI cell count of 2.7×10^6 ($\pm 3.7 \times 10^5$) cells/mm², followed by the addition of washed cells of the oxygen sensitive *Desulfovibrio desulfuricans* (DSM 6949) to a concentration of 5×10^5 cells/ml. The biofilm reactors were then kept for 4 days on a parallel shaker to allow growth and establishment of *Desulfovibrio desulfuricans* in the biofilm. Afterwards the reactors were connected to a drinking water tap and the flow through was adjusted to 75 ml/hour. Microscopical investigation of DAPI stained glass slides revealed cell counts of 5.7×10^6 ($\pm 2.1 \times 10^5$) cells/mm² before the start of the flow, which decreased to 3.8×10^6 ($\pm 3.9 \times 10^5$) cells/mm² within 1 day, to 9.1×10^5 ($\pm 8.9 \times 10^4$) cells/mm² within 25 days and to 3.8×10^5 ($\pm 2.3 \times 10^4$) cells/mm² after 63 days. The number of hybridization positive cells as detected by use of the *Bacteria*-specific oligonucleotide probe EUB338 labeled with Cy3 declined from 73.6% (± 2.1%) to 57.1% (± 3.1%) after 25 days and to 50.7% (± 2.6%) after 63 days. The

surface of the glass slides was covered by small cell clusters and by single cells, other parts of the surface were completely bare. *Desulfovibrio desulfuricans* was detectable after in situ hybridization with probe DSV1292 within cell clusters of *Aquabacterium commune* for up to 25 days (Fig. 14). A statistical evaluation of the number of cells hybridizing with probe DSV1292 was not possible, because of the low number and random distribution of clusters over the growth substratum. After 63 days no signals after hybridization with probe DSV1292 were detectable.

5.2 Survival of SRB in aerobic biofilms grown in centrifuge tube bioreactors

In a second series of experiments newly isolated SRB from the Berlin drinking water system were inoculated together with different important aerobic bacteria isolated from the same system. In situ hybridization was used for the determination of the number of SRB and to analyse if the concomitant inoculation had any influence on the spatial distribution of the SRB. Furthermore first tests were performed for the determination of culturability of SRB from the biofilm and from the effluent of the system.

5.2.1 Inoculation and flow conditions

Experiments were performed in 15 ml polypropylene centrifuge tubes with glass slides as substratum. Inoculation was performed in modified R2A with the following combinations of organisms: strain B3 with strain zt3l (assay a), strain B3 with strain zt10e (b), *Aquabacterium commune* with strain zt3l (c), and *Aquabacterium commune* with strain zt10e (d) in modified R2A. Cell numbers were adjusted by the determination of the OD 588 based on calibration curves to approximately 10^6 cells/ml for aerobic bacteria and to 10^8 cells/ml for SRB. Pumping was started after 21 hours with 4 volume exchanges within 30 minutes. As medium for continuous operation 150fold diluted modified R2A was used, the flow was adjusted to 30 ml/h. On day 20 the medium was changed to drinking water with flow rates of 240 ml/h for 11 days and of 540 ml/h for further 32 days.

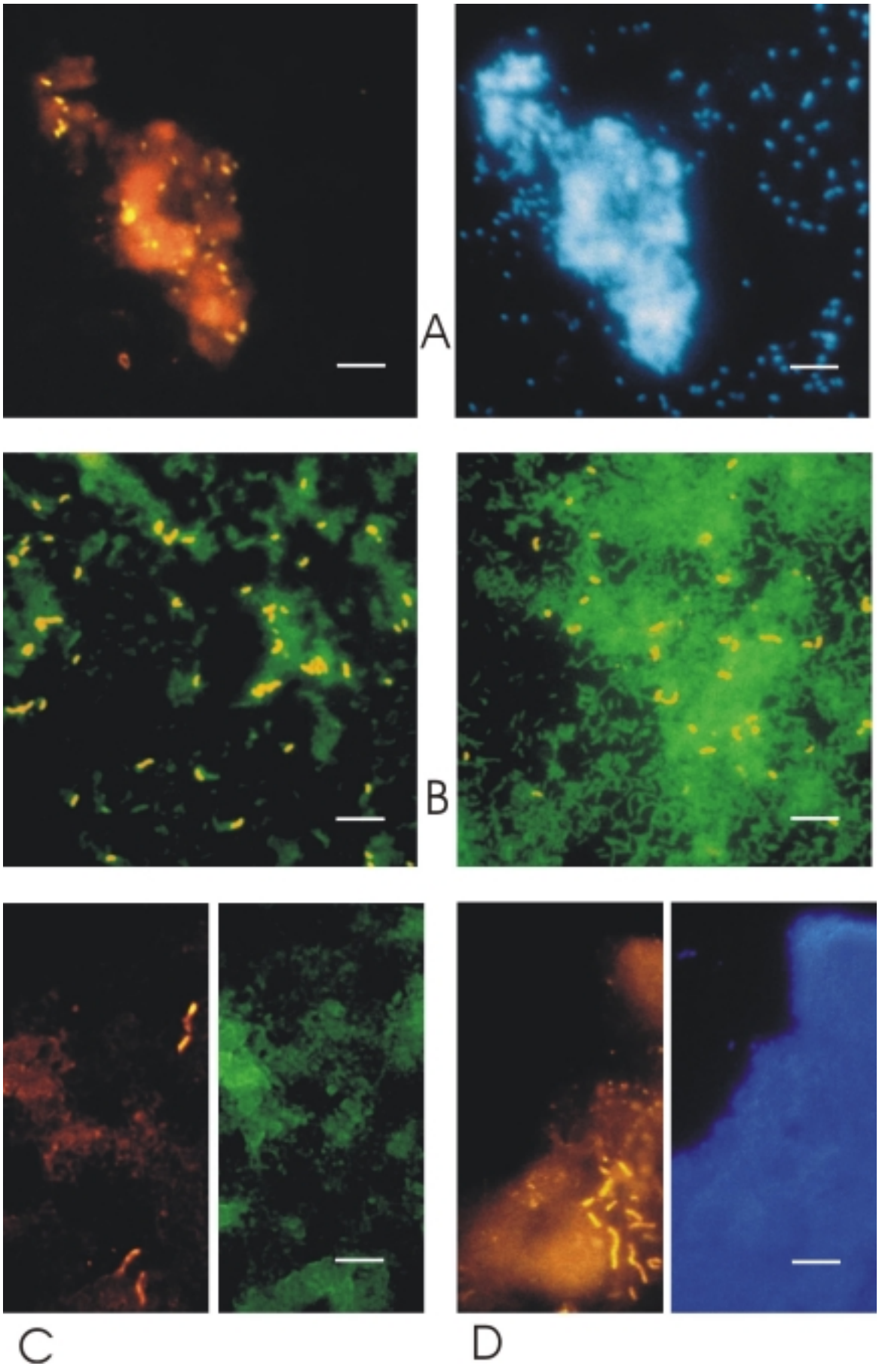

A
B
C
D

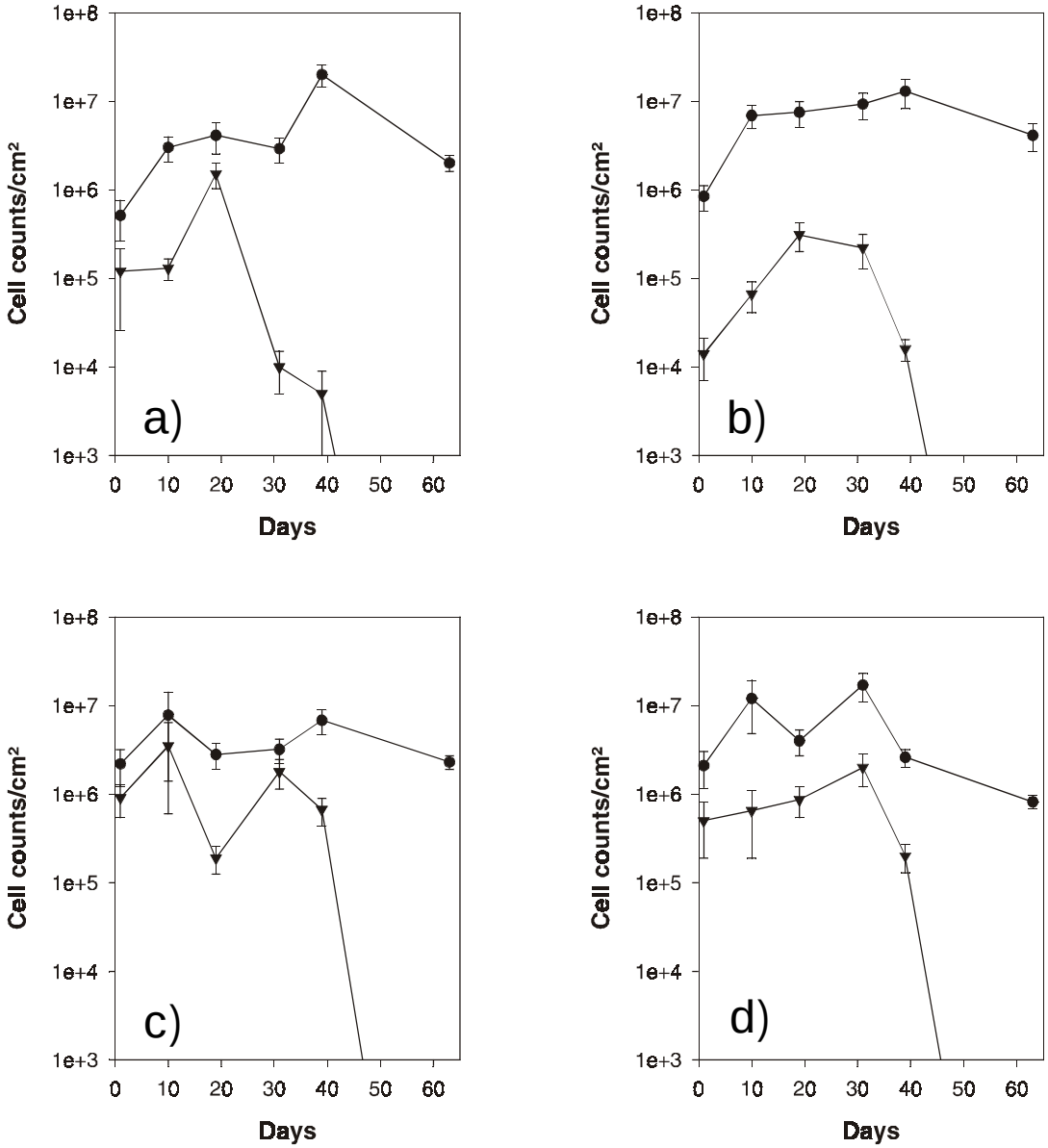
Figure 14: (A) In situ hybridization of pregrown biofilms on glass slides of *Aquabacterium commune* inoculated with *Desulfovibrio desulfuricans*, afterwards exposed to drinking water. 25-day-old biofilm hybridized with Cy3-labeled probe DSV1292 (left) and corresponding DAPI-staining (right) showing the spatial distribution of *Desulfovibrio desulfuricans* within a cell-cluster of *Aquabacterium commune*. (B) Biofilm grown on glass slides in a centrifuge tube reactor: In situ hybridization with Cy3 labeled probe 3L468 and FLUOS labeled probe beta8b after 1 day (left) and after 7 days (right), demonstrating the random distribution of strain zt3l within the biofilm population. (C) and (D) Biofilms grown in a combination of biofilm reactor and continuous flow microchamber: In situ hybridization of biofilms grown in microchambers with Cy3-labeled probe 10E458 (left) and with FLUOS labeled probe BET42a (right) after 14 days growth and 10 days starvation of the biofilm, indicating the survival of strain zt10e. (D) in situ hybridization of detached biofilms from a stage 2 biofilm reactor with Cy3-labeled probe 10E458 (left) and corresponding DAPI-staining (right), showing the establishment of strain zt10e in pregrown aerobic biofilms. For closer explanation see text.

5.2.2 Determination of cell counts by in situ hybridization and DAPI-staining

In the first 11 days of the experiment hybridization and DAPI-staining of the biofilm slides revealed a similar course of increase for aerobic as well as for anaerobic bacteria. After 19 days a bacterial contamination was observed in all four biofilm reactors. Additionally a too low pH of 6.0 was determined in the effluent. Therefore the biofilm reactors were connected to unsterile drinking water on day 20.

After one day hybridization of the glass slides with the respective probes (BET42a, 3L468, 10E458) revealed counts of BET42a-positive cells from 5.1×10^5 ($\pm 2.5 \times 10^5$) cells/cm^2 (strain B3 with strain zt3l, Fig. 15a) up to 2.2×10^6 ($\pm 9.8 \times 10^5$) cells/cm^2 (*Aquabacterium commune* with strain zt3l, Fig. 15c). Cell counts of SRB ranged from 1.4×10^4 ($\pm 7 \times 10^3$) cells/cm^2 (strain B3 with strain zt10e, Fig. 15b) up to 9.1×10^5 ($\pm 3.7 \times 10^5$) cells/cm^2 (*Aquabacterium commune* with strain zt3l, Fig. 15c). All cell counts increased up to day 10, although sometimes very slightly (Fig. 15).

Figure 15: Biofilms grown on glass slides in centrifuge tube reactors. a) strain B3 combined with strain zt3l, b) strain B3 with strain zt10e, c*) Aquabacterium commune* with strain zt3l and d) *Aquabacterium commune* with strain zt10e. Error bars represent standard error. (●), DAPI cell counts per cm^2, (▼) hybridization positve cells of SRB after hybridization with the Cy3-labeled specific probe, 3L468 or 10E458. e) trend calculated from the results of the 4 assays (----) DAPI cell counts, (⋯⋯) hybridization positive cells of SRB.


1e+8
1e+7
1e+6
1e+5
1e+4
1e+3
Cell counts/cm²
0 10 20 30 40 50 60
Days
a)
b)
c)
d)
e)

Up to day 19 the number of aerobic and anaerobic bacteria in biofilms, as determined by hybridization and DAPI-staining of glass slides, increased further in the assays a and b (Fig. 15a and 15b), although there was only a slight increase of aerobic bacteria. In the assays c and d the number of cells hybridizing with probe BET42a decreased as well as the 3L468-positive cells in the assay c. Only the number of 10E458-positive cells increased slightly (Fig. 15b and 15d). This dissimilarity of the different assays was also found in the further course of the experiment between day 19 and 39 despite the medium change to drinking water (Fig. 15). Nevertheless, the number of SRB decreased in all four assays from day 31 on and a corresponding decline of the signal intensity was observable. On day 63 hybridization-positive cells of SRB were no longer detectable in the biofilm although cell-envelopes were still visible (Fig. 15). After 63 days cell counts after DAPI-staining ranged between 8.2×10^5 ($\pm 1.4 \times 10^5$) cells/cm^2 and 4.1×10^6 ($\pm 1.4 \times 10^6$) cells/cm^2 (Fig. 15b and 15d). During the 63 days of the experiment, the examination of the spatial distribution of SRB in the biofilm revealed no species specific features.

5.2.3 Determination of culturability of cells from biofilms and effluents

On day 10 additionally the following characteristics were examined: total cell numbers in the effluent by DAPI-staining, the culturability of aerobic cells in the effluent and in biofilms and the culturability of anaerobic cells in the effluent and in biofilms (Tables 15 and 16). The percentage of culturable cells in the effluent was normalized over total cell counts detected by DAPI-staining. The number of culturable cells of aerobic bacteria varied between 30.6% for strain B3 to values of 2.1% for *Aquabacterium commune* (Table 15).

The percentage of culturable cells from biofilm material was normalized over the cell counts obtained after hybridization with the specific probes. Culturability of aerobic bacteria was much lower compared to the effluent and ranged between 0.5% and 0.004%, the culturability of SRB was as low as 0.002% (Table 16). This is possibly not only due to the applied oxygen stress, the low culturability of the aerobic bacteria hints on additional problems, which may be caused by the low nutrient concentration and the low pH. Nevertheless, the number of hybridization-positive SRB increased in three of the four assays up to day 19 (Fig. 15a,15b, 15d).

Table 15: Cell counts and culturability of aerobic and anaerobic cells in the effluent from centrifuge tube bioreactors after 10 days.

Combination of strains	[a]Cell counts in the effluent per ml (± SE)	Culturability of aerobic bacteria [cfu/ml]	Culturability of an-aerobic bacteria [cfu/ml]	[b]% culturability aerobic bact.	[b]% culturability anaeerobic bact.
strain B3 and zt3l	1.1×10^6 (± 6.2×10^4)	2.2×10^5	1	20	0.00009
strain B3 and zt10e	1.7×10^6 (± 1.3×10^5)	5.2×10^5	4.1×10^2	30.6	0.02
A. commune and strain zt3l	1.4×10^5 (± 2.0×10^4)	3.0×10^3	1.5×10^2	2.1	0.11
A. commune and strain zt10e	2.6×10^5 (± 4.7×10^4)	8.9×10^5	2.4×10^2	4.6	0.09

[a] determined by DAPI-staining
[b] normalized over total cell counts detected by DAPI-staining

Table 16: Cell counts and culturability of cells from scraped off biofilms grown in centrifuge tube bioreactors for 10 days.

Combination of strains	[a]Cell counts of beta bacteria per cm^2 (± SE)	[b]Cell counts of SRB per cm^2 (± SE)	Culturability of aerobic bacteria [cfu/cm^2]	Culturability of SRB [cfu/cm^2]	[c]% culturability aerobic bact.	[d]% culturability SRB.
strain B3 and zt3l	3.0×10^6 (± 9.5×10^5)	1.3×10^5 (± 3.5×10^4)	1.4×10^4	1.7×10^1	0.5	0.01
strain B3 and zt10e	6.9×10^6 (± 2.0×10^6)	6.6×10^4 (± 2.5×10^4)	1.7×10^4	1.2×10^2	0.3	0.2
A. commune and strain zt3l	7.8×10^6 (± 6.4×10^5)	3.5×10^6 (± 2.9×10^6)	1.9×10^3	5.3×10^1	0.03	0.002
A. commune and strain zt10e	1.2×10^7 (± 6.0×10^6)	2.0×10^6 (± 8.0×10^5)	4.8×10^4	1.3×10^2	0.004	0.007

[a] determined after hybridzation with probe BET42a
[b] determined after hybridization with the strain specific probes 3L468 and 10E458
[c] normalized over cell counts determined by hybridzation with probe BET42a
[d] normalized over cell counts determined by hybridzation with the strain specific probes 3L468 and 10E458

5.2.4 Common aspects of the assays

The trend calculated from the results of all four assays (Fig. 15e) indicates a rather balanced picture of the cell counts in the biofilm. Cell counts of aerobic bacteria showed a stable level throughout the whole experiment, while the number of hybridization-positive SRB in all assays began to decrease after 31 days and was no longer detectable after 63 days. The examination of the spatial distribution of SRB in the biofilm revealed no species specific features during the experiment (Fig. 14).

One problem of the experiment was the contamination of the assays, detected after day 19, probably due to the opening of the biofilm reactors for the sampling of the glass slides. Another problem was the uneven growth on the two sides of the substratum because an exact orientation parallel to the flow was not possible. Therefore a new construction for the observation of the survival of SRB in oxic biofilms was developed, which consisted of a combination of a biofilm reactor and a continuous flow microchamber.

5.3 Survival of starvation and establishment of strain zt10e in biofilms of strain B3 and *Aquabacterium commune*

Intentions of this experiment were the comparison of the concomitant inoculation of SRB with aerobic bacteria with the ability of SRB to establish in already existing aerobic biofilms and evaluation of the effect of starvation on SRB in mixed species biofilms. Other points were the detection of the correlation between total cell counts by DAPI-staining and the culturability of cells in the effluent and between hybridization-positive and culturable cells from detached biofilms.

In this experiment the combination of a biofilm reactor with a continuous flow microchamber offered various advantages. The maintenance of sterile conditions was simplified by working with a closed system. Luer connectors simplified sampling and the exchange of tubes. Biofilm development was improved by maximization of the growth surface, using glass beads as immobilization material and thus facilitating the detection of high cell counts in the effluent. Direct investigation of the biofilm was possible in the microchambers, providing a useful general picture of the state of the biofilm. Nevertheless, the system had disadvantages as well. Cell counts and culturability were predominantly investigated by the sampling of effluent, as the investigation of the biofilm led to its irreversible destruction. The spatial distribution of

SRB in the biofilm was therefore examined only once, after hybridization of the biofilms grown in microchambers at the end of the experiment.

5.3.1 Inoculation and flow conditions

The already described reactors (Fig. 1) were inoculated via a side port septum adjacent to the inlet pipe. After determination of the OD 588 based on calibration curves the cell numbers were adjusted to approximately 10^6 cells/ml for strain B3 and *Aquabacterium commune* and to 10^7 cells/ml for strain zt10e. Pumping was started after 18 hours with 4 volume exchanges within 30 minutes. During the first 14 days 50fold diluted R2A served as medium for all 8 reactors, the flow was adjusted to 14 ml/h analogous to 2 volume exchanges per hour. Sampling of stage 1 was started 1 day after the start of the continuous flow, of stage 2 after 8 days.

Experiments were performed in 4 parallels. For 14 days 4 biofilm reactors (stage 1) were operated as mixed species reactors, inoculated with the aerobic bacteria strain B3 and *Aquabacterium commune* and with the sulfate-reducing strain zt10e (Fig. 16). Starvation was introduced to this stage after 14 days, the 50fold diluted R2A medium was then substituted with freshwater buffer. In these reactors concomitant inoculation as well as the effect of starvation was examined. 4 additional reactors (stage 2) served for 15 days as reactors for the development of aerobic biofilms consisting of strain B3 and *Aquabacterium commune* (Fig. 16). After 14 days the flow-direction was changed and a two-stage reactor system was established with freshwater buffer running at the former flow-rate through stage 1. After supplementation of the effluent from stage 1 with R2A to a final concentration of a 20fold dilution, which was performed avoiding changes of the flow-through, the medium was led through stage 2. Thus, the establishment of SRB in already existing biofilms could be examined. For two of these 4 two-stage reactors the experiment was terminated after a running time of 24 days, i.e. 10 days after the onset of starvation, concluding examinations of the other two reactors were performed after 31 days.

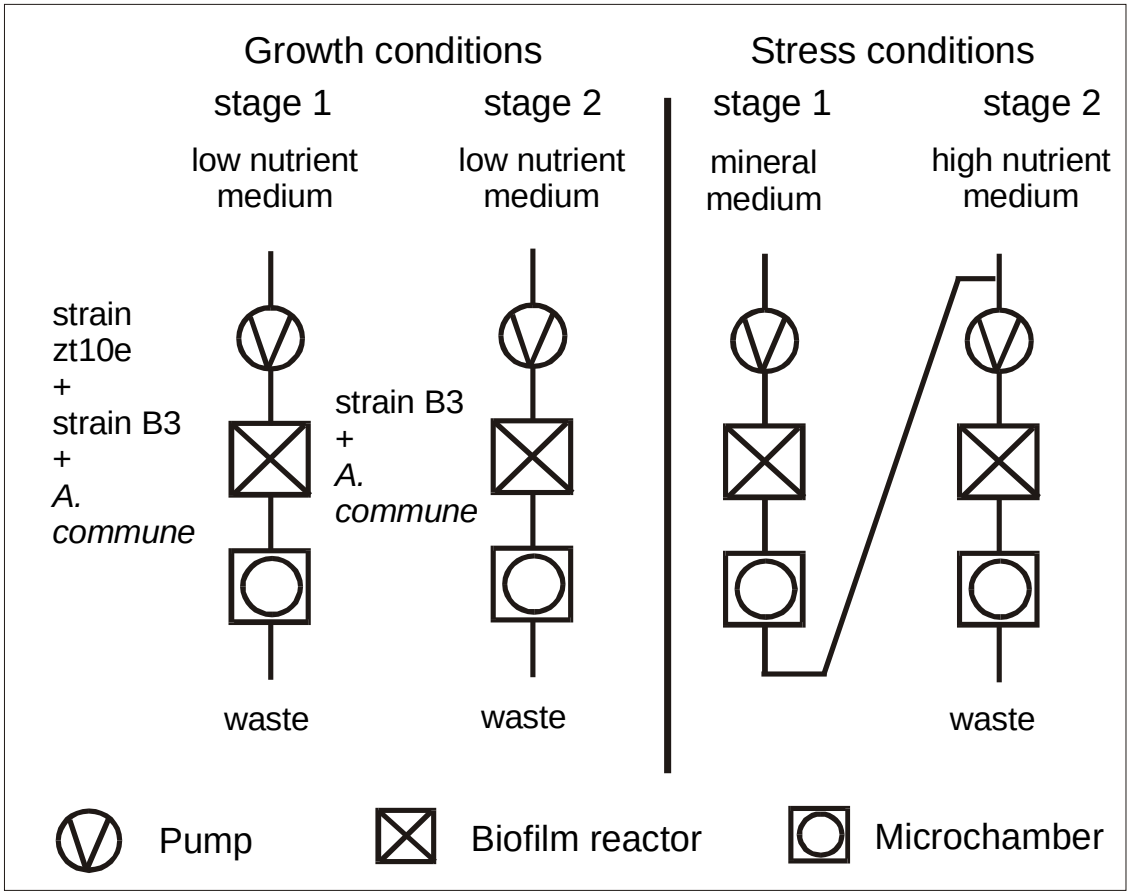


Figure 16: Scheme of the medium flow through the combined biofilm reactors during the growth phase (14 days from the start of the experiment) and the stress phase. For further explanation see text.

5.3.2 Total cell counts and culturable cell counts in the effluent

5.3.2.1 Development of aerobic bacteria

In stage 1 aerobic bacteria reached a stable level of approximately 2 x 10^7 cells/ml very fast as revealed by DAPI-staining (Fig. 17). The onset of starvation was followed by a decrease of one order of magnitude, remaining on a constant level despite the lack of nutrients. The percentage of culturable cells was between 25.8% up to 33.6% for 14 days. After switching to starvation conditions culturability presented a less constant picture, with values of culturability varying between 6.3% and 34.7% (Table 17).

Table 17: Percentage of culturable cells in the effluent of a two-stage bioreactor system

Day	% culturability of cells in the effluent[a] (± SE)			
	aerobic cells in stage 1	aerobic cells in stage 2	anaerobic cells in stage 1	anaerobic cells in stage 2
1	33.2 (± 5.5)%		0.416 (± 0.047)%	
4	29.9 (± 4.6)%		0.485 (± 0.066)%	
7	33.6 (± 7.5)%	67.6 (± 11,1)%	0.784 (± 0.126)%	
10	25.8 (± 2.6)%	28.1 (± 8.1)%	0.114 (± 0.029)%	
14	28.9 (± 0.6)%	21.0 (± 2.1)%	0.032 (± 0.014)%	
17	12.7 (± 0.7)%	48.8 (± 22.4)%	0.016 (± 0.011)%	0.0022 (± 0.0014)%
21	33.8 (± 14.2)%	78.9 (± 23.7)%	0.017 (± 0.014)%	0.0004 (± 0.0002)%
24	7.7 (± 0.4)%	40.4 (± 16.7)%	0.015 (± 0.014)%	0.0021 (± 0.0012)%
28	34.7 (± 26.0)%	21.6 (± 8.8)%	0.011 (± 0.002)%	0.0013 (± 0.0004)%
31	6.3 (± 2.1)%	15.7 (± 3.9)%	0.004 (± 0.004)%	0.0647 (± 0.0313)%

[a] normalized over total cell counts detected by DAPI-staining

Sampling of stage 2 began 7 days after start of the continuous flow with cell counts by DAPI-staining a little below the cell counts detected in the effluent of stage 1. Remarkably, the conditions were almost identical, the only difference being the presence of the SRB in stage 1. The cell counts increased until day 14, when they were on the same level as stage 1. From there on a steady rise was observable which led to a cell count of 2.6×10^8 ($\pm 5.5 \times 10^7$) cells/ml on day 31, probably due to the higher nutrient concentration (R2A 20fold diluted) used in this stage. The number of culturable cells in this assay was rather unsteady. The percentage of culturable cells was 67.6% of the total cells on day 7, decreasing to 20.9 % on day 14. After the switch to a higher nutrient concentration, the number of culturable cells began to rise again and reached 78.8% on day 21. After reaching this point the percentages decreased once more and reached subsequently 15.7% on the last day of the experiment (Table 17).

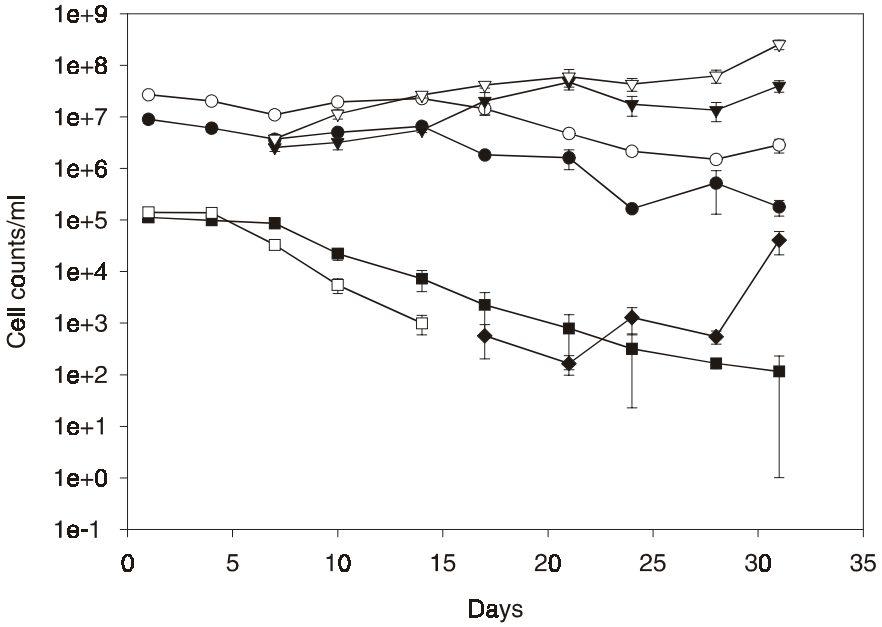


Figure 17: Effluent from a combination of biofilm reactor and continuous flow microchamber. Unfilled symbols (❍) indicate cell counts per ml after DAPI-staining, filled symbols (●) represent the number of culturable cells per ml of cells of aerobic bacteria (strain B3 and *Aquabacterium commune*) in the effluent from stage 1, (▽, ▼) cells of aerobic bacteria (strain B3 and *Aquabacterium commune*) in the effluent from stage 2, (□, ■) cells of strain zt10e from the effluent of stage 1, and (◆) of cells of strain zt10e from the effluent of stage 2. Error bars represent standard error. For further explanation see text.

5.3.2.2 Development of anaerobic bacteria

Cell counts of strain zt10e after DAPI-staining were only measurable until day 14, when they reached the detection limit. Because of the high proportion of aerobic cells in the effluent direct detection of strain zt10e was difficult and led to values which were even below the number of detected culturable cells. As the values of culturable cells of strain zt10e seemed to be in good accordance with the direct counts, the number of culturable cells was normalized to the total cell counts by DAPI-staining (Table 17).

In stage 1 the number of cells of strain zt10e started to decrease on day 7 at a constant rate unaltered until the end of the experiment independently of the change of the nutrient concentration on day 14 (Fig. 17). After 31 days 1.2×10^2 ($\pm$ 1.1×10^2) culturable cells/ml of strain zt10e were still detectable (Fig. 17).

Detection of strain zt10e by DAPI-staining in the effluent of stage 2 showed similar difficulties as described for stage 1, resulting in the determination of only the number of culturable cells of strain zt10e. The number of culturable cells of zt10e from the

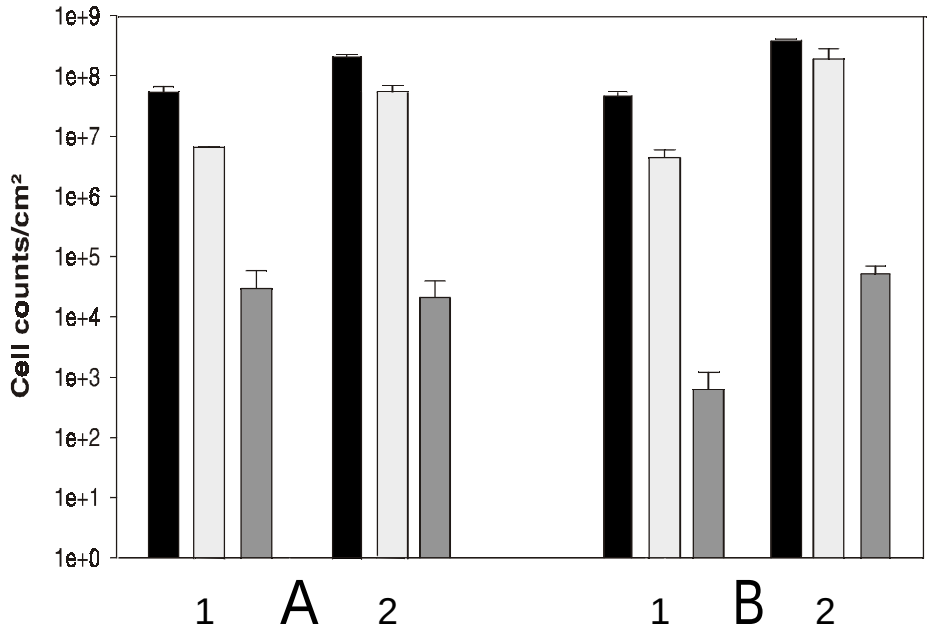
stage 2 effluent was 20% to 25% below the numbers obtained from the effluent from stage 1 during the first 7 days after the change of the flow direction. This was followed by an increase to cell numbers of 1.0×10^3 ($\pm 7.0 \times 10^2$) culturable cells/ml on day 24, which was higher than the values obtained from stage 1 (Fig. 17). On day 31 the number of culturable cells in the effluent of stage 2 reached a level corresponding with the number of cells in the effluent of stage 1 at the beginning of the experiment (Fig. 17), thus suggesting the establishment of strain zt10e in the already developed biofilm.

5.3.3 Investigation of the biofilm composition

5.3.3.1 Cell counts as revealed by DAPI-staining and cultivation

After a total time of 24 (sample A) and 31 (sample B) days, respectively, the experiment was terminated for two of the 4 two-stage reactors thus allowing the investigation of the biofilm composition by DAPI and by aerobic and anaerobic cultivation. DAPI-cell counts revealed only little differences between the two sampling dates with values of 5.5×10^7 ($\pm 1.4 \times 10^7$) cells/cm^2 (sample A) and 4.9×10^7 ($\pm 8.1 \times 10^6$) cells/cm^2 (sample B) for biofilms from stage 1 and 2.3×10^8 ($\pm 1.9 \times 10^7$) cells/cm^2 (sample A) and 4.0×10^8 ($\pm 3.5 \times 10^7$) cells/cm^2 for biofilms from stage 2 (Fig. 18). Remarkable was the small change in cell counts of stage 1 biofilms between the two sampling dates, although the medium contained no nutrients.

Figure 18: Cell counts per cm^2 of a detached biofilm grown in a combination of biofilm reactor and continuous flow microchamber. Characters indicate the sampling date A after 24 days and B after 31 days. Numbers indicate the reactor stage 1 or 2: : ■ DAPI cell counts, : □ culturable cell counts of aerobic bacteria (strain B3 and *Aquabacterium commune*), : ▭ culturable cell counts of strain zt10e. Error bars represent standard errors. For further explanation see text.



Culturability of anaerobic bacteria showed a less balanced picture, but confirmed the establishment of strain zt10e in an already developed biofilm (Fig. 18). Nevertheless, changes in the number of culturable cells of strain zt10e were observable in biofilms from stage 1 at the different sampling dates (Fig. 18). Although the total cell counts remained on a nearly identical level, strain zt10e from stage 1 biofilms lost its culturability between day 24 and day 31 at a higher rate than in the effluent. This could also be observed after calculation of the percentages of culturable cells after aerobic and anaerobic cultivation as shown in Table 18.

Table 18: Percentages of culturable cells from detached biofilm.

Reactor stage	Sample	% culturability of cells from detached biofilms[a] (± SE)	
		aerobic cultivation	anaerobic cultivation
1	A	12.8 (± 0.4)	0.055 (± 0.053)
2	A	26.3 (± 7.3)	0.010 (± 0.008)
1	B	9.5 (± 3.3)	0.013 (± 0.012)
2	B	50.5 (± 25.2)	0.013 (± 0.005)

[a] normalized over total cell counts detected by DAPI-staining

The percentage of cells culturable after aerobic cultivation seemed to be influenced by the nutrient conditions present in the different stages. Only minor changes occured between the two sampling dates in the stage 1 biofilms, which were held under starvation conditions for 10 days before the first and for 7 more days before the second sampling date. Culturability of aerobic bacteria from biofilms sampled from stage 2 of the bioreactors was clearly higher and increasing between the two sampling dates.

5.3.3.2 Hybridization of detached biofilm material

The hybridization of the detached biofilm material was performed as described for liquid cultures with the probes beta3, specific for strain B3, beta8a and beta8b, specific for *Aquabacterium commune* and with the probe 10E458, specific for strain zt10e. Strain B3 proved to be dominant in the biofilm, therefore only the percentages of *Aquabacterium commune* and strain zt10e are given, normalized over the total cell counts determined by DAPI-staining (Table 19).

Table 19: Composition of biofilm population

Reactor stage	Sample	% of hybridization positive cells from biofilms[a] (± SE)	
		Probe beta8a and beta8b	Probe 10E458
1	A	0.458 (± 0.197)	0.328 (± 0.156)
2	A	2.522 (± 0.359)	0.022 (± 0.014)
1	B	0.324 (± 0.075)	0.131 (± 0.044)
2	B	1.331 (± 0.136)	0.079 (± 0.061)

[a] normalized over total cell counts detected by DAPI-staining

Remarkable was the low percentage of *Aquabacterium commune* within the biofilm community, as it is the in situ dominant strain in young biofilms from the natural habitat, the Berlin drinking water system. Additionally the percentage is still decreasing in both stages, no matter the different nutrient conditions. At least strain zt10e behaved as expected, showing a decrease in stage 1 (starvation conditions) and an increase in stage 2 (high nutrient conditions) between the two sampling dates.

The percentage of culturable cells of zt10e normalized to the number of hybridization positive cells showed a different course, as a decrease was detectable

for both stages between the two sampling dates. For stage 1 the percentage decreased from 17.2 (± 16.7)% on day 24 to 1.0 (± 0.9)% on day 31. Parallel was the decrease in stage 2 from 43.3 (± 37.8)% to 17.1 (± 6.0)% culturable cells of the hybridization positive cells of strain zt10e. A divergent behaviour was found, when the percentage of culturable cells of strain zt10e was normalized to the total cell counts by DAPI (Table 18), probably due to the different development of aerobic bacteria.

5.3.3.3 Spatial distribution of strain zt10e after hybridization of biofilms grown in microchambers

The investigation of the spatial distribution of SRB within the biofilms made several problems obvious. The high cell counts of aerobic bacteria made their determination by direct methods very difficult, after calculation total cell counts were as high as 5000 cells on an area of 0.01 mm^2 in stage 1 biofilms. As the cells were very densely packed, direct observation of hybridization positive cells was nearly impossible, especially in biofilms from stage 2. For the biofilms from stage 2 cell counts as high as 30000 cells per 0.01 mm^2 could be calculated, while cell counts of strain zt10e were much lower. Occasionally hybridization positive cells of strain zt10e could be observed, but were mostly covered by aerobic bacteria. Thus a statistic evaluation of the cell counts was impossible. Pictures taken from the biofilms revealed no particular spatial distribution of strain zt10e within the biofilm (Fig. 14D).

Discussion

1. Characterization of raw water biofilms

In this approach to characterize the microbial population of a groundwater biofilm, different in situ methods were combined to describe cell density, phylogenetic affiliation and exopolymeric substances. Of special interest was the presence of sulfate-reducing bacteria in the biofilm.

1.1 Population density and composition

Substrata used in this study were identical to those used by Kalmbach (1998) for the investigation of the Berlin ground and drinking water system, allowing a comparison of the results. Hydrophobic PE (Pringle and Fletcher, 1982) is known to enhance bacterial attachment and growth (Schoenen and Wehse, 1988; van der Kooij and Veenendahl, 1993). PE-LD allowed light microscopic examination of the biofilms, but was only applicable in the Robbins devices, as it is a flexible material. The handling of the PE-HD slides was obstructed by the detachment of upper layers of the very thick biofilm during fixation and dehydration, the results obtained with this material are therefore not discussed. Although PE is increasingly used in the drinking water distribution system of the Berliner Wasserbetriebe (Berliner Wasserbetriebe, personal communication), glass slides were used in most of the experiments in this study because of the easy handling, the possible microscopic examination of the biofilms and the inert nature of the material.

Bacterial densities on 90 days old biofilms on glass and PE-LD slides from the Berlin groundwater differed only inconsiderably from values obtained for 70 days old biofilms from the Berlin drinking water system (Kalmbach et al., 1997a). Similarities were also found with regard to the influence of the growth surfaces. Total cell counts on PE-LD were twofold higher than on glass, confirming the observation that bacteria isolated from freshwater attach preferentially to hydrophobic surfaces (Pringle and Fletcher, 1983; Kalmbach et al., 1997a). The phylogenetic composition showed the beta-*Proteobacteria* to be the most important group within this habitat, as was already shown for other drinking (Kalmbach et al., 1997a; Kalmbach 1998) and freshwater habitats (Weiss et al., 1996; Alfreider et al., 1996; Pernthaler et al., 1998). The different substrata exposed in the Berlin groundwater also showed the already described higher phylogenetic diversity compared to drinking water biofilms,

represented by higher percentages of bacteria affiliated to the alpha- and gamma-subclasses of *Proteobacteria*, especially on glass slides (Kalmbach et al., 1997a). The population shift compared to the drinking water is probably due to the drinking water treatment process, where important ions like iron and manganese are almost completely precipitated. Results on the number of hybridization positive cell counts in Berlin ground water biofilms after hybridization of PE-LD and glass slides with probe EUB338 also resembled results already obtained by Kalmbach et al. (1997), showing similar values to 70 days old biofilms from Berlin drinking water.

1.2 In situ characterization of biofilm exopolymers

The lectin staining of the biofilm revealed the presence and the spatial relationships of extracellular polymeric components within the groundwater biofilm. The staining with lectins specific for various binding sites indicated that sufficient quantities of EPS were present and could easily be visualized. The EPS is able to bind various nutrients, metals and contaminants (Geesey and Jang, 1989; Wolfaardt et al., 1995), which seems to be the case in the typical spiral bands excreted from the terminal *Gallionella* cells. The EPS is a major component of most biofilms (Characklis and Cooksey, 1983) and plays an important role in development and persistence of biofilms as structural component as well as a nutrient source (Neu and Lawrence, 1997).

1.3 Presence of sulfate-reducing bacteria in Berlin groundwater biofilm

In situ hybridization of the groundwater biofilm with probes specific for different sulfate-reducing bacteria revealed only a low number of sulfate-reducing bacteria to be detectable by this method, independent of the exposure time of the biofilm. Hybridization-positive cells could be found with the probes SRB385Db and with the newly developed probes 3L468 and 10E458, although the detection was infrequent and the hybridization signal intensity was low, making a statistical evaluation very difficult. One reason might be the absence of target sequences, as only two out of six newly isolated organisms reacted with previously developed probes (Devereux et al., 1992; Rabus et al., 1996; Manz et al., 1998). This shows again the limitations of the current database used in this study. 13803 partial and complete 16S rRNA sequences still correspond only to a small part of the estimated species number ranging from 40 000 (Bull et al., 1992) to more than 1 000 000 species (American

Society of Microbiology, 1997). A concentration step and the application of molecular methods like selective PCR-amplification with specific primers as described by Fry et al. (1997) or the PCR-based detection of genes coding for the dissimilatory sulfite reductase (Wagner et al., 1998) might be necessary to improve the detection of sulfate-reducing bacteria in this habitat, though limiting the examination of the spatial distribution. In 90 days old biofilms an additional reason for the limited SRB-detection might be low metabolic activity of the sulfate-reducing bacteria, resulting in low hybridization signal intensities due to a decreasing rRNA content as described by Poulsen et al. (1993). In drinking water biofilms the proportion of bacteria containing a sufficient amount of ribosomes to yield positive hybridization signals declined to a stable level of 50% of the total population after 35 days (Kalmbach et al., 1997a), resembling values, which were obtained for the groundwater biofilms. Metabolic potential of sulfate-reducing bacteria, and therefore the hybridization signal intensity, might be even lower in this oligotrophic, oxic, for the growth and survival of sulfate-reducing bacteria unfavorable habitat. Even activation of cultures of sulfate-reducing bacteria by means of the probe active count (PAC) method for 48 h, 96 h and 144 h, respectively, did not yield an improved detection of sulfate-reducing bacteria. The original direct viable count (DVC) method of Kogure et al. (1979) worked with a incubation period of 8 h, while the PAC method (Kalmbach et al., 1997a) showed the best results after an incubation time of 16 h for drinking water biofilms. A change of antibiotics (Joux and LeBaron, 1997) resulted in the extension of the incubation period for marine bacteria to 24 h without elongation. Although the incubation period in this study was lengthened to 144 h, this extension still seemed not to be sufficient for the activation of sulfate-reducing bacteria from a natural habitat.

2. Isolation and characterization of sulfate-reducing bacteria from ground- and drinking water systems

2.1 Phylogenetic affiliation

A variety of sulfate-reducing bacteria was isolated from different locations in the Berlin ground- and drinking and the Duisburg drinking water system. Phylogenetic classification of these sulfate-reducing bacteria was performed by 16S rDNA sequence analysis, which has a variety of advantages, including providing insights into the evolutionary origins of sulfate reduction in distantly related species (Castro et al., 2000). Comparative sequence analysis revealed four of the newly isolated

sulfate-reducing bacteria to be members of the Gram-negative *Desulfovibrionaceae*, while two clustered within the genus *Desulfosporosinus*, indicating a possible predominance of the *Desulfovibrionaceae*. A predominance of members of the *Desulfovibrionaceae* in the population of sulfate-reducing bacteria was demonstrated for habitats like anoxic groundwater (Fry et al., 1997), oil field water (Voordouw et al., 1996) or the upper layers of lake sediment (Sass et al., 1997).

Members of the sulfate-reducing genus *Desulfovibrio* are well known to survive oxygen stress. Most sulfate-reducing isolates from the oxic/anoxic interface of the sediment of an oligotrophic lake have been found to be affiliated to this genus (Sass et al., 1997). Their presence could also be demonstrated in habitats like the oxic zone of a stratified fjord (Teske et al., 1996) or the chemocline of a hypersaline cyanobacterial mat community (Minz et al., 1999).

The four isolated members of the *Desulfovibrionaceae* were found to be clustering in two different groups. Strain zt3l and strain Mlhm clustered within a group of the *Desulfovibrionaceae*, whose members have only been isolated recently. The first representative, strain STL10 (GenBank acc. no. X99501) was isolated from oligotrophic Lake Stechlin (Sass et al., 1997), indicating adaptation of the species to conditions with intermittent periods of oxygen exposure and starvation. Another member of this group, R-LacA1 (GenBank acc. no. AJ012593), was isolated from rice paddy soil (Wind et al., 1999), a habitat, which is at least periodically in contact with oxygen (S. Stubner, personal communication). Only clone B4 (GenBank acc. no. AJ133797) was isolated from an anoxic habitat, a ditch sediment.

Parallels were found for strain zt10e and strain GWE2. Here as well close relatives were isolated from rice field soil, particularly *Desulfovibrio burkinensis* (GenBank acc. no. AF053752) (Outtara et al., 1999) and R-SucA1 (GenBank acc. no. AJ012592) (Wind et al., 1999). Other relatives of strain zt10e and strain GWE2 were obtained from habitats as diverse as an anaerobic waste water reactor and an estuarine sediment (Qatibi et al., 1991; Ollivier et al., 1988).

The spore-forming strains, strain 5apy and strain Blif, clustered within the genus *Desulfosporosinus*. Close relatives of these strains were isolated from habitats like shallow groundwater (Robertson et al., 2000), soil (DSMZ-catalogue) and surface sediment (Newman et al., 1997). As members of the genera *Desulfosporosinus* and *Desulfotomaculum* are highly sensitive towards oxygen (Cypionka et al., 1985), it can be assumed that they were present in the habitat in form of spores. Sass et al. (1997)

isolated non-sporulated members of this genus from the sediment of lake Stechlin, but only from the anoxic layers of the sediment.

2.2 Phenotypic characterization

In traditional classification schemes various properties like cell shape, motility, presence of desulfoviridin, catalase or cytochromes, optimal temperature, type of oxidation and utilization of electron donors and electron acceptors were used for the description of this complex physiological bacterial group (Castro et al., 2000). Investigation of some of these properties was performed with all strains, utilization of electron and acceptors was only investigated for the two strains isolated from the Berlin groundwater, strain zt3l and strain zt10e.

Cell morphology differed considerably between the close relatives strain zt3l and strain Mlhm and between strain 5apy and strain Blif, only strain zt10e and strain GWE2 showed similarities. For all other closely related isolates size or cell shape showed differences making their close relation as revealed by 16S rDNA sequencing unlikely. But the simple morphologies of bacteria, without remarkable characteristics except the size, are of no use in defining their phylogeny, as well as bacterial physiologies are only of limited use for a classification (Woese, 1987). Other characteristics like the presence of desulfoviridin, an enzyme typical for the *Desulfovibrionaceae* (Postgate 1959), which could be detected in all isolates belonging to the *Desulfovibrionaceae*, and of the spore forming capacity of members of the genus *Desulfosporosinus* are better tools to give hints on the classification of the isolates.

For strain zt3l and zt10e additional characteristics like the utilization of electron acceptors and donors and the growth rates were detemined, showing close similarities to their next relatives (Qatibi et al., 1991; Olivier et al., 1988; Sass et al., 1997; Outtara et al., 1999; Wind et al., 1999). The growth rate of strain zt3l was almost identical to the rate of strain STL 10 (Sass 1997), obtained at 30°C, while the growth rate of *Desulfovibrio fructosovorans* when grown with fructose at 30°C was close to one third of the value obtained for strain zt10e grown on ethanol (Ollivier et al., 1988).

2.3 Relation of isolated sulfate-reducing bacteria to oxygen

The newly isolated sulfate-reducing bacteria came from ground- and drinking water habitats containing considerable amounts of oxygen, thus rising questions on the survival of sulfate-reducing bacteria under oxic conditions. For this reason oxygen-dependent respiration of strain zt3l and strain zt10e was tested. Aerobic respiration has already been demonstrated for different sulfate-reducing species (Dilling and Cypionka, 1990; Dannenberg et al., 1992; Krekeler et al., 1997; Kuhnigk et al., 1996; Sass et al., 1997), during which they were able to survive and to form ATP (Dilling and Cypionka, 1990). Both strains from the Berlin groundwater were able to respire oxygen with different substrates, an ability which might be essential for their survival in this habitat, as the best accession to substrates is given adjacent to the water, though it transports not only substrates but also oxygen.

3. Additional characteristics of in situ dominant drinking water bacteria

Eight different bacteria belonging to the beta-subgroup of the *Proteobacteria* were recently shown to represent the predominant, frequent in situ bacterial species in biofilms obtained from the Berlin drinking water distribution system (Kalmbach et al., 1997b). Three of the most abundant strains, *Aquabacterium citratiphilum* (B4), *Aquabacterium parvum* (B6) and *Aquabacterium commune* (B8) were described in closer detail (Kalmbach et al., 1999). Nevertheless, some characteristics, which may play a role in the groundwater habitat, as well as another abundant species were not included into the description.

3.1 Significance of the oxidation of ferrous iron coupled to the reduction of nitrate

The bacterial process of anaerobic microbial oxidation of ferrous iron coupled to the reduction of nitrate was discovered only recently (Straub et al., 1996), and the aerobic oxidation of ferrous iron could be demonstrated at neutral pH (Benz et al., 1998). Since nitrate-dependent iron oxidation closes the iron cycle within the anoxic zone for example in sediments and aerobic iron oxidation enhances the reoxidation of ferrous to ferric iron in the oxic zone, both processes increase the importance of iron as transient electron carrier. In anoxic zones, as they are potentially existing in groundwater biofilms, the iron cycle could be closed by anaerobically Fe-(III)

reducing bacteria, an ability also known to be present in sulfate-reducing bacteria (Lovley et al, 1993). The close relationship of the in situ abundant species in the Berlin drinking water with species performing anaerobic nitrate-dependent oxidation of ferrous iron (Buchholz-Cleven et al., 1997), induced the investigation whether the Berlin drinking water species were able to perform this bacterial process as well. It was performed by strain B2, which showed an abundance of 2.5% (mean value of 10 months) in the drinking water, of 0% in the ground water and of 1% in the distribution system (Kalmbach 1998; Kalmbach et al., 1997b). As the nitrate content in the Berlin raw and drinking water is fairly low (0.01 – 0.014 mM), the process is probably insignificant in this habitat.

3.2 Detection of *N*-Acyl homoserine lactones

N-Acyl homoserine lactones molecules have been shown to act as mediators of autoinduction, a form of intercellular communication, in which cells monitor the level of self-produced autoinducer signal molecules, typically acylated homoserine lactone derivatives (Fuqua et al., 1994). AHLs are membrane-permeant signal molecules that accumulate as a function of cell density, and at some threshold level, described as bacterial quorum, activate expression of target genes. Therefore, quorum-dependent gene expression occurs preferentially at high cell densities (Fuqua at al., 1994). High density of bacteria, as it is typical for biofilms, has consequently led to the investigation whether quorum sensing genes and AHL production are present within biofilms. The presence of AHL was proven in river water biofilm (McLean et al., 1997), and the mutation in autoinducer synthesis led to formation of biofilms with abnormal structures (Davies et al., 1998). Nevertheless, the role of cell-to-cell signalling for the physiology of biofilms has only partly been explained (Davies et al., 1998). The investigation of the production of AHLs by the eight frequent in situ bacterial species from the Berlin drinking water system revealed the unmistakable release of detectable amounts of this molecules by strain B1 and strain B3. Nevertheless, from the absence of signal one cannot conclude that the other tested bacteria do not produce one or more AHLs. Such organisms may produce signals that are not detectable by the reporter strain because of structural differences, which may inhibit recognition, or the organisms may produce insufficient amounts, below the detection level of the reporter strain (Shaw et al., 1997).

3.3 Monospecies biofilms

For further biofilm and coculture experiments strain B3 and *Aquabacterium commune* were selected out of the eight isolates from the Berlin drinking water. Strain B3 had a broad substrate spectrum, was able to produce detectable amounts of an autoinducer and was present in ground and drinking water, with a high abundancy within the distribution net (Kalmbach et al., 1997b). *Aquabacterium commune* was selected as it was the in situ dominant bacterial species in all parts of the Berlin water distribution net (Kalmbach et al., 1997b).

The biofilm building behaviour of aerobic drinking water bacteria was tested in monospecies experiments with strain B3 and *Aquabacterium commune* by direct microscopical investigation of their reactions in continuous-flow microchambers. The flow chamber allowed direct observation of the bacteria under sterile conditions over long time periods. Monospecies biofilms of strain B3 and *Aquabacterium commune* differed neither in the initial colonization stage nor in subsequent biofilm accumulation. Each species accumulated in small clusters, which expanded rapidly in both the horizontal and vertical direction, leaving bare substratum between the clusters. This kind of microcolony-formation in the initial stages of biofilm building was demonstrated for various aerobic and anaerobic species, although their character and their development differed (Szewzyk and Schink, 1988; Dalton et al., 1996) and was negated for different *Pseudomonas* species (Siebel and Characklis, 1991; Dalton et al., 1996). Influence on the following formation of a stable biofilm community could be exerted by the substrate concentration, as higher concentration induce thicker biofilms (Szewzyk and Schink, 1988; Wimpenny and Colasanti, 1997). Nevertheless, the formation of thick, homogeneous biofilms was not realized with the substrate concentrations used. Instead the biofilms resembled the water-channel model developed by Costerton and coworkers (1994), where bacteria grow in matrix-enclosed microcolonies interspersed with less-dense regions of the matrix that include highly permeable water channels (Lawrence et al., 1991; de Beer et al., 1994; Massol-Deyá et al., 1995). This kind of biofilm architecture is probably a general microbial strategy to deal with the problem of limiting diffusive transport in thick biofilms. Nevertheless, oxygen profiles measured in cell clusters of such biofilms revealed oxygen penetration only into the upper parts of the clusters, leaving their lower parts anaerobic (de Beer et al., 1994), a possible explanation for the survival and activity of sulfate-reducing bacteria in biofilms (Kühl and Jørgensen,

1992; Ramsing et al., 1993; Santegoeds et al., 1998). The biofilm organization of the monospecies biofilms, in terms of structure and spatial arrangement, remained stable after 10 to 14 days, a value also found for a degradative biofilm community (Wolfaardt et al., 1994).

The influence of starvation, probably the common lot of bacteria in nature (Matin, 1992), was investigated by changing the substrate in the biofilm experiments to a mineral medium without any energy or carbon source. Bacteria respond to such a changing of the environmental conditions by a number of adaptive survival mechanisms, including phenotypic switching, often manifested both physiologically and structurally. A common structural response to changes in the nutrient level is the variation of the cell size, which has been demonstrated for several bacterial species (Kjelleberg et al., 1983; Herman and Costerton, 1993; James et al., 1995a) and was also found for strain B3 and *Aquabacterium commune*. Additionally, the nutrient status also affects bacterial adhesion (Marshall et al., 1971). Kjelleberg and coworkers (1985) demonstrated that marine bacterial communities growing in low-nutrient medium displayed greater relative adhesion than those grown in high-nutrient medium, and a range of different bacteria displayed enhanced adhesion after starvation (Dawson et al., 1981; Kjelleberg and Hermansson, 1984; Camper et al., 1993; James et al, 1995a). But increased bacterial attachement as a low-nutrient response is not a universal phenomenon, as some pseudomonads detached under nutrient-limited conditions (Delaquis et al., 1989). However, the attachement as well as the biofilm structure of strain B3 and *Aquabacterium commune*, the objects of this investigation, showed no changes during a starvation period of 4 weeks and a significant detachment of cells was not observed. A reason might be that population expansion is less important for survival under starvation conditions, leading to an aggregative behaviour.

4. Behaviour of selected sulfate-reducing bacteria under oligotrophic conditions and oxygen stress in particle-free systems

Two newly isolated sulfate-reducing bacteria from Berlin groundwater and two sulfate-reducing bacteria from culture collections were stressed under oligotrophic conditions and oxygen stress in particle-free systems, which resembled conditions as they are relevant in ground- and drinking water systems. The two newly isolated sulfate-reducing bacteria, strain zt3l and strain zt10e, and their closest relatives have

already been discussed above. The other two organisms belong to the genera *Desulfomicrobium* and *Desulfovibrio*. *Desulfomicrobium baculatum* (DSM 1743) was first isolated from an estuarine sediment, which indicates oxygen tolerance. *Desulfovibrio desulfuricans* (DSM 6949), isolated from strictly anaerobic sheep-rumen, was found to be the most oxygen-sensitive sulfate-reducing bacteria in our experiments. However, a very close relative was isolated from microbial aggregates of the river Elbe, suggesting a high oxygen tolerance of this isolate (GenBank acc. no. AF150723, Böckelmann, personal communication). The observations made on the newly isolated strains as well as on strains from the culture collection emphasize the fact, that physiological characteristics or ecological functions of a bacterium can not be linked to the behaviour of its closest relatives.

4.1 Influence of culture conditions

Investigations on the survival of SRB under oxic, oligotrophic conditions in aquatic environments performed so far demonstrated a survival period of 72 hours before the experiments were terminated (Hardy and Hamilton, 1981). The results of our study suggest that some SRB are able to survive much longer periods of similar conditions without changes in the culturablity. In addition to the strain specific influence, culturability of stressed sulfate-reducing bacteria was significantly influenced by the medium used in the experiments. Starvation under carbon limitation in mineral medium resulted in prolonged culturability compared to the Berlin drinking water stress microcosm, although chemical parameters obtained for the Berlin drinking water show values for organic carbon, nitrate and phosphate which support bacterial growth (LeChevallier et al., 1991; Miettinen et al., 1997). These results are in good accordance with findings of Botzenhart and Kufferath (1976), who reported improved survival and growth of various aerobic gram-negative bacteria in carbon free mineral medium compared to drinking water.

Coculture experiments with the autochthonous drinking water species *Aquabacterium commune* were performed in mineral medium to elucidate the presumed protective function of aerobic bacteria on the survival of sulfate-reducing bacteria. A significant increase of the survival time of sulfate-reducing bacteria compared to pure culture tests was found, although the number of anaerobic bacteria surpassed the number of aerobic bacteria. Usually a higher number of aerobic bacteria was found in aerobic or periodically oxic habitats, apparently accounting for

the improved survival of the anaerobes. The effect was explained by the protection of the anaerobic bacteria against oxygen by the high oxygen uptake of growing, oxygen-consuming, aerobic bacteria (Gerritse et al., 1992). Nevertheless, a protective effect can also be attributed to autochthonous drinking water bacteria, even if their metabolic activity is low due to starvation conditions. But how low is this activity? The best indication of inactivation is the lysis of a great percentage of the initially inoculated *Aquabacterium commune* cells. However, the remaining cells could still be active and perform metabolism and therefore oxygen respiration for example on airborne organic substances (Geller, 1983) or on the debris of already lysed cells (Horn and Hempel, 1997). In experiments with lower initial cell counts (10^5 cells/ml of sulfate-reducing bacteria and *Aquabacterium commune*) only few debris is available, as the number of *Aquabacterium commune* cells remains on a stable level. But the effect on the sulfate-reducing bacteria was difficult to determine. Only *Desulfomicrobium baculatum* showed a culturability comparable to experiments with high initial cell concentration, while culturability of *Desulfovibrio desulfuricans* and strain zt3l ceased after a considerable shorter period. One reason might be the lower activity of *Aquabacterium commune* cells due to lack of cell debris, another might be the reduced respiration capacity of the low number of sulfate-reducing bacteria.

4.2 Measurement of the metabolic potential by in situ hybridization

In situ hybridization was first introduced 1989 by DeLong et al. (1989) as a tool for the identification of whole bacterial cells and for the quantification of the ribosome content of growing cells. In the past, FISH signal intensities of bacterial cells have been used as measurement for the metabolic activity or potential of slow growing (Poulsen et al., 1993) or starving bacteria (Givskov et al., 1994; Weichart et al., 1997). Nevertheless, ribosomes are known to undergo various changes, e.g. degradation (Davis et al., 1986), fragmentation (Kalpaxis et al., 1998) and conformational modifications (Andrieux and Cozzone, 1984; Öfverstedt et al., 1994) during starvation, the impact of these events on the intensity of the fluorescence signal is still fairly unknown. Additionally, although starving organisms may contain small numbers or ribosomes, the cellular ribosomal content is in large excess over the apparent demand for protein synthesis (Flärdh et al., 1992). This disproportion of the number of ribosomes may give misleading results, when they are used as sole indicators of the physiological state of individual cells (Møller et al., 1995). As a

consequence, no universal correlation between ribosome content and metabolic potential or viability of starving populations could be given until now (Givskov et al., 1994; Flärdh et al., 1992). However, the pattern of intensity decrease in some of the assays suggests a loss of culturability after reaching an almost constant, strain specific level.

Strain specific probes were used for the differentiation between sulfate-reducing bacteria and *Aquabacterium commune* in coculture tests, whilst in pure culture assay the *Bacteria*-specific probe EUB338 was used for the determination of the FISH signals. A comparison of values determined by testing the identical samples with the strain-specific or domain-specific probe resulted in significantly different signal intensities, putatively caused by ribosomal higher order structure (Frischer et al., 1996) and by the variable accessibility of rRNA target sites (Fuchs et al., 1998). Nevertheless, by the normalization of the fluorescence intensity values on the brightest signal obtained after hybridization of log-phase cells a comparison of the FISH dynamics was possible.

The results obtained by use of in situ hybridization and DNA-staining by DAPI lead to the conclusion that a distinction of the physiological state in culturable and nonculturable is not possible by this means, which is a common problem of all methods proposed to assess viability of nonculturable cells (McDougald et al., 1998). Methods which have been used as indications of metabolic activity included investigation of substrate responsiveness by the direct viable count method (DVC) (Kogure et al., 1979), of respiration by reduction of tetrazolium salts (Rodriguez et al.,1992) and of membrane permeability (Matsuyama, 1984). However, the number of metabolically active cells differed depending on the method used and no correlation to culturable cell counts could be made (Kalmbach et al., 1997a). Results of Joux et al. (1997) suggested a progressive physiological cell alteration throughout the transition between culturable and prelytic cells. Nevertheless, the measurement of the signal intensity of hybridized sulfate-reducing bacteria after stress response confirmed this method to be suitable for obtaining general information on the metabolic potential of a distinct population.

4.3 Influence of the culture age on culturability after stress

Aside from strain-specific properties (Oliver, 1993) and the medium composition (Botzenhart and Kufferath 1976), the loss of culturability is also influenced by the

culture age (Jenkins et al., 1990; Oliver et al., 1991). This influence also affected the behaviour of strain zt3l, which showed a considerably slower decrease of culturability when stressed after entering the stationary phase. The stationary phase response of bacteria involves drastic changes in cellular physiology and morphology: structural changes in the cell envelope, an altered membrane composition and differences in DNA supercoiling and compactness (Siegele and Kolter, 1992). Depending on medium composition, stationary phase cells synthesize storage compounds, such as glycogen and polyphospates (Preiss, 1989) or polyglucose in some sulfate-reducing bacteria (van Niel et al., 1996), and they produce protective substances, such as trehalose (Hengge-Aronis et al., 1991). The synthesis of a set of stationary phase or postexponential proteins is induced, regardless of the class of nutrient for which the cells are starved (Groat et al., 1986). A role in stress protection has been proposed for these proteins (Matin, 1991), since stationary phase cells are extremely resistant to heat shock (>50°C) and high concentrations of H_2O_2 and NaCl (Jenkins et al., 1990). The ability to survive under these very diverse stress conditions indicates that stationary cells posess systems not expressed in exponentially growing cells for DNA repair and the protection of membranes and proteins (Hengge-Aronis, 1993).

Another question is, why cells of strain zt3l set under stress conditions 96 hours after the beginning of the stationary phase showed a faster decline of culturability compared to cells grown to the stationary phase. A speculative explanation for this behaviour could be the consumption of protective storage polymers during the anaerobic starvation (van Niel et al., 1996). A further possibility could be the inability to produce oxygen-protective enzymes when starved prior to oxygen stress, but a convincing explanation for this behaviour is not yet available.

4.4 Resuscitation of ‘non-culturable’ sulfate-reducing bacteria

In most natural or man-made oligotrophic habitats less than 1% of the bacterial population can usually be enumerated by cultivation methods (Staley and Konopka, 1985), which might be either due to unfavorable culture conditions or to the formation of VBNC cells. The formation of VBNC cells has been proposed as a survival strategy (Oliver, 1993), analogous to the stress responses of the differentiating bacteria (e.g. spore formation) (Kjelleberg et al., 1993). Thus the VBNC response may be a genetically programmed physiological response of some bacteria which enhances survival during environmental stress. The cells should therefore also have

the ability to reverse this program and resuscitate when conditions become favorable for growth. But although it is generally accepted that certain conditions can induce the VBNC response in bacteria (Oliver, 1993), reports of resuscitation have been met with scepticism, because in most of these reports resuscitation could only be accomplished with cultures which had been VBNC only for a short period of time (Roszak et al., 1984; Nilsson et al., 1991; Ravel et al., 1995). In other reports resuscitation was due to regrowth of a few viable cells remaining in the cultures (Weichart et al., 1992; Morgan et al., 1991). But resuscitation could also be demonstrated under conditions, where regrowth of viable cells is less likely to have ocurred (Oliver et al., 1995). Nevertheless, the VBNC state may also be a moribund condition in which cells progressively debilitated, showing signs of metabolic activity for some time, until death finally occurs.

Culturability of sulfate-reducing bacteria could be ceased after exerting stress to different strains in form of oxic, oligotrophic conditions. For the investigations whether cells of *Desulfovibrio desulfuricans* (DSM 6949) reached a VBNC-state the most commonly used approch to determine the amount of VBNC cells in a natural habitat (Oliver, 1993), the DVC method, was applied. The difference between culturable cell counts and elongated cells determined by the DVC method is commonly considered the portion of the bacterial population in a VBNC state (Roszak and Colwell, 1987; Byrd et al., 1991; Oliver, 1993).

The original DVC method as proposed by Kogure et al., (1979) has been modified for the application in different habitats. Kalmbach et al. (1997a) adapted the method for application in drinking water biofilms, which involved the selection of suitable antibiotics, the evaluation of antibiotic concentrations, incubation media and incubation periods. Furthermore, enumeration of elongated cells was complemented by in situ hybridization, which provided informations on the phylogenetic affilitation and the ribosome content of the investigated bacteria. The term probe active counts (PAC) was introduced for the enumeration of hybridization-positive cells prior to and after incubation (Kalmbach et al., 1997).

Adaptation of this method for the use on pure cultures of sulfate-reducing bacteria in sulfate reducer medium was performed with regard to the selection of the best possible antibiotic. In contrast to the use of this method in the natural environment, the groundwater biofilm, in situ hybridization was omitted, because DAPI-staining was a sufficient method to detect elongated cells, and no phylogenetic distinction

was necessary. While Kalmbach et al. (1997a) found the use of pipimedic acid sufficient to prevent cell division in drinking water biofilms, Joux and LeBaron (1997) proposed the use of an antibiotic cocktail in a marine habitat, since high numbers of marine bacterial isolates were found to be resistant to one of several gyrase inhibitors. Therefore both were tested for their effectiveness on *Desulfovibrio desulfuricans* (DSM 6949), where the antibiotic cocktail showed a higher effectiveness.

After examination of the general applicability of this method on *Desulfovibrio desulfuricans*, experiments were performed with populations which contained less than 1 culturable cell per ml in agar shakes. Besides the influence of different nutrient concentrations also the influence of *N*-oxohexanoyl homoserinelactone (HLA) and of a mixture of different *N*-acyl homoserine lactones (HSL) was tested. HLA was shown to influence the speed of recovery of cell suspensions of the ammonia oxidizer *Nitrosomonas europaea* after starvation (Batchelor et al., 1997), implying an effect of high cell densities. Cell density was also found to be responsible for the recovery of 'dormant' cells of *Micrococcus luteus* (Votyokova et al., 1994). As two of the in situ dominant aerobic bacteria in the Berlin drinking water were also able to produce quorum sensing AHLs their influence on sulfate-reducing bacteria was tested as well.

The results obtained in these experiment indicate, that no VBNC-cells according to the definition of Roszak and Colwell (1987), Byrd et al. (1991) and Oliver (1993) were present in the population of *Desulfovibrio desulfuricans* after stress, as no elongated cells were found. Nevertheless, the cells seemed not to be dead, since they yielded a DAPI signal and were visible for a considerable time, although the question of bacterial death is a question of definition. Recent studies proposed that bacterial death is concomitant with the degradation of DNA and RNA (Weichart et al., 1997) or should be equaled with the loss of morphological integrity (Servais et al., 1985; Gonzales et al., 1992). Applying these concepts to the present study, the non-elongated but DAPI-positive cells are clearly not dead and might fit the definition of a dormant cell, which are temporarily inactive or resting microbes (Mason et al., 1986). Whether they are moribund or might recover and resume growth under certain conditions is a point of future research.

The lysis of cells of 'non-culturable' *Desulfovibrio desulfuricans* tested for viability seemed to be accelerated by the addition of high nutrient concentrations, though delayed when a mixture of different *N*-acyl-homoserine lactone analogs was added to

the assays. A corresponding reaction to elevated nutrient concentations was demonstrated for cells of *Vibrio vulnificus*, where a full-strength medium was inhibitory to cell division in VBNC populations, but no explanation for this behaviour was offered (Whitesides and Oliver, 1997).

The additon of a mixture of different *N*-acyl-homoserine lactone analogs in combination with nutrients seemed to induce a divison of parts of the 'non-culturable' *Desulfovibrio desulfuricans* cells regardless of the presence of antibiotics. An influence of autoinducer-like molecules has been reported for the control of cell division of *Escherichia coli*, leading to the expression of genes which might induce the formation of short minicells (Sitnikov et al., 1996). The effect of this autoinducer-like molecules was potentiated by *N*-acyl homoserine lactones. Nevertheless, the reaction of the 'non-culturable' *Desulfovibrio desulfuricans* cells to the addition of HSL remains enigmatic, as the antibiotics seemed not to inhibit the division and no cell elongation was detectable. The cells are therefore still not viable and any form of resuscitation has to be excluded, though they kept their morphological integrity considerable longer than cells in assays without the mixture of different *N*-acyl-homoserine lactone analogs. The most likely reason was that the mixture of different *N*-acyl-homoserine lactone analogs consisted of AHL-analogs with high molecular weights and with two hydroxy-groups. Additionally, any explanation is complicated by the fact that no detection of determinable amounts of *N*-acyl homoserine lactones produced by *Desulfovibrio desulfuricans* was possible by use of the biological test system. As a definite explanation of this process is not realizable by the available means, further research should be focussed on the role of quorum sensing molecules in the possible resuscitation of sulfate-reducing bacteria.

5. Behaviour of SRB in artificial aerobic biofilms

5.1 Bacterial interactions with regard to biofilms

In the last years it has become increasingly clear, that biofilms constitute a distinct growth phase of bacteria that is profoundly different from the planktonic growth phase (Costerton et al., 1995). Although the majority of research on bacterial interactions has utilized planktonic communities, the characteristics of biofilm growth (cell positions that are relative stable and local areas of hindered diffusion) suggest that interspecies interactions may be more significant in biofilms (James et al, 1995b). One of the most important points for the survival of sulfate-reducing bacteria in

aerobic biofilms as demonstrated in this study is probably microbial interaction, since it can have a profound influence on the structure and physiology of microbial communities. Microbial systems are commonly classified based on the effect of the interaction on each population in a binary system (reviewed in James et al., 1995b). *Neutralism* occurs when neither population is affected by the presence of the other. *Competition* refers to an interaction where two populations are competing for a growth-limiting nutrient and which is detrimental to both populations. When one population benefits from the presence or activity of the other while the benefactor is unaffected, the phenomenon is termed *commensalism*. An interaction where both populations benefit is *mutualism*, while *ammensalism* refers to an interaction, where one population has an indirect, negative impact on another. Direct negative interaction include *predation* and *parasitism*. But although this binary system classification is useful for defining interactions, in natural communities interactions can be complex and include mixed interactions, where more than one type of interaction occurs between two species, as well as interactions involving more than one species. Additionally, biofilm specific interactions have to be considered.

Bacterial interactions begin to influence a biofilm during the initial stages of biofilm development. The formation of a biofilm begins with the adsorption of molecules to a surface (i.e. conditioning film formation), followed by bacterial adhesion and colonization. It was demonstrated, that the adhesion of one bacterial species to a surface can have a negative, positive or neutral influence upon another species, depending on the species involved and the nature of the substratum (McEldowney and Fletcher, 1987). In this study no differences could be detected between the adhesion of pure cultures of strain B3 and *Aquabacterium commune*, respectively, between mixed cultures of both strains and between mixed cultures of one or both aerobic bacteria with a sulfate-reducing species on glass surfaces.

Further biofilm specific interactions affect the biofilm thickness and stability. Biofilms formed by microbial communities (i.e. mixed-species biofilms) are often thicker and more stable than monospecies biofilms (Siebel and Characklis, 1991). This enhancement of stability is possibly due to the copious production of extracellular polymeric substances which enhances the stability of other species within a biofilm and/or that stabilizing interactions occur between polymers of the different species (McEldowney and Fletcher, 1987). A similar effect may be attributed to strain B3 and *Aquabacterium commune*, since both produce large amounts of EPS

(S. Kalmbach, personal communication). This kind of biofilm stabilization can be considered a commensal interaction, where one species benefits from the ability of another to form a stable film.

Another type of commensalism most likely to be present in the biofilm systems in this study involves the consumption of oxygen by aerobic and/or facultative anaerobic microorganisms, allowing the growth of anaerobes. This interaction has been demonstrated for chemostat systems (Gottschal and Szewzyk, 1985) and is possible in biofilm systems, where oxygen gradients are created (Costerton et al., 1994; de Beer et al., 1993) and can play an important role in microbially induced corrosion, due to the growth of sulfate-reducing bacteria in anaerobic microniches within the biofilm (Hamilton, 1985). Possibly it was a factor in the increase of sulfate-reducing bacteria in the biofilm experiment 5.2 in the first ten days and in biofilm experiment 5.3 in stage 2 biofilms in the last seven days of the investigation, since sulfate-reducing bacteria are known to show only limited growth under aerobic conditions (Marschall et al., 1993; Krekeler et al., 1997; Sass et al., 1998a), as discussed below. But especially biofilms in 5.2 were rather thin, making the presence of anaerobic microniches questionable. The investigation of similar biofilms with oxygen microsensors (Revsbech, 1989) could be a helpful tool to explain this process.

5.2 Stress conditions exerted on the biofilm community

Growth of microorganisms is a function of different factors, such as substrate and inhibitory agents concentration, temperature, pH and hydrodynamics conditions. The effect of different substrate concentrations in form of strict carbon starvation - use of mineral medium without carbon source as medium for biofilms - has already been discussed for pure cultures. Exertion of low nutrient concentrations - use of Berlin drinking water as medium - on biofilms pregrown under higher nutrient conditions resulted in biofilms with cell densities and activities similar to biofilms grown exclusively in Berlin drinking water (Kalmbach at al., 1997a). An explanation for this change of the biofilm conditions could be the lysis of starved cells or a higher detachment rate, probably due to the increased shear forces caused by the higher flow through adjusted in drinking water systems.

An additional factor investigated in this study was the influence of pH on the growth of a biofilm community. The pH minimum for growth of strain B3 has not been

determined, but the minimum of *Aquabacterium commune* is at pH 6.5 for growth in batch culture (Kalmbach et al., 1999). Nevertheless, the cell counts of both species increased considerably in the first eleven days of biofilm experiment 5.2, where the pH of the efffluent was only 6.0. Surface growth was also demonstrated to be the major factor affecting the pH response of a *Nitrobacter* species, which allowed nitrite oxidation in continuous culture down to a pH of 4.5 compared to 5.5 in batch cultures (Keen and Prosser, 1987). The influence of the pH on the *Nitrobacter* was additionally affected by a reduced nutrient concentration, a factor probably also influencing the biofilms of strain B3 and *Aquabacterium commune*, respectively, since 150fold diluted R2A was used as medium.

5.3 Survival and growth of sulfate-reducing bacteria in biofilms

Biofilms are defined as matrix-enclosed bacterial populations adherent to each other and/or to surfaces or interfaces. This definition includes microbial aggregates and floccules and also adherent populations within the pore spaces of porous media (Costerton et al., 1995). Therefore microbial populations in habitats as diverse as sediments, microbial mats, sludge flocs and aerobic waste water biofilms, possibly containing sulfate-reducing bacteria, have to be considered when discussing the significance of sulfate reducers in aerobic biofilm environments. In sediments and microbial mats, sulfate reduction may be the most important metabolic process (Jørgensen, 1982; Skyring et al., 1983), while in aerobic wastewater treatment systems sulfate reduction can account for up to 50% of the mineralization of organic matter (Kühl and Jørgensen, 1992; Lens et al., 1995).

The best investigated habitats by in situ hybridzation are sludge flocs and aerobic waster water biofilms (Ramsing et al., 1993; Santegoeds et al., 1998; Manz et al., 1998; Schramm et al., 1999). Manz and coworkers (1998) demonstrated that 5.1% of the total population within the aerobic compartment of a waste water treatment plant were hybridizable with probes specific for the sulfate-reducing genera *Desulfovibrionaceae* and *Desulfobacteriaceae*, a number similar to that obtained by Schramm et al. (1999). In an aerobic waste water biofilm the number of sulfate-reducing bacteria was estimated to be less than 1% of the total microbial population (Santegoeds et al., 1998), a number also found in heterotrophic biofilms grown on stainless steel coupons (Power at al., 1999). In contrast, in this investigation a maximum of 27% hybridizable sulfate-reducing bacteria was found for the average of

the four biofilm assays described under 5.2, but only 0.02% to 0.33% were found in the biofilm experiment 5.3. These numbers seemed to be influenced by the length of the investigation period, as highest numbers of sulfate-reducing bacteria in experiment 5.2 were found in the beginning of the assays, which decreased in the following weeks, a behaviour transferable on the biofilms presented in 5.3. Nevertheless, only few data are available on the number of culturable cells in such systems (Lens et al., 1995) and none correlating the percentage of culturable cells to the hybridizable sulfate-reducing population. In experiment 5.2 an average of 0.001% of the sulfate-reducing bacteria correlated to the total population was still culturable after 11 days, in experiment 5.3 values between 0.01% and 0.06% were obtained after 24 and 31 days, respectively. In coculture experiments of *Aquabacterium commune* with strain zt3l and strain zt10e performed in batch cultures under starvation conditions 56% and 87%, respectively, of the sulfate-reducing bacteria were still culturable after 11 days. However, after 31 days culturability was lost completely. The decrease of culturability in this experiment also showed a steep decline, not found in the biofilm experiment 5.3, where culturable cell counts of sulfate-reducing bacteria in the effluent of the starvation stage were determined. A tentative explanation of this behaviour is the consumption of storage polymers combined with aerobic respiration by sulfate reducers in aerobic starvation cultures (van Niel et al., 1996; van Niel and Gottschal, 1998), since the exhaustion of the storage polymer could lead to a decrease of culturability. The enzymes responsible for this process were relatively stable under starvation conditions but were susceptible to inactivation as soon as substrates were added (van Niel and Gottschal, 1998). Other enzymes like lactate dehydrogenase or hydrogenase, necessary for metabolic processes, were demonstrated to be oxygen sensitive as well (Stams and Hansen, 1982; Fitz and Cypionka, 1991). This could be an explanation for the decrease of culturable cell counts in the biofilm experiment: the rate of inactivation could be higher than the rate of growth of sulfate reducers, thus causing a gradual decline of the number of culturable cells in the effluent.

Growth of sulfate-reducing bacteria has been shown to be limited under aerobic conditions in several studies (Marschall et al., 1993; Krekeler et al., 1997; Sass et al., 1998a), only one doubling could be observed in homogenously aerated cultures at 0.5 to 2% oxygen (Marschall et al., 1993). Nevertheless, their ability to grow in sulfate-free oxygen-sulfide gradients has been demonstrated (Cypionka et al., 1985;

Marschall et al., 1993), as well as their ability to respire oxygen at considerable rates (Dilling and Cypionka, 1990; Dannenberg et al., 1992; Krekeler et al., 1997; Sass et al., 1998a; Fröhlich et al., 1999). Together with their aerotactical response this might provide an efficient strategy for removing oxygen from a habitat (Krekeler et al., 1998; Eschemann et al., 1999). This conclusion is also supported by Johnson et al. (1997), although they found *Desulfovibrio desulfuricans* Hildenborough to show positive aerotaxis and even aerobic growth at an oxygen concentration of 0.02 to 0.04%. The increase of sulfate-reducing bacteria in the biofilm experiments presented in this study might be connected to such a behaviour, as the strains zt3l and zt10e were able to perform aerobic respiration. An additional requirement might be the presence of the aerobic drinking water bacteria which are able to deplete at least parts of the oxygen present in the system, as already discussed above.

5.4 Spatial distribution of sulfate-reducing bacteria within the biofilm

In situ hybridization of sulfate-reducing bacteria in the aerobic biofilms revealed no particular spatial arrangement of the cells within the microbial community. This is in contrast to results obtained by Ramsing et al. (1993), who found a negative correlation between the vertical distribution of positively stained cells of sulfate-reducing bacteria and the measured O_2 profiles in a 4 mm thick, photosynthetic biofilm from the trickling filter of a sewage treatment plant. Other investigations, performed on activated sludge flocs and young, aerobic waste water biofilms (Manz et al., 1998; Santegoeds et al., 1998) found no particular spatial arrangement of the sulfate-reducing bacteria. A possible reason for the differences in the various investigations might be the age of the biofilms or sludge flocs, possibly in combination with the thickness. In this investigation, even after establishment of sulfate-reducing bacteria in already developed biofilms no distinct three-dimensional arrangement was detectable. The demonstration of the ability of sulfate-reducing bacteria to invade already developed biofilms particularly contradicts an investigation also performed on heterotrophic biofilms in a laboratory reactor (Power et al., 1999) urging a more detailed investigation, since only few data are available on this subject.

6. Outlook

Several sulfate-reducing bacteria were isolated from the Berlin groundwater, containing considerable amounts of oxygen, and from the oxygen saturated drinking water distribution systems of Berlin and Mülheim. Their ability to survive under conditions allowing their transport within these environments was demonstrated. This induces questions concerning their role in the system, especially with regard to the materials used in the distribution systems. Iron pipes are known to be susceptible to tuberculation and the role of sulfate-reducing bacteria in the formation of tubercles has not been completely elucidated. The influence of the isolated sulfate-reducing species on corrosion is therefore an interesting question, which should be addressed in the future.

The decrease of culturability after exposition to starvation and/or oxygen stress was a common feature of the Gram-negative sulfate-reducing bacteria investigated in this study. This leads to the question, if the inability to grow is an unchangeable condition or if the ‘non-culturable’ cells are still metabolically active, allowing a kind of resuscitation. The conditions necessary to induce the resuscitation and if they are present in natural environments might give additional information on the spreading of sulfate-reducing bacteria.

Furthermore, one of the basic questions of microbial ecology, the interactions between sulfate reducers and aerobic organisms ought to be approached with regard to physiological and biochemical questions. The form of bacterial interaction might be important for the question if and how sulfate-reducing bacteria are active over longer time periods within an aerobic biofilm.

References

Abdollahi, H. and Wimpenny, J.W. (1990) Effects of oxygen on the growth of *Desulfovibrio desulfuricans*. J.Gen.Microbiol. **136**:1025-1030.

Adrian, L., Manz, W., Szewzyk, U. and Görisch, H. (1998) Physiological characterization of a bacterial consortium reductively dechlorinating 1,2,3- and 1,2,4-Trichlorobenzene. Appl.Environ.Microbiol. **64:**496-503

Alfreider, A., Pernthaler, J., Amann, R., Sattler, B. Glöckner, F.-O. Wille, A. and Psenner, R.. (1996) Community analysis of the bacterial assemblages in the winter cover and pelagic layers of a high mountainlake by in situ hybridization. Appl.Environ.Microbiol. **62**:2138-2144.

Alm, E.W., Oerther, D.B., Larsen, N., Stahl, D.A. and Raskin, L. (1996) The oligonucleotide database. Appl.Environ.Microbiol. **62:**3557-3559

Amann, R.I., Binder, B.J., Olson, R.J., Chisholm S.W., Devereux, R. and Stahl, D.A. (1990) Combination of 16S rRNA targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl.Environ.Microbiol. **56:**1919-1925

American Society of Microbiology (1997) The microbial world: foundation of the biosphere. Report from the American Society of Microbiology, Washington, DC

Andrieux, E. and Cozzone, A.J. (1984) Conformational changes in bacterial polysomes induced by amino acid starvation. Int.J.Biochem. **16**:113-115

Bak, F. and Pfennig, N. (1991) Sulfate-reducing bacteria in littoral sediment of Lake Constance. FEMS Microbiol.Ecol. **85**:43-52

Batchelor, S.E., Cooper, M., Chhabra, S.R., Glover, L.A., Stewart, G.S., Williams, P., and Prosser J.I. (1997) Cell density-regulated recovery of starved biofilm populations of ammonium-oxidizing bacteria. Appl.Environ.Microbiol. **63**:2281-2286.

Beijerinck, W.M. (1895) Ueber Spirillum desulfurcans als Ursache von Sulfatreduction. Centralbl.Bakteriol. II. Abt. **1**:1-9, 49-59, 104-114.

Benz, M., Brune, A. and Schink, B. (1998) Anaerobic and aerobic oxidation of ferrous iron at neutral pH by chemoheterotrophic nitrate-reducing bacteria. Arch.Microbiol. **169**:159-165.

Botzenhart, K. and Kufferath, R. (1976) Über die Vermehrung verschiedener Enterobacteriaceae sowie *Pseudomonas aeruginosa* und *Alkaligenes spec.* in destilliertem Wasser, entionisiertem Wasser, Leitungswasser und Mineralsalzlösung. Zbl.Bakt.Hyg., I. Abt. Orig. B **163**:470-485

Bryant, R.D., Jansen, W., Boivin, J. and Laishley, E.J.C. (1991) Effect of hydrogenase and mixed sulfate-reducing bacterial populations on the corrosion of steel. Appl.Environ.Microbiol. **57**:2804-2809.

Buchholz-Cleven, B.E., Rattunde, B. and Straub, K.L. (1997) Screening for genetic diversity of isolates of anaerobic Fe(II)-oxidizingbacteria using DGGE and whole-cell hybridization. System.Appl.Microbiol. **20**:301-309.

Bull, A.T. and Slater, J.H. (1982) Microbial interactions and communitystructure. in: Microbial interactions and communities, Vol 1, (Eds:A.T.Bull and J.H.Slater), Academic Press, **1**:13-44.

Byrd, J.J., Xu, H.S. and Colwell, R.R. (1991) Viable but nonculturable bacteria in drinking water. Appl.Environ.Microbiol. **57**:875-878.

Camper, A.K., Hayes, J.T., Sturman, P.J., Jones, W.I. and Cunningham, A.B. (1993) Effects of motility and adsorption rate coefficient on transport of bacteria through saturated porous media. Appl.Environ.Microbiol. **59**:3455-3462

Canfield, D.E. and DesMarais, D.J. (1991) Aerobic sulfate reduction in microbial mats. Science **251**:1471-1473

Castro, H.F., Williams, N.H. and Ogram, A. (2000) Phylogeny of sulfate-reducing bacteria. FEMS Microbiol.Ecol. 31:1-9.

Cha, C., Gao, P., Chen Y.C., Shaw, P.D. and Farrand, S.K. (1998) Production of acyl-homoserine lactone quorum-sensing signals by gram-negative plant-associated bacteria. Mol.Plant Microbe Interact. **11**:1119-1129

Chilton, M., Courier, T.C., Farrand, S.K., Bendich, A.J., Gordon, M.P. and Nester, E.W. (1974) *Agrobacterium tumefaciens* DNA and PS8 Bacteriaophage DNA not detected in crown gall tumors. Proc.Natl.Acad.Sci. **71**:3672-3676

Characklis, W.G. and Cooksey, K.E. (1983) Biofilms and microbial fouling. Adv.Appl.Microbiol. **29**:93-138.

Coallier, J., Prévost, M., Pompré, A. and Duchesne, D. (1994) The optimization and application of two direct viable count methods for bacteria in distributed drinking water. Can.J.Microbiol. **40**:830-836.

Costerton, J.W., Nickel, J.C. and Ladd, T.I. (1986) Suitable methods for the comparative study of free-living and surface-associated bacterial populations. in: Bacteria in Nature - Methods and Special Applications in Bacterial Ecology **2**:49-84.

Costerton, J.W., Cheng, K.J., Geesey, G.G., Ladd, T.I., Nickel, J.C., Dasgupta, M. and Marrie, T.J. (1987) Bacterial biofilms in nature and disease. Ann.Rev.Microbiol. **41**:435-464.

Costerton, J.W., Lewandowski, Z., DeBeer, D., Caldwell, D.E., Korber, D.R. and James G. (1994) Biofilms, the customized microniche. J.Bacteriol. **176**:2137-2142.

Costerton, J.W., Lewandowski, Z., Caldwell, D.E., Korber, D.R. and Lappin-Scott, H.M. (1995) Microbial biofilms. Annu.Rev.Microbiol. **49**:711-745.

Coutinho, C.M., Magalhaes, F.C. and Araujo-Jorge, T.C. (1994) Ultrastructure of sulphidogenic biofilms rich in sulphate-reducingbacteria causing corrosion in the offshore oil extraction platformsoff Brazil's atlantic coast. J.Gen.Appl.Microbiol. **40**:227-241.

Coutinho, C.M., Magalhaes, F.C. and Araujo-Jorge, T.C. (1994) Morphology of the surface coat and extracellular matrix of sulphidogenic biofilms enriched in sulphate-reducing bacteria involved in biocorrosion processes in the offshore oil extraction industry off. J.Gen.Appl.Microbiol. **40**:271-276.

Cypionka, H. (1994) Sulfate transport. Methods Enzymol. **243**:3-14

Cypionka, H., Widdel, F. and Pfennig, N. (1985) Survival of sulfate-reducing bacteria after oxygen stress, and growth in sulfate-free oxygen-sulfide gradients. FEMS Microbiol.Ecol. **31**:39-45

Dalton, H.M., Goodman, A.E. and Marshall, K.C. (1996) Diversity in surface colonization behavior in marine bacteria. J.Ind.Microbiol. **17**:228-234.

Dannenberg, S., Kroder, M., Dilling, W. and Cypionka, H. (1992): Oxidation of H_2, organisc compounds and inorganic sulfur compounds coupled to reduction of O_2 or nitrate by sulfate-reducing bacteria. Arch.Microbiol. **158**:93-99.

Davies, D.G., Parsek, M.R., Pearson, J.P, Iglewski, B.H., Costerton, J.W. and Greenberg, E.P. (1998) The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science **280**:295-298

Davis, B.D, Luger, S.M. and Tai, P.C. (1986) Role of Ribosome degradation in the death of starved *Escherichia coli* cells. J.Bacteriol. **166**:439-445

Dawson, M.P., Humphrey, B.A. and Marshall, K.C. (1981) Adhesion: a tactic in the survival strategy of a marine vibrio during starvation. Curr.Microbiol. **6**:195-199.

de Beer, D., van den Heuvel, J.C. and Ottengraf, S.P. (1993) Microelectrode measurements of the activity distribution in nitrifying bacterial aggregates. Appl.Environ.Microbiol. **59**:573-579.

de Beer, D., Stoodley, P., Roe, F. and Lewandowski, Z. (1994) Effects of biofilm structures on oxygen distribution and mass transport. Biotechnol.Bioengin. **43**:1131-1138.

Delaquis, P.J., Caldwell, D.E., Lawrence, J.R. and McCurdy, A.R. (1989) Detachment of *Pseudomonas fluorescens* from biofilms on glass surfaces in response to nutrient stress. Microb.Ecol. **18**:199-210.

DeLong, E.F., Wickham, G.S. and Pace, N.R. (1989) Phylogenetic stains: ribosomal RNA based probes for the identification of single cells. Science **243**:1360-1363

Deutsche Sammlung von Mikroorganismen und Zellkulturen (1993) Catalogue of Strains, 5th Edn., Braunschweig

Devereux, R., Kane, M.D., Winfrey, J. and Stahl, D.A. (1992) Genus- and group-specific hybridization probes for determinative and environmental studies of sulfate-reducing bacteria. System.Appl.Microbiol. **15**:601-609.

Dilling, W. and Cypionka, H. (1990) Aerobic respiration in sulfate-reducing bacteria. FEMS Microbiol.Lett. **71**:123-128.

Donlan, R.M., Pipes, W.O. and Yohe, T.L. (1994) Biofilm formation on cast iron substrata in water distribution systems. Water Res. **28**:1497-1503.

Eschemann, A., Kühl, M. and Cypionka, H. (1999) Aerotaxis in *Desulfovibrio*. Environ.Microbiol. **1**:489-494

Fitz, R.M. and Cypionka, H. (1991) Generation of a proton gradient in *Desulfovibrio vulgaris*. Arch. Microbiol. **155**:444-448

Flärdh, K., Cohen, P.S. and Kjelleberg, S. (1992) Ribosomes exist in large excess over the apparent demand for protein sysnthesis during carbon starvation in marine *Vibrio* sp. Strain CCUG 15956. J.Bacteriol. **174**:6780-6788

Ford, T. and Mitchell, R. (1990) The ecology of microbial corrosion. Adv.Microb.Ecol. **11**:231-262.

Frischer, M.E., Stewart, G.J. and Paul, J.H. (1994) Plasmid transfer to indigenous marine bacterial populations by natural transformation. FEMSMicrobiol.Ecol. **15**:127-136.

Fröhlich, J., Sass, H., Babenzien, H-D., Kuhnigk, T., Varma, A., Saxena, S., Malepa, C., Pfeiffer, P. and König, H. (1999) Isolation of *Desulfovibrio intestinalis* sp. nov. from the hindgut of the lower termite *Mastotermes darwiniensis.* Can.J.Microbiol. **45**:145-152

Fründ, C. and Cohen, Y. (1992) Diurnal cycles of sulfate reduction under oxic conditions in cyanobacterial mats. Appl.Environ.Microbiol. **58**:70-77

Fry, N.K., Fredrickson, J.K., Fishbain, S., Wagner, M. and Stahl D.A. (1997) Population structure of microbial communities associated with two deep, anaerobic, alkaline aquifers. Appl.Environ.Microbiol. **63**:1498-1504.

Fuchs, B.M., Wallner, G., Beisker, W., Schwippl, I., Ludwig, W. and Amann, R. (1998) Flow cytometric analysis of the in situ accessibility of *Escherichia coli* 16SrRNA for fluorescently labeled oligonucleotide probes. Appl.Environ.Microbiol. **64**:4973-4982

Fukui, M. and Takii, S. (1990) Survival of sulfate-reducing bacteria in oxic surface sediment of a seawater lake. FEMS Microbiol.Ecol. **73**:317-322

Fuqua, W.C., Winans, S.C. and Greenberg, E.P. (1994) Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. J.Bacteriol. **176**:269-275.

Geesey, G.G. and Jang, L. (1989) Interactions between metal ions and capsular polymers. in: Metal ions and bacteria (eds.Beveridge, T.J.and Doyle, R.J.) John Wiley & Sons, Chapter 11, 325-357.

Geller, A. (1983) Growth of bacteria in inorganic medium at different levels of airborne organic substances. Appl.Environ.Microbiol. **46**:1258-1262.

Gerritse, J., Schut, F. and Gottschal, J.C. (1992) Modelling of mixed chemostat cultures of an aerobic bacterium, *Comamonas testosteroni*, and an anaerobic bacterium, *Veillonella alcalescens*: Comparison with experimental data. Appl.Environ.Microbiol. **58**:1466-1476

Gibson, G.r., Parkes, R.J. and Herbert, R.A. (1987) Evaluation of viable counting procedures for the enumeration of sulfate-reducing bacteria in estuarine sediment. J.Microbiol.Meth. **7**:201-211.

Givskov, M., Eberl, L., Møller, S., Poulsen, L.K. and Molin, S. (1994) Responses to nutrient starvation in *Pseudomonas putida* KT2442: Analysis of general cross-protection, cell shape, and macromolecular content. J.Bacteriol. **176**:7-14

Gonzales, J.M., Iriberri, J., Egea, L. and Barcina, I. (1992) Characterization of culturability, protistan grazing, and death of enteric bacteria in aquatic environment. Appl.Environ.Microbiol. **58**:998-1004.

Gottschal, J.C. and Szewzyk, R. (1985) Growth of a facultative anaerobe under oxygen-limiting conditions in pure culture and in co-culture with a sulfate-reducing bacterium. FEMS Microbiol.Ecol. **31**:159-170.

Groat, R.G., Schultz, J.E., Zychlinsky, E., Bockman, A. and Matin, A. (1986) Starvation protiens in Escherichia coli: Kinetics of synthesis and role in starvation survival. J.Bacteriol. **168**:486-493.

Hamilton, W.A. (1985) Sulphate-reducing bacteria and anaerobic corrosion. Ann.Rev.Microbiol. **39**:195-217.

Hamilton, W.A. (1990) Sulphate-reducing bacteria and their role in biocorrosion. In: Biofouling and Biocorrosion in Industrial Water Systems (Flemming, H.-C. and Geesey, G.G., Eds.) 187-193, Springer-Verlag Berlin, Heidelberg

Hardy, J.A. and Hamilton, A. (1981) The oxygen tolerance of sulfate-reducing bacteria isolated from North Sea waters. Curr.Microbiol. **136**:1025-1030

Heimbrook, M.E., Wang, W.L.L. and Campbell, G. (1989) Staining bacterial flagella easily. J Clinical Microbiol. **27**:2612-2615

Heinzmann, B. and Wascher, K. (1997) Aufbereitung von Oberflächenwasser zur Grundwasseranreicherung in Jungfernheide, Berlin. gwf Wasser/Abwasser **138**:464-472

Hengge-Aronis, R., Klein, W., Lange, R., Rimmele, M. and Boos, W. (1991) Trehalose synthesis genes are controlled by the putative sigma factor encoded by *rpoS* and are involved in stationary-phase thermotolerance in *Escherichia coli*. J.Bacteriol. **173**:7918-7924

Hengge-Aronis, R. (1993) Survival of hunger and stress: the role of *rpoS* in early stationary phase gene regulation in *E. coli*. Cell, **72**:165-168

Herman, D.C. and Costerton, J.W. (1993) Starvation-survival of a p-nitrophenol-degrading bacterium. Appl.Environ.Microbiol. **59**:340-343.

Horn, H. and Hempel, D.C. (1997) Growth and decay in an auto-/heterotrophic biofilm. Wat.Res. **31**:2243-2252.

James, G.A., Korber, D.R., Caldwell, D.E. and Costerton, J.W. (1995a) Digital image analysis of growth and starvation response of a surface colonizing *Acinetobacter* sp. J.Bacteriol. **177**:907-915.

James, G.A., Beaudette, L. and Costerton, J.W. (1995b) Interspecies bacterial interactions in biofilms J.Ind.Microbiol. **15**:257-262

Jenkins, D.E., Chaisson, S.A. and Matin, A. (1990) Starvation-induced cross protection against osmotic challenge in *Escherichia coli*. J.Bacteriol. **172**:2779-2781.

Jørgensen, B.B. (1977) Bacterial sulfate reduction within reduced microniches of oxidized marine sediments. Mar.Biol. **41**:7-17.

Jørgensen, B.B. (1982) Mineralization of organic matter in the sea bed - the role of sulphate reduction. Nature **296**:643-645.

Jørgensen, B.B. and Bak, F. (1991) Pathways and microbiology of thiosulfate transformations and sulfate reduction in a marine sediment(Kattegat, Denmark). Appl.Environ.Microbiol. **57**:847-856.

Johnson, M.S., Zhulin, I.B., Gapuzan, M-E.R., and Taylor, B. (1997) Oxygen-dependent growth of the obligate anaerobe Desulfovibrio vulgaris Hildenborough. J.Bact. **179**:5598-5601

Joux, F., LeBaron, P. and Troussellier, **M.** (1997) Succession of cellular states in a *Salmonella typhimurium* population during starvation in artificial seawater microcosms. FEMS Microbiol.Ecol. **22**:65-76.

Joux, F. and LeBaron, P. (1997) Ecological implications of an improved direct vable count method for aquatic bacteria. Appl.Environ.Microbiol. **63**:3643-3647

Jukes, T.H. and Cantor, C.R. (1969) Evolution of protein molecules, p. 21-132. *In* H.N. Munro (ed.), Mammalian protein metabolism. Academic Press, New York, N.Y.

Kalmbach, S., Manz, W. and Szewzyk, U. (1997a) Dynamics of biofilm formation in drinking water: phylogenetic affiliation and metabolic potential of single cells assessed by formazan reduction and in situ hybridization. FEMS Microbiol.Ecol. **22**:265-279

Kalmbach, S., Manz, W. and Szewzyk, U. (1997b) Isolation of new bacterial species from drinking water biofilms and proof of their in situ dominance with highly specific 16S rRNA probes. Appl.Environ.Microbiol. **63**:4164-4170.

Kalmbach, S. 1998. Polyphasic characterization of the microbial population of drinking water biofilms. Thesis, Technische Universität Berlin, (www.dissertation.de)

Kalmbach, S., Manz, W., Wecke, J. and Szewzyk, U. (1999) *Aquabacterium* gen. nov., with description of *Aquabacterium citratiphilum* sp. nov., *Aquabacterium parvum* sp. nov. and *Aquabacterium commune* sp. nov., three in situ dominant bacterial species from the Berlin drinking water system. Int.J.Syst.Bacteriol. **49**:769-777

Kalpaxis, D.L., Karahalios, P. and Papapetropoulou, M. (1998) Changes in ribosomal activity of *Escherichia coli* cells during prolonged culture in sea salts medium. J.Bacteriol. **180**:3114-3119

Keen, G.A. and Prosser, J.I. (1988) The surface growth and activity of *Nitrobacter*. Microb.Ecol. **15**:21-39.

Kjelleberg, S., Humphrey, B.A. and Marshall, K.C. (1982) Effect of interfaces on small, starved marine bacteria. Appl.Environ.Microbiol. **43**:1166-1172.

Kjelleberg, S., Humphrey, B.A. and Marshall, K.C. (1983) Initial phases of starvation and activity of bacteria at surfaces. Appl.Environ.Microbiol. **46**:978-984

Kjelleberg, S., and Hermansson, M. (1984) Starvation induced effects on bacterial surface characteristics. Appl.Environ.Microbiol. **48**:497-503

Kjelleberg, S., Marshall, K.C. and Hermansson, M. (1985) Oligotrophic and copiotrophic marine bacteria - observations related to attachment. FEMS Microbiol.Ecol. **31**:89-96.

Kogure, K., Simidu, U., and Taga, N. (1979) A tentative direct microscopic method for counting living marine bacteria. Can.J.Microbiol. **25**:415-420.

Krekeler, D. and Cypionka, H. (1995) The preferred electron acceptor of *Desulfovibrio desulfuricans* CSN. FEMS Microbiol.Ecol. **17**:271-278.

Krekeler, D., Sigalevich, P., Teske, A., Cypionka, H. and Cohen, Y. (1997) A sulfate-reducing bacterium from the oxic layer of a microbial mat from Solar Lake (Sinai), *Desulfovibrio oxyclinae* sp. nov. Arch.Microbiol. **167**:369-375.

Krekeler, D., Teske, A. and Cypionka, H. (1998) Strategies of sulfate-reducing bacteria to escape oxygen stress in a cyanobacterial mat. FEMS Microbiol.Ecol. **25**:89-96.

Kühl, M. and Jørgensen, B.B. (1992) Microsensor measurements of sulfate reduction and sulfide oxidation in compact microbial communities of aerobic biofilms. Appl.Environ.Microbiol. **58**:1164-1174

Kuhnigk, T., Branke, J., Krekeler, D., Cypionka, H. and König, H. (1996) A feasible role of sulfate-reducing bacteria in the termite gut. System.Appl.Microbiol. **19**:139-149.

Laanbroek, H.J. and Pfennig, **N.** (1981) Oxidation of short-chain fatty acids by sulfate-reducing bacteria in freshwater and in marine sediments. Arch.Microbiol. **128**:330-335.

Lawrence, J.R., Korber, D.R., Hoyle, B.D., Costerton, J.W. and Caldwell, D.E. (1991) Optical sectioning of microbial biofilms. J.Bact. **173**:6558-6567.

LeChevallier, M.W., Babcock, T.M. and Lee, R.G. (1987) Examination and characterization of distribution system biofilms. Appl.Environ.Microbiol. **53**:2714-2724.

LeChevallier, M.W., Schulz, W. and Lee, R.G. (1991) Bacterial nutrients in drinking water. Appl.Environ.Microbiol. **57**:857-862

Lens, P., Massone, A., Rozzi, A. and Verstraete, W. (1995) Effect of sulfate concentration and scraping on aerobic fixed biofilm reactors. Water Res. **29**:857-870.

Lovley, D.R., Roden, E.E., Phillips, E.J.P. and Woodward, J.C. (1993) Enzymatic iron and uranium reduction by sulfate-reducing bacteria. Mar. Geol. **113**:41-53

Manz, W., Amann, R., Ludwig, W., Wagner, M. and Schleifer, K.H. (1992) Phylogenetic oligodeoxynucleotide probes for the major subclasses of Proteobacteria: problems and solutions. System.Appl.Microbiol. **15**:593-600.

Manz, W., Szewzyk, U., Ericsson, P., Amann, R., Schleifer, K.H. and Stenström, T.A. (1993) In situ identification of bacteria in drinking water and adjoining biofilms by hybridization with 16S and 23S rRNA-directed fluorescent oligonucleotide probes. Appl.Environ.Microbiol. **59**:2293-2298.

Manz, W., Eisenbrecher, M., Neu, T.R. and Szewzyk, U. (1998) Abundance and spatial organization of Gram-negative sulfate-reducing bacteria in activated sludge investigated by in situ probing with specific 16S rRNA targeted oligonucleotides. FEMS Microbiol.Ecol. **25**:43-61

Marschall, C., Frenzel, P. and Cypionka, H. (1993) Influence of oxygen on sulfte reduction and growth of sulfate-reducing bacteria. Arch.Microbiol. **159**:168-173.

Marshall, K.C., Stout, R. and Mitchell, R. (1971) Mechanism of the initial events in the sorption of marine bacteria to surfaces. J.Gen.Microbiol. **68**:337-348

Marshall, K.C. (1988) Adhesion and growth of bacteria at surfaces in oligotrophic habitats. Can.J.Microbiol. **34**:503-506.

Mason, C.A., Hamer, G. and Bryers, J.D. (1986) The death and lysis of microorganisms in environmental processes. FEMS Microbiol.Rev. **39**:373-401

Massol-Deyá, A., Whallon, J., Hickey, R.F. and Tiedje, J.M. (1995) Channel structures in aerobic biofilms of fixed-film reactors treating contaminated groundwater. Appl.Environ.Microbiol. **61**:769-777.

Matin, A. (1992) Physiology, molecular biology and applications of the bacterial starvation response. J.Appl.Bacteriol.Symp.Suppl. **73**:49S-57S.

Matsuyama, T. (1984) Staining of living bacteria with rhodamine 123. FEMS Microbiol. Letters **21**:153-157

McCoy, W.F., Bryers, J.D., Robbins, J. and Costerton, J.W. (1981) Observations of fouling biofilm formation. Can.J.Microbiol. **27**:910-917.

McDougald, D., Rice, S.A., Weichart, D., Kjelleberg, S. (1998) Nonculturability: adaptation or debilitation? FEMS Microbiol. Ecol. **25**:1-9

McEldowney, S. and Fletcher, M. (1987) Adhesion of bacteria from mixed cell suspension to solid surfaces. Arch.Microbiol. **148**:57-62.

McKenzie, J. and Hamilton, W.A. (1992) The assay of in-situ activities of sulphate-reducing bacteria in a laboratory marine corrosion model. Int.Biodeterioration Biodegradation **29**:285-297.

McLean, R.J., Whiteley, M., Stickler, D.J. and Fuqua, W.C. (1997) Evidence of autoinducer activity in naturally occurring biofilms. FEMS Microbiol.Lett. **154**:259-263.

Miettinen, I.T., Vartiainen, T. and Martikainen, P.J. (1996) Contamination of drinking water. Nature **381**:654-655.

Miettinen, I.T., Vartiainen, T. and Martikainen, P.J. (1997) Phosphorus and bacterial growth in drinking water. Appl.Environ.Microbiol. **63**:3242-3245

Minz, D., Fishbain, S., Green, S.J., Muyzer, G., Cohen, Y., Rittmann, B. and Stahl, D.A. (1999) Unexpected population distribution in a microbial mat community: sulfate-reducing bacteria localized to the highly oxic chemocline in contrast to a eukaryotic preference for anoxia. Appl.Environ.Microbiol. **65**:4659-4665.

Møller, S., Kristensen, C.S., Poulsen, L.K., Carstensen, J.M. and Molin, S. (1995) Bacterial growth on surfaces: Automated image analysis for quantification of growth rate-related parameters. Appl.Environ.Microbiol. **61**:741-748

Morgan, J.A., Rhodes, G. and Pickup, R.W. (1993) Survival of nonculturable *Aeromonas salmonicida* in lake water. Appl.Environ.Microbiol. **59**:874-880.

Neu, T.R., and Lawrence, J.R. (1997) Development and structure of microbial biofilms in river water studied by confocal laser scanning microscopy. FEMS Microbiol.Ecol. **24**:11-25

Newman, D.K., Kennedy, E.K., Coates, J.D., Ahmann, D., Ellis, D.J., Lovley, D.R. and Morel, F.M. (1997) Dissimilatory arsenate and sulfate reduction in *Desulfotomaculum auripigmentum* sp. nov. Arch.Microbiol. **168**:380-388.

Nilsson, L., Oliver, J.D. and Kjelleberg, S. (1991) Resuscitation of *Vibrio vulnificus* from the viable but nonculturable state. J.Bact. **173**:5054-5059.

Odom, J.M. (1993) Industrial and environmental activities of sulfate-reducing bacteria. In: Odom, J.M. and Singleton, R.jr. (Eds.): The sulfate-reducing bacteria: contemporary perspectives. Springer Verlag, New York, Berlin, Heidelberg, pp. 161-188

Öfverstedt, L.-G., Zhang, K., Tapio, S., Skoglund, U. and Isakson, L.A. (1994) Starvation in vivo for aminoacyl-tRNA increases the spatial separation between the two ribosomal subunits. Cell **79**:629-638

Okabe, S., Hirata, K., Ozawa, Y. and Watanabe, Y. (1996) Spatial microbial distributions of nitrifiers and heterotrophs in mixed population biofilms. Biotechnol.Bioeng. **50**:24-35

Oliver, J.D. (1991) Formation of nonculturable *Vibrio vulnificus* cells and its relationship to the starvation state. Appl.Environ.Microbiol. **57**:2640-2644.

Oliver, J.D. (1993) Formation of viable but nonculturable cells. in: Starvation in bacteria (ed.: S.Kjelleberg), Plenum Press New York 239-272.

Oliver, J.D. and Bockian, R. (1995) In vivo resuscitation, and virulence towards mice, of viable but nonculturable cells of *Vibrio vulnificus*. Appl.Environ.Microbiol. **61**:2620-2623.

Ollivier, B., Cord-Ruwisch, R., Hatchikian, E.C., and Garcia, J.L. (1988) Characterization of *Desulfovibrio fructosovorans* sp.nov. Arch.Microbiol. **149**:447-450

Outtara, A.S., Patel, B.K.C., Cayol, J.L., Cuzin, N., Traore, A.S. and Garcia, J.L. (1999) Isolation and characterization of *Desulfovibrio burkinensis* sp. nov. from an African ricefield, and phylogeny of *Desulfovibrio alcoholovorans*. Int.J.Syst.Bacteriol. **49**:639-643

Pedersen, K. (1990) Biofilm development on stainless steel and PVCsurfaces in drinking water. Water Res. **24**:239-243.

Pernthaler, J., Glöckner, F.-O., Unterholzner, S., Alfreider, A., Psenner, R. and Amann R. (1998) Seasonal Community and Population Dynamics of Pelagic Bacteria and Archaea in a High Mountain Lake. Appl.Envir.Microbiol. **64**:4299-4306.

Pfennig, N. and Wagener, S. (1986) An improved method of preparing wet mounts for photomicrographs of microorganisms. J.Microbiol.Meth. **4**:303-306.

Postgate, J., (1959) A diagnostic reaction of Desulphovibrio desulphuricans. Nature **163**:481-482

Poulsen, L.K., Ballard, G. and Stahl, D.A. (1993) Use of rRNS fluorescence in situ hybridization for measuring the activity of single cells in young and established biofilms. Appl.Environ.Microbiol. **59**:1354-1360

Power, M.E., Araujo, J.C., van der Meer, J.R., Harms, H. and Wanner, O. (1999) Monitoring sulfate-reducing bacteria in heterotrophic biofilms. Wat.Sci.Tech. **39**:49-56

Preiss, J. (1989) Chemistry and metabolism of intracellular reserves. in: Bacteria in nature.Vol.3., (eds: J.S.Poindexter, E.R.Leadbetter),Plenum Press, New York **3**:189-258.

Pringle, J.H. and Fletcher M., (1983) Influence of substratum wettability on attachment of freshwater bacteria to solid surfaces. Appl.Environ.Microbiol. **45**:811-817.

Qatibi, A.I., Nivière, V. and Garcia, J.L. (1991) *Desulfovibrio alcoholovorans* sp.nov., a sulfate-reducing bacterium able to grow on glycerol, 1,2- and 1,3-propanediol. Arch.Microbiol. **155**:143-148

Rabus, R., Fukui, M., Wilkes, H. and Widdel, F. (1996) Degradative capacities and 16S rRNA-targeted whole-cell hybridization of sulfate-reducing bacteria in an anaerobic enrichment culture utilizing alkylbenzenes from crude oil. Appl.Environ.Microbiol. **62**:3605-3613.

Ramsing, N.B., Kühl, M. and Jørgensen, B.B. (1993) Distribution of sulfate-reducing bacteria, O_2, and H_2S in photosynthetic biofilms determined by oligonucleotide probes and microelectrodes. Appl.Environ.Microbiol. **59**:3840-3849

Ravel, J., Knight, I.T., Monahan, C.E., Hill, R.T. and Colwell, R.R. (1995) Temperature induced recovery of *Vibrio cholerae* from the viable but nonculturable state: growth or resuscitation? Miocrobiol. **141**:377-383.

Revsbech, N.P. (1989) Diffusion characteristics of microbial communities determined by use of oxygen microsensors. J.Microbiol.Meth. **9**:111-122.

Ridgway, H.F. and Olson, B.H. (1981) Scanning electron microscope evidence for bacterial colonization of a drinking-water distribution system. Appl.Environ.Microbiol. **41**:274-287.

Robertson, W.J., Franzmann, P.D. and Mee, B.J. (2000) Desulfosporosinus-like sulphate reducing bacteria from a shallow aquifer contaminated with gasolene. J.Appl.Microbiol. **88**:248-259

Rodriguez, G.G., Phipps, D., Ishiguro, K. and Ridgway, H.F. (1992) Use of a fluorescent redox probe for direct visualization of actively respiring bacteria. Appl.Environ.Microbiol. **58**:1801-1808.

Rogers, J., Dowsett, A.B., Dennis, P.J., Lee, J.V. and Keevil, C.W. (1994) Influence of temperature and plumbing material selection on biofilm formation and growth of *Legionella pneumophila* in a model potable water system containing complex microbial flora. Appl.Environ.Microbiol. **60**:1585-1592.

Roszak, D.B., Grimes, D.J. and Colwell, R.R. (1984). Viable but nonrecoverable stage of *Salmonella enteritidis* in aquatic systems. Can.J.Microbiol. **30**:334-338.

Roszak, D.B. and Colwell, R.R. (1987) Metabolic activity of bacterial cells enumerated by direct viable count. Appl.Environ.Microbiol. **53**:2889-2893.

Saitou, N. and Nei, M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol.Biol.Evol. **4**:406-425

Santegoeds, C.M., Ferdelman, T.G., Muyzer, G. and De Beer, D. (1998) Structural and functional dynamics of sulfate-reducing populations in bacterial biofilms. Appl.Environ.Microbiol. **64**:3731-3739

Sass, H., Cypionka, H. and Babenzien, H-D. (1997a) Vertical distribution of sulfate-reducing bacteria at the oxic-anoxic interface in sediments of the oligotrophic lake Stechlin. FEMS Microbiol.Ecol.**22**:245-255

Sass, H. (1997b) Vorkommen und Aktivität sulfatreduzierender Bakterien in der Oxykline limnischer Sediment. Dissertation Universität Oldenburg.

Sass, H., Cypionka, H. and Babenzien, H-D. (1998a) Psychrotolerant sulfate-reducing bacteria from an oxic feshwater sediment, description of *Desulfovibrio cuneatus* sp.nov. and *Desulfovibrio litoralis* sp. nov. System.Appl.Microbiol. **21**:212-219

Sass, H., Wieringa, E., Cypionka, H., Babenzien, H-D. and Overmann, J. (1998b) High genetic and physiological diversity of sulfate-reducing bacteria isolated from an oligotrophic lake sediment. Arch.Microbiol. **170**:243-251

Schaechter, M., Maaloe, O., and Kjeldgaard, N.O. (1958) Dependency on medium and temperature of cell size and chemical composition during balanced growth of Salmonella typhimurium. J.Gen.Microbiol. **19**:592-606

Schaule, G., Flemming, H.C. and Ridgway, H.F. (1993) Use of 5-cyano-2,3-ditolyl tetrazolium chloride for quantifying planktonic and sessile respiring bacteria in drinking water. Appl.Environ.Microbiol. **59**:3850-3857.

Schoenen, D. and Wehse, A. (1988) Mikrobielle Kontamination des Wassers durch Rohr- und Schlauchmaterialien 1. Mitteilung: Nachweis von Koloniezahlveränderungen. Zbl.Bakt.Hyg.B. **186**:108-117.

Schramm, A., Santegoeds, C.M., Nielsen, H.K., Ploug, H., Wagner, M., Pribyl, M., Wanner, J., Amann, R. and de Beer, D. (1999) On the occurence of anoxic microniches, denitrification, and sulfate reduction in aerated activated sludge. Appl.Environ.Microbiol. **65**:4189-4196.

Servais, P., Billen, G. and Rego, J.V. (1985) Rate of bacterial mortality in aquatic environments. Appl.Environ.Microbiol. **49**:1448-1454.

Shaw, P.D., Ping, G., Daly, S.L., Cha, C., Cronan, J.E.jr., Rinehart, K.L. and Farrand S.K. (1997) Detecting and characterizing N-acyl homoserine lactone signal molecules by thin-layer chromatography. Proc.Natl.Acad.Sci. **94**:6036-6041.

Siebel, M.A. and Characklis, W.G. (1991) Observations of binary population biofilms. Biotechnol.Bioengin. **37**:778-789.

Siegele, D.A. and Kolter, R. (1992) Life after log. J.Bacteriol. **174**:345-348.

Sitnikov, DM., Schineller, J.B. and Baldwin, T.O. (1996) Control of cell division in *Escherichia coli*: Regulation of transcription of *ftsQA* involves both *rpoS* and SdiA-mediated autoinduction. Proc.Nat.Acad.Sci. **93**:336-341.

Skyring, G.W., Chambers, L.A. and Bauld, J. (1983) Aust.J.Mar.Freshwater Res. **34**:359

Smith, R.L. and Klug, M.J. (1981) Reduction of sulfur compounds in the sediments of a eutrophic lake basin. Appl.Environ.Microbiol. **41**:1230-1237.

Staley, J.T. and Konopka, A. (1985) Measurement of in situ activities of nonphotosynthetic microorganisms in aquatic and terrestrial habitats. Annu.Rev.Microbiol. **39**:321-346

Stams, A.J.M. and Hansen, T.A. (1982) Oxygen-labile L(+) lactate dehydrogenase activity in Desulfovibrio desulfuricans. FEMS Microbiol.Lett. **13**:389-394.

Stookey, L.L. (1970) Ferrozine - a new spectrophotometric reagent for iron. Anal.Chem. **42**:779-781.

Straub, K.L., Benz, M., Schink, B. and Widdel, F. (1996). Anaerobic,nitrate-dependent microbial oxidation of ferrous iron. Appl.Environ.Microbiol. **62**:1458-1460.

Strunk, O., and Ludwig, W. (1995) ARB - a software environment for sequence data. Department of Microbiology, Technical University of Munich, Munich, Germany.

Szewzyk, U. and Schink, B. (1987) Surface colonization by and lifecycle of *Pelobacter acidigallici* studied in a continuous-flowmicrochamber. J.Gen.Microbiol. **134**:183-190.

Tatnall, R. (1990) Case histories: Biocorrosion. In: Biofouling and Biocorrosion in Industrial Water Systems (Flemming, H.-C., and Geesey, G.G., Eds.) 187-193, Springer-Verlag Berlin, Heidelberg

Teske, A., Waver, C., Muyzer, G. and Ramsing, N.B. (1996) Distribution of sulfate-reducing bacteria in a stratified fjord (Mariager Fjord, Denmark) as evaluated by most-probale number counts and denaturing gradient gel electophoresis of PCR-amplified ribosomal DNA fragments. Appl.Environ.Microbiol. **62**:1405-1415

Tschech A., and Pfennig, N. (1984) Growth yield increase linked to caffeate reduction in *Acetobacterium woodii*. Arch.Microbiol. **137**:163-167

Tuovinen, O.H. and Hsu, J.C. (1982) Aerobic and anaerobic microorganisms in tubercles of the Columbus, Ohio, water distribution system. Appl.Environ.Microbiol. **44**:761-764

Tuovinen, O.H., Button, K.S., Vuorinen, A., Carlson, L., Mair, D.M. and Yut, L.A. (1980) Bacterial, chemical, and minerological characteristics of tubercles in distribution pipelines. J.Am.WaterWorks Assoc. **72**:626-635.

van der Kooij, D. and Veenendaal, H.R. (1992) Assessment of the biofilmformation characteristics of drinking water. AWWA WQTC, November 1992,Toronto

van der Kooij, D. and Veenendaal, H.R. (1993) Biofilm development on surfaces in drinking water distribution systems. in: Proc.Int.water Supply Congr. Budapest; Special subject "Biological activity in distribution systems“

van Loosdrecht, M.C., Lyklema, J., Norde, W. and Zehnder, A.J. (1990) Influence of interfaces on microbial activity. Microb.Rev. **54**:75-87.

van Niel, E.W., Pedro, G., Willems, A., Collins, M.D., Prins, R.A. and Gottschal, J.C. (1996) The role of polyglucose in oxygen-dependent respiration by a new strain of *Desulfovibrio salexigens*. FEMS Microbiol.Ecol. **21**:243-253.

van Niel, E.W. and Gottschal, J.C. (1998) Oxygen consumption by *Desulfovibrio* strains with and without polyglucose. Appl.Environ.Microbiol. **64**:1034-1039.

Voordouw, G., Armstrong, S.M., Reimer, M.F., Fouts, B., Telang, A., Shen, Y. and Gevertz, D. (1996) Characterization of the 16S rRNA genes form oil field microbial communities indicates the presence of a variety of sulfate-reducing, fermentative, and sulfide-oxidizing bacteria. Appl.Environ.Microbiol. **62**:1623-1629.

Votyakova, T.V., Kaprelyants, A.S. and Kell, D.S. (1994) Influence of viable cells on the resuscitation of dormant cells in Micrococcus luterus cultures held in an extended stationary phase: the population effect. Appl.Environ.Microbiol. **60**:3284-3291.

Wagner, M.A., Roger, A.M., Flax, J.L., Brusseau, G.A. and Stahl, D.A. (1998) Phylogeny of dissimilatory sulfite reductases supports an early origin of sulfate respiration. J.Bacteriol. **180**:2975-2982

Wall, J.D., Rapp-Giles, B.J., Brown, M.F. and White, J.A. (1990) Response of *Desulfovibrio desulfuricans* colonies to oxygen stress. Can.J.Microbiol. **36**:400-408.

Weichart, D., McDougald, D., Jacobs, D. and Kjelleberg, S. (1997) In situ analysis of nucleic acids in cold-induced nonculturable *Vibrio vulnificus*. Appl.Environ.Microbiol. **63**:2754-2758

Weichart, D., Oliver, J.D. and Kjelleberg, S. (1992) Low temperature induced non-culturability and killing of *Vibrio vulnificus*. FEMSMicrobiol.Lett. **100**:205-210.

Weiss, P., Schweitzer, B., Amann, R. and Simon, M. (1996) Identification in situ and dynamics of bacteria on limnetic organic aggregates (lake snow). Appl.Environ.Microbiol. **62**:1998-2005.

Whitesides, M.D. and Oliver, J.D. (1997) Resuscitation of *Vibrio vulnificus* from the viable but nonculturable state. Appl.Environ.Microbiol. **63**:1002-1005.

Widdel, F. and Bak, F. (1992) Gram-negative mesotrophic sulfate-reducing bacteria. In: The Prokaryotes: a Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification and Applications (Balows, A., Trüper, H.G., Dworkin, M., Harder, W. and Schleifer, K.-H., Eds.), 2nd Edn., 3353-3378, Springer Verlag, New York

Wind, T., Stubner, S. and Conrad, R. (1999) Sulfate-reducing bacteria in rice field soil and on rice roots. System.Appl.Microbiol. **22**:269-279.

Wimpenny, J.W. and Colasanti, R. (1997) A unifying hypothesis for the structure of microbial biofilms based on cellular automaton models. FEMS Microbiol.Ecol. **22**:1-16.

Woese, C.R. (1987) Bacterial evolution. Microbiol.Rev. **51**:221-271.

Wolfaardt, G.M., Lawrence, J.R., Robarts, R.D., Caldwell, S.J. and Caldwell, D.E. (1994) Multicellular organization in a degradative biofilm community. Appl.Environ.Microbiol. **60**:434-446.

Wolfaardt, G.M., Lawrence, J.R., Robarts, R.D. and Caldwell, D.E. (1995) In situ characterization of biofilms exopolymers involved in the accumulation of chlorinated. Microb.Ecol. **35**:213-223.

ZoBell, C.E. (1943) The effect of solid surfaces upon bacterial activity. J.Bacteriol. **46**:39-56.