sommigen plooien staal
met hun blote handen
anderen doorschouwen kosmische verbanden
of snijden fluitend
gezwellen uit rotte ingewanden
sommigen verkneukelen zich
met formules jonglerend terwijl ze
atomaire deeltjes de daver op het lijf jagen
anderen klimmen in masten of bergen
verbouwen in een handomdraai
een vogelnest tot een villa
of ze vinden pillen uit
die de mensen eeuwig doen leven
machines om nooit meer
te moeten beven
ik kan niets van dat alles
ik vind geen ster die mij
aan een touwtje door het heelal leidt
ik ben misschien maar een gekwelde kinkel
leunend tegen de muur van een fruitwinkel?

(J. Joos, 1957)

Gelukkig stond er snel een heuse melkweg van “sterren met touwtjes” voor me klaar: Jaco, Niels, Ludo, Brigitte, Karolien, Dirk, mama & papa, Evi, Koen, Lieveke, Shirley, Ann, Evelyne, Philippe, Veerle, Maarten, Zita, Safiyh, Winnie, Leen, Myriam, David, Queenie, Wouter, Tania, Kelly, Thomas, Jan, Barbara, Staf, Sandra, Ann P., Johan, de “Metalbioreduction” partners (Ioannis, Bernard, Fabienne, Cathérine, Mireille, Caroline, …) en de rest van het firmament, die ik hier vergeet te vermelden …
dankjewel!

Abbreviations

A	alanine
AAS	atomic absorption spectrometry
AMD	acid mine drainage
APS	adenosine-5′-phosphosulfate
ARDRA	amplified ribosomal DNA restriction analysis
ATP	adenosine triphosphate
BAC	bacterial artificial chromosome
BEN	Belgian EMBnet Node
BLAST	basic logical alignment tool
BMSR	bio metal sludge reactor
bp	base pairs
C	cytidine
CGA	community genome array
CLSM	confocal laser scanning microscopy
cPCR	competitive PCR
C-source	carbon source
COD	chemical oxygen demand
DGGE	denaturing gradient gel electrophoresis
DIG	dioxygenine
dm	dry matter
DNA	deoxyribonucleic acid
dNTP	2′-deoxyribonucleoside-5′-triphosphate
DOC	dissolved organic carbon
dUTP	2'-deoxyuridine- 5'-triphosphate
DSMZ	Deutsche sammlung von mikroorganismen und zellculturen
DSR	dissimilatory sulfite reductase
EDTA	ethylenediaminetetraacetic acid
EMBOSS	European molecular biology open software suit
ERIC-PCR	enterobacterial repetitive intergenic consensus- polymerase chain reaction
FGA	functional gene array
FISH	fluorescence in situ hybridization
G	guanine
GC-FID	gas chromatography- flame ionization detection
GC-RGD	gas chromatography- reductive gas detection
GST	genomic sequence tag
HM	heavy metal
HMM	heavy metal and metalloid
HRC®	hydrogen release compound
ICP-AEMS	induction coupled plasma- atomic emission mass spectrometry
ISMP	*in situ* metal precipitation

kb	kilo base pairs
LPS	lipopolysaccharide
MCR	methyl- coenzyme M reductase
MERESAFIN	metal removal by sand filter inocultation
MeS	metal sulfide
MP	methane producers
MPN	most probable number
MTs	metallothioneins
N/P	NH_4Cl/ KH_2PO_4 nutrient solution
N- source	nitrogen source
PCR	polymerase chain reaction
PLFA	phospholipid ester linked fatty acids
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rpm	rounds per minute
rRNA	ribosomal ribonucleic acid
RSGP	reverse sample genome probing
RST	ribosomal sequence tag
RT-PCR	reverse transcriptase- polymerase chain reaction
SARST	serial analysis of RSTs
SEM	scanning electron microscopy
sp.	species
SRB	sulfate reducing bacteria
SRP	sulfate reducing prokaryotes
SSCP	single- strand- conformation- polymorphism
T	thymine
Taq	*Thermus aquaticus*
TE	Tris- EDTA
TGGE	temperature gradient gel electrophoresis
Tris	Tris-(hydroxymethyl)aminomethane
UPGMA	unweighed pair group method of arithmetic averages
UV	ultra-violet

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Summary

The presence of toxic heavy metals in the environment greatly affects the quality of water resources and poses an eminent risk for food-chains. Metal precipitation with lime or more expensive chemicals, which is the classical treatment for the removal of heavy metals from water bodies, has major drawbacks such as high operational and maintenance costs, and the production of large quantities of contaminated sludge that require safe disposal. *In situ* metal precipitation (ISMP) by means of bacterial sulfate reduction has promise as an alternative technique over chemical methods for the treatment of heavy metals and metalloids contaminated groundwater. Under anaerobic conditions, sulfate reducing bacteria (SRB) oxidize simple organic compounds or hydrogen by utilizing sulfate as an electron acceptor and generate sulfide which can react with dissolved metal ions to form metal sulfide precipitates. In addition, SRB are able to precipitate certain heavy metals and metalloids by changing their speciation via direct and indirect reduction mechanisms. By the formation of an *in situ* anaerobic reactive zone or biobarrier in the path of the groundwater plume, dissolved heavy metals will precipitate as metal sulfides or due to changes of their speciation, thus preventing further spreading of the contamination to neighboring areas. The molecular follow- up of the SRB communities and their activity is an important tool in the optimization and control of such an ISMP process.

In the present study, we evaluated the perspectives of ISMP as a treatment technology for mildly acidic metal (Zn, Ni, Co, Cd, Fe) and sulfate contaminated groundwater at the site of a nonferrous company by combining physical-chemical follow-up of the bacterial sulfate reduction and metal removal with molecular monitoring of the indigenous SRB community.

For the molecular analysis of the SRB communities, we based our investigations on both the 16S rRNA gene and the *dsr*- gene, encoding the dissimilatory sulfite

reductase (Dsr), that is considered as a specific functional biomarker for sulfate reducing bacteria. A series of eight primer sets, targeting the 16S rRNA gene of different SRB-subgroups and –genera, was optimized or newly designed. PCR with these primers allowed a profound insight in the indigenous SRB community composition present in different environmental samples with a heavy metal and metalloid contamination. In addition, a DGGE (Denaturating Gradient Gel Electrophoresis)-based approach was developed to rapidly assess the diversity of *dsrB* genes, encoding the β-subunit of the Dsr protein. This method allowed to differentiate between *dsrB* genes of SRB at the genus and species level, and was successfully used to explore the *dsrB* diversity within different SRB-communities as a phylogenetic marker. Although this approach turned out to be useful to get a first insight in the composition of the indigenous SRB community, we concluded that to optimize bioremediation strategies such as ISMP, the study of SRB communities based on the diversity of *dsrB* genes should focus on the metabolically active bacteria, rather than on the total population. In addition, rather than solely concentrating on the SRB, an analysis of the total microbial community composition remains important especially when the SRB-activity is affected by synergistic or competing interactions with other microorganisms, such as methanogens, fermenting bacteria, acetogens, etc.

The batch experiments performed during this study aimed at determining if indigenous communities of SRB were present in our environmental samples, and to define optimal conditions for stimulating their activities to obtain efficient *in situ* precipitation of the contaminating heavy metals. Molecular analysis showed that the SRB community was predominated by *Desulfosporosinus* strains, and that their activity was most efficient when lactate, molasses or HRC® were added as carbon source and electron donor. Subsequently, these substrates were tested in column experiments that had as objective to examine the ISMP process under more realistic aquifer mimicking conditions. Special attention was given to the sustainability of the precipitation process under circumstances of a changed COD

to [SO_4^{2-}] ratio, or when the substrate supply was interrupted. As expected from the batch experiments, all three carbon sources supported sulfate reduction and metal precipitation. However, these processes eventually declined whether or not the substrate supply was maintained: the sulfate removal rate suddenly dropped and thereupon, metal concentrations in the column's effluent dramatically increased. Calculation of Ni and Co mass balances indicated that most of this increase was due to the release of retained metals into solution. The origin of this sudden shortcoming of the ISMP process in the presence of substrates is not immediately clear. Analysis of archaeal and eubacterial 16S rDNA sequences did not reveal the presence of non-sulfate reducers (methanogens, iron reducers, acetogens, fermenting bacteria) that might have outplayed the SRB community. It was postulated that the SRB- community became inhibited by the formed metal sulfides. Since it was observed that re-establishment of sulfate reduction was easily obtained when part of the metal precipitates were dissolved, the metal sulfides were, although inhibitory, not lethal to the SRB community. In the case of intentional interruption of substrate supply, the ISMP process failed most likely because the growth and activity of the indigenous SRB community delayed or even stopped due to the lack of a carbon and electron donor, which was also accompanied by a release of metals in the column's effluent.

Our results indicate that the ISMP process is highly dependent on SRB-stimulation by substrate amendments and suggests that this remedial approach might not be a sustainable strategy for long-term application, this unless substrate amendments are continued, environmental conditions are strictly controlled, or the with metals enriched aquifer material is removed at the end of the remediation process. Therefore, both a pre and post analysis of both the feasibility and long-term sustainability of the ISMP process is required before it can be implemented as a remediation strategy. In other words, additional tests are required to investigate what will happen when at the end of the remediation process clean groundwater will pass through the reactive zone, while no more C-sources are amended and all indigenous carbon is consumed. Also, the effects of

dramatic changes in sulfate- or HM concentrations on the SRB community composition and activity and the concomitant ISMP process need to be investigated in more detail before ISMP can be implemented as a reliable bioremediation and site management strategy.

Samenvatting

Door de aanwezigheid van toxische zware metalen en metalloïden (ZMM) in het milieu kan de kwaliteit van het (grond)water worden aangetast, zodat deze ZMM een grote bedreiging voor voedselketens gaan vormen. Traditioneel worden zware metalen uit het gecontamineerde water verwijderd door precipitatie met calciumoxide of andere, duurdere chemicaliën; deze behandeling brengt echter hoge operationele en onderhoudskosten met zich mee en vereist bovendien een oplossing voor de aanzienlijke hoeveelheden gecontamineerd slib die hierbij geproduceerd worden. *In situ* metaalprecipitatie (ISMP) met behulp van sulfaatreducerende bacteriën is een veelbelovend alternatief voor de chemische behandeling van met ZMM vervuild grondwater. Sulfaatreducerende bacteriën (SRB) zijn anaërobe bacteriën die voor hun energievoorziening de oxidatie van eenvoudige organische componenten of waterstof koppelen aan de reductie van sulfaat. Hierbij wordt er sulfide vrijgesteld dat met de in het grondwater opgeloste metaalionen reageert onder de vorming van metaalsulfide precipitaten. Bovendien zijn SRB via directe of indirecte reductiemechanismen in staat om de speciatie van metalen en metalloïden te veranderen en ze te doen neerslaan. Door in de baan van de grondwaterstroom een *in situ* reactieve anaërobe zone of biowand te creëren waarin de opgeloste zware metalen neerslaan als metaalsulfiden of als gevolg van speciatieverandering, kan aldus de verspreiding van de contaminanten naar omliggende gebieden vermeden worden. Voor de optimalisatie, opvolging en bijsturing van zulk een ISMP proces is de moleculaire evaluatie van de SRB populaties en hun sulfaatreductie activiteit een zeer belangrijk hulpmiddel.

Tijdens dit doctoraatsonderzoek evalueerden we de perspectieven van ISMP als een kosten-efficiënte *in situ* remediatie strategie voor de behandeling van een licht zuur, met zware metalen (Zn, Ni, Co, Cd, Fe) en sulfaat vervuild grondwater

van een non-ferro-industriële site. Voor de opvolging van de bacteriële sulfaatreductie en de metaalverwijdering werden fysico-chemische technieken gecombineerd met moleculaire technieken waarmee de endogene SRB gemeenschap in beeld werd gebracht.

Voor de moleculaire analyse van de SRB gemeenschap baseerden we ons op zowel het 16S rRNA gen, hetwelk universeel in alle bacterien aanwezig is, als op het *dsr*- gen dat codeert voor het dissimilatie sulfiet reductase (Dsr) en beschouwd wordt als een specifieke functionele merker voor sulfaat reducerende bacteriën. Acht primersets werden geoptimaliseerd of ontworpen voor de detectie van het 16S rRNA gen van verschillende SRB-subgroepen en -genera. De PCR resultaten, verkregen met deze primers, gaven een eerste inzicht in de samenstelling van de endogene SRB gemeenschap in de met verschillende ZMM gecontamineerde aquifer en grondwater stalen. Bovendien werd een DGGE (Denaturing Gradient Gel Electrophoresis) methode ontwikkeld om snel een beeld te krijgen van de diversiteit van *dsr*B genen, welke coderen voor de β-subeenheid van het Dsr enzym. Het was mogelijk om met deze methode *dsrB* genen te onderscheiden tot op het niveau van SRB-genera en –species; de methode werd met succes toegepast om in verschillende SRB gemeenschappen de *dsrB* diversiteit te achterhalen. Deze aanpak bleek goed bruikbaar te zijn om een beeld te krijgen van de samenstelling van de endogene SRB gemeenschappen. Echter, voor de optimalisatie van bioremediatie strategieën zoals ISMP zou het beter zijn om voor de analyse van SRB aan de hand van hun *dsrB* genen de nadruk te leggen op de metabolisch actieve populaties in plaats van op de totale SRB gemeenschap. Bovendien blijft ook de analyse van de gehele microbiële gemeenschap uiterst belangrijk, vooral wanneer de SRB-activiteit beïnvloed wordt door synergistische of competitieve interacties met andere micro-organismen, zoals methanogenen, fermenteerders, acetogenen, enz.
In een eerste stap om de haalbaarheid van *in situ* metaalprecipitatie (ISMP) met behulp van sulfaatreducerende bacteriën te evalueren werden batch experimenten

uitgevoerd, waarmee we wilden nagaan of er daadwerkelijk een endogene SRB-gemeenschap in onze milieustalen aanwezig was en wat de optimale condities waren om de SRB activiteit te stimuleren en een efficiënte *in situ* precipitatie van de aanwezige metalen te bekomen. Moleculaire analyses toonden aan dat de SRB gemeenschap voornamelijk bestond uit *Desulfosporosinus* stammen en dat hun sulfaatreducerende activiteit het meest efficiënt was wanneer lactaat, melasse of HRC® als koolstofbron en elektrondonor werden toegevoegd. Om het ISMP proces te bestuderen in een aquifer systeem dat de *in situ* situatie beter benadert werden deze substraten werden vervolgens toegediend aan kolomopstellingen die met aquifer materiaal en grondwater van de site opgestart werden. Tijdens de kolomexperimenten ging onze aandacht vooral uit naar de duurzaamheid van het metaal verwijderings en precipitatieproces bij veranderlijke COD/ SO_4^{2-} ratio of bij onderbreking van de substraattoevoer. Zoals ook uit de batchexperimenten was gebleken, werd voor alle drie de toegepaste koolstofbronnen een duidelijke stimulatie van de sulfaatreductie en metaalprecipitatie waargenomen. Echter, voor alle koolstofbronnen vielen deze processen na verloop van tijd stil, zelfs indien de substraat toevoer gehandhaafd bleef: de sulfaatverwijdering nam plots af, waarop de metaalconcentratie in het effluent van de kolommen significant steeg. Via berekening van massabalansen voor Ni en Co werd aangetoond dat het merendeel van deze stijging te wijten was aan de vrijstelling van voordien geprecipiteerde metalen in het grondwater. De oorzaak van deze plotse achteruitgang van het ISMP proces, zelfs in aanwezigheid van een geschikte koolstofbron, was niet meteen duidelijk. Immers, via 16S rRNA gen sequentie analyse van de aanwezige bacteriën en *Archaea* konden geen niet-SRB stammen (methanogenen, ijzerreduceerders, acetogenen, fermenteerders) opgespoord worden die de SRB gemeenschap hadden kunnen uitschakelen. Er wordt daarom geopperd dat de SRB gemeenschap geïnhibeerd werd door de gevormde metaalprecipitaten. Deze gevormde metaalneerslag was echter niet lethaal aangezien het sulfaatreductie proces voor alle geteste koolstofbronnen opnieuw op gang kwam nadat een deel van deze neerslag terug in oplossing was gegaan.

In het geval dat de toediening van de substraten werd onderbroken, werd het stilvallen van het ISMP proces veeleer veroorzaakt doordat de groei en activiteit van de endogene SRB vertraagde en zelfs stopte als gevolg van een gebrek aan koolstof en elektrondonor, wat uiteindelijk resulteerde in een vrijstelling van metalen.

Onze resultaten wijzen erop dat het ISMP proces sterk afhankelijk is van SRB stimulatie door substraattoediening; ze suggereren ook dat deze remediatie strategie op lange termijn niet kan werken tenzij er continu substraat wordt toegevoegd, de omgevingscondities strikt worden geregeld, of dat het met ZMM aangerijkte aquifer materiaal wordt verwijderd op het einde van het saneringsproces. Alvorens ISMP kan worden toegepast is het dan ook van belang dat er zowel een pre- als een post- saneringsanalyse uitgevoerd wordt, niet alleen naar de haalbaarheid van het proces maar zeker ook naar de duurzaamheid ervan. Met andere woorden, bijkomende testen zijn nodig om te onderzoeken wat er gebeurt als er op het einde van de sanering zuiver grondwater door de reactieve zone stroomt, terwijl er geen C- bron meer wordt toegevoegd en alle endogene koolstof is opgebruikt. Ook moet het effect van drastische wijzigingen in sulfaat- en zware metaal- concentraties op de samenstelling en activiteit van de SRB gemeenschap en het bijhorende ISMP proces nader bestudeerd worden vooraleer ISMP op een betrouwbare manier kan worden toegepast voor de bioremediatie en beheer van de verontreinigde site.

CHAPTER 1

Introduction

Part of this chapter has been accepted for publication in *Handbook of Remediation of Contaminated Soils*

1.1. General introduction

Most heavy metals exist naturally in the earth's crust at trace concentrations, sufficient to provide living systems with essential nutrients but too low to cause toxicity. Exceptions are soils that are naturally rich in heavy metals such as the African copper belt, arsenopyrite minerals or the serpentine soils of e.g. New Caledonia, which are characterized by the presence of ecosystems comprised of life forms (plants, microorganisms) that are well adapted to increased levels of bioavailable heavy metals. Since the industrial revolution, pollution by heavy metals has substantially increased through industrial effluents and landfill leaching, mining activities, use of fertilizers and pesticides in agriculture, burning of waste and fossil fuels, and municipal waste treatment. Since heavy metals cannot be degraded they are persistent and accumulate over time in the environment, including the food chain. Exposure to heavy metals through ingestion or uptake of drinking water and food can result in their accumulation in different trophic levels and eventually in plants, animals and humans (Mulligan *et al.*, 2001). There these metals exert inhibitory actions, e.g. by blocking essential functional groups of proteins, displacing essential metal ions, or modifying the active conformations of biological molecules (Hassen *et al.*, 1998). These phenomena can result in acute or chronic toxicity, as well as genotoxicity, mutagenesis or carcinogenesis. Among the heavy metals, cadmium, copper, lead, mercury, nickel, zinc together with the metalloid arsenic are considered to be the

most hazardous (Cameron, 1992). Because of their threat to human health and the extend of the problems related to both natural and anthropogenic contamination by heavy metals and metlloids, major efforts are undertaken to develop remediation technologies for treatment of metal-contaminated soils, sediments and groundwater, which are based either on physical or chemical principles, or on biological processes.

1.1. 1. Physicochemical methods

Conventionally, heavy metal pollution in soils and waters is removed by methods based on physical and/ or chemical processes (Mulligan *et al.*, 2001). Pump and treat, using precipitation or flocculation techniques followed by sedimentation and disposal of the resulting contaminated sludge is frequently used for treating heavy metal contamination in water. Other methods for heavy metal removal from water involve ion exchange, reverse osmosis and microfiltration. For the *in situ* treatment of groundwater, a reactive barrier may be installed which removes the heavy metals either by chemical means, i.e. reduction by ferrous iron, or by biological means (Benner *et al.*, 2002; Nyman *et al.*, 2002; Scherer *et al.*, 2000).
Polluted soil can be treated *ex situ* by physical separation (e.g. froth flotation, fluidized bed separation, hydrocyclones), pyrometallurgical separation (i.e. volatilization in high temperature furnaces) or electrokinetic processes, involving passage of a low intensity electric current between a cathode and an anode imbedded in the contaminated soil which results in migration of the metals, followed by their removal. The latter technique can also be used *in situ*. Soil washing ('chemical leaching') and *in situ* soil flushing involve the addition of water with or without additives including chelating agents such as EDTA, inorganic or organic acids, biosurfactants, etc., in order to desorp and leach the heavy metals. Soil washing can be followed by returning the clean soil to the original site. Chemical treatments of soils and wastewaters by reductive or oxidative reaction mechanisms are used to detoxify, precipitate or solubilize

metals or to neutralize soil pH. However, these reactions are not specific and other metals can be converted into a more toxic or mobile form, resulting in novel contamination problems. Also, other essential trace elements and nutrients will be removed with these processes, leaving a treated soil that has no environmental value.

Other *in situ* methods for the treatment of contaminated soils include the isolation and containment of contaminants. This is used in order to prevent further movement of the contaminants, to reduce the permeability of the waste and to increase the strength or bearing capacity of the waste; these methods include vertical and horizontal containment by physical barriers made of steel, cement, bentonite and grout walls (Rumer and Ryan, 1995). Another method is solidification/ stabilization, which contains the contaminants in an area by mixing or injecting agents. Solidification techniques such as encapsulation and vitrification block the contaminants in a solid matrix (Conner, 1990) while stabilization reduces the contaminant's mobility by formation of chemical bonds. An example is beringite, a waste product from the burning of coal refuse, used to immobilize heavy metals in a contaminated soil, thereby decreasing their phytotoxic effects (Vangronsveld *et al.*, 1995; Vangronsveld *et al.*, 1996) and allowing the establishment of vegetation on heavily contaminated sites.

1.1.2. Biological treatment

Due to the fact that conventional methods are often expensive, lack specificity or even give rise to more environmental problems, alternative cost effective technologies that are generally based on biological processes using microorganisms and/ or higher organisms, such as plants, are being developed as treatment alternatives.

Phytoremediation uses plants and their associated microorganisms to assimilate and remove contaminants from the environment. Phytoremediation of heavy metals comprises several processes (Salt *et al.*, 1995). Rhizofiltration is based on

the ability of plant roots to adsorb, precipitate and concentrate toxic metals and is mainly being developed to treat water contaminated with heavy metals and/ or radionuclides. Phytoextraction of heavy metals involves uptake by metal accumulating plants, like e.g. by hyperaccumulator plants such as *Thlaspi* sp. and *Alyssum* sp. into the harvestable parts of roots and shoots. Phytostabilization is an ideal method to reduce the spreading of heavy metals due to their leaching to the groundwater or their dispersion by wind erosion. Finally, phytovolatilization involves the transformation of toxic elements into relative harmless and gaseous forms which are volatilized by the plant's tissues (Rugh *et al.*, 1998; Terry and Zayed, 1998). Although phytoremediation is a promising remediation method, it is limited to shallow depths of contamination and longer times are required compared to other methods (Cunningham *et al.*, 1995). The general potential of phytoremediation was recently reviewed (Adriano *et al.*, 2004; van der Lelie *et al.*, 2001; Vassilev *et al.*, 2004).

Bioremediation exploits microorganisms to deal with heavy metal pollution in a variety of schemes, such as bioleaching, biosorption, oxidation/ reduction reactions, bioprecipitation and biomethylation. These technologies rely on the genetic and biochemical capacities of microorganisms to protect themselves against the toxic effects of heavy metals. An understanding of the mechanisms how bacteria cope with toxic concentrations of heavy metals is therefore essential in order to exploit them for detoxification and removal of heavy metals.

1.2. Heavy metal resistance in bacteria

Microorganisms have co- existed with metals since the beginning of life. This is reflected by the presence of a wide range of metals in the active sites of many enzymes, and the chemical properties of metal cations which have been recruited for catalyzing key metabolic reactions and for maintaining protein structures. These metals are considered as essential micronutrients because they are required in minute amounts for normal cell metabolism. However, other metals have not

been found to be involved in biologically relevant functions and when present at high concentration, they can damage cell membranes, block and inactivate enzymes, and damage DNA- structure. The same is true for essential nutrients, which also become toxic above a threshold concentration. From a physiological point of view, metals fall into three main categories: (i) essential and basically non- toxic (e.g. Ca and Mg), (ii) essential, but harmful at high concentrations (typically Fe, Mn, Zn, Cu, Co, Ni, Cr and Mo), and (iii) toxic and non-essential (e.g. Hg, Pb or Cd) (Valls and de Lorenzo, 2002).

Over geological timescales, bacteria evolved in order to inhabit ecological niches containing high concentrations of heavy metals. In addition to natural heavy metal rich biotopes, anthropogenic activities leading to increased atmospheric release as well as deposition into aquatic and terrestrial environments have created novel metal-loaded niches with a strong selective pressure for metal endurance (Gadd and White, 1993). For protection against the toxic effects of heavy metals, bacteria can adopt diverse chromosomal, transposon, and plasmid-mediated resistance systems which provide them with a certain range of metal tolerance. The physiological role of plasmid-encoded resistance determinants is generally to confer resistance, while the role of chromosomally encoded systems may also include metal ion homeostasis of the essential trace elements. For example, copper and zinc ions are required for growth and therefore a fine balance between this uptake, internal availability and efflux is required to provide metal ion homeostasis (Rensing *et al.*, 1999).

Overall, bacteria can adopt two major strategies for heavy metal tolerance. In some bacteria, metal tolerance is the outcome of their metabolism or is an intrinsic property related to their cell wall structure or the presence of extra-cellular polymeric substances. Other bacteria have developed specific resistance mechanisms to protect themselves against the toxic effects of heavy metals. These mechanisms include active transport, mediated by efflux pumps, intra- and extracellular sequestration, enzymatic transformation to other, less toxic

chemical species by redox reactions, methylation, or alkylation/ dealkylation, and reduction in the sensitivity of cellular targets to metal ions (Gadd, 1992).
Almost all microbe-metal interactions have been examined in the context of environmental biotechnology as a possible means for the removal, recovery or detoxification of heavy metals or radionuclides (Gadd, 2000a). Advances in understanding the roles of microorganisms in such processes, together with the ability to fine-tune their activities using molecular biological tools, has led to the development of novel or improved metal bioremediation processes (Lloyd and Lovley, 2001).

1.2.1. Efflux- based heavy metal resistance

Non- essential metals can enter the cell through the normal nutrient transport systems. Once their concentrations exceed a certain threshold limit, microorganisms use active transport or efflux systems, which can be chromosomal or plasmid- encoded, to remove these toxic metals from their cytoplasm. These mechanisms are highly specific for the cation or anion they export (Bruins *et al.*, 2000; Nies and Silver, 1995; Silver *et al.*, 1989). There are several types of efflux based mechanisms described, from which the most intensively studied are (i) CBA efflux pumps, driven by protein complexes of the resistance-nodulation-cell division (RND) protein complex family, which export superfluous cations, and (ii) P-type ATPases, which are the basic defense against heavy metal cations (Nies, 2003). Often, the efflux of heavy metals is combined with a form of heavy metal sequestration or precipitation, this to avoid reentry of the toxic metals.
Ralstonia metallidurans, formerly known as *Alcaligenes eutrophus* and thereafter as *Ralstonia eutropha*, is a β-proteobacterium that seems to thrive well in industrial sediments, soils and wastes with a high heavy metal content (Mergeay *et al.*, 1985). The type strain, CH34, which was isolated from the decantation tank of a zinc factory in Liège, Belgium, was found to be highly resistant to Zn(II), Cd(II), and Co(II) and later Ni(II), Cu(II), CrO_4^{2-}, Hg(II) and Pb(II) (Mergeay *et al.*,

1985). CH34 carries two indigenous megaplasmids, pMOL28 (180 kb) and pMOL30 (~250 kb) bearing resistance determinants to combinations of cobalt and nickel (*cnr*), cadmium, zinc and cobalt (*czc*), mercury (*mer*), thallium (*tll*), copper (*cop*) and lead (*pbr*) (Borremans *et al.*, 2001; Diels *et al.*, 1985; Taghavi *et al.*, 1997a). The genetical, physiological and biotechnological study of heavy metal resistance characteristics of *R. metallidurans* CH34 has highly contributed to the knowledge of these mechanisms, and is still increasing thank to the progress made with the sequencing of its genome (see http://www.jgi.doe.gov). A catalog of the *R. metallidurans* CH34 resistance genes was recently published (Mergeay *et al.*, 2003), which was linked to a global analysis of its metal induced proteome (Noel-Goris *et al.*, 2004). Recently, genome sequencing of *Pseudomonas putida* KT2440 revealed an unexpected capacity for tolerance and homeostasis of heavy metals and metalloids (Canovas *et al.*, 2003). However, like for *R. metallidurans* CH34, further studies on the transcriptome and proteome level are required to better understand these processes. Efflux mediated heavy metal resistance in prokaryotes has been recently reviewed in detail in a special issue of FEMS Microbiology Reviews (Vol. 27, 2003) and will not be described here (Mergeay *et al.*, 2003; Mukhopadhyay *et al.*, 2002; Nies, 2003; Solioz and Stoyanov, 2003).

1.2.2. Enzymatic reduction

Toxicity of metals and metalloids such as mercury, arsenic, uranium and chromium often depends on their oxidation state. As a resistance mechanism, many microorganisms have been found that are able to change the metal oxidation state through enzymatic reduction or oxidation. Several such resistance systems, which do not support bacterial growth, have been studied in detail using molecular biological tools, e.g. resistance to Hg(II), As(V) and Cr(VI). These systems were recently reviewed and will not be discussed here (Barkay *et al.*, 2003; Mukhopadhyay *et al.*, 2002; Silver, 2003). However, up to now, the molecular basis of respiratory metal reduction processes is not understood in such

fine detail, although rapid advances are expected in this area with the imminent availability of complete genome sequences for key metal- reducing bacteria, in combination with genomic and proteomic tools (Lloyd, 2003).

1.2.3. Dissimilatory metal-reduction

As part of their respiratory chain, dissimilatory metal reducing bacteria couple the oxidation of H_2 or organic substrates to the reduction of ferric iron, manganese or other metals in order to conserve energy for growth support (Lovley, 1993).

Dissimilatory Fe(III)-reducing communities are often responsible for the majority of organic matter oxidized in anoxic non-sulfidogenic environments (Nealson and Saffarini, 1994). These microorganisms can also play an important role in the fate of heavy metals present in their environment through direct enzymatic reduction and also via indirect reduction mechanisms, catalyzed by biogenic Fe(II) (Lloyd, 2003). The reduction of Cr(VI), Co(III), U(VI) and Tc(VII) by Fe(III)-reducing microorganisms and metal reducing enzymes have been proposed as a concept for remediating heavy metal- and radionuclide-contaminated waters and sediments (Lloyd and Lovley, 2001).

Several types of Fe(III)-reducing bacteria, which have been isolated from various environments, were shown to constitute a phylogenetically diverse group (Lonergan *et al.*, 1996). Recent reports demonstrate that the phylogenetic diversity of Fe(III)-reducing bacteria extends beyond the *Proteobacteria*. These include e.g. *Geovibrio ferrireducens* (Caccavo Jr. *et al.*, 1996) and the related thermophile *Deferribacter thermophilus* (Green *et al.*, 1997), *Geothrix fermentans* (Coates *et al.*, 1999), *Ferribacter limneticum*, *Sulfurospirillum barnesii* (Oremland *et al.*, 1994), the acidophilic sulfur-oxidizing bacterium *Thiobacillus ferrooxidans* (Pronk *et al.*, 1992), and several hyperthermophilic Archaea and Bacteria (Vargas *et al.*, 1998).

Geobacter metallireducens (Lovley *et al.*, 1993c) and *Shewanella oneidensis* (formerly *Alteromonas putrefaciens*) (Myers and Nealson, 1988), which both belong to the delta subdivision of the *Proteobacteria*, were among the first bacteria studied in pure culture that were able to gain energy for growth by coupling the oxidation of organic matter and/ or H_2 to the reduction of iron and manganese, which served as electron acceptors. It is now clear that dissimilatory iron and manganese reduction is not limited to these genera but is broadly distributed among the known bacterial taxa.

The mechanisms by which dissimilatory Fe(III) reducing microorganisms transport electrons to Fe(III) and Mn(IV) have been studied in most detail for *S. oneidensis* and *G. sulfurreducens* (Figure 1.1).

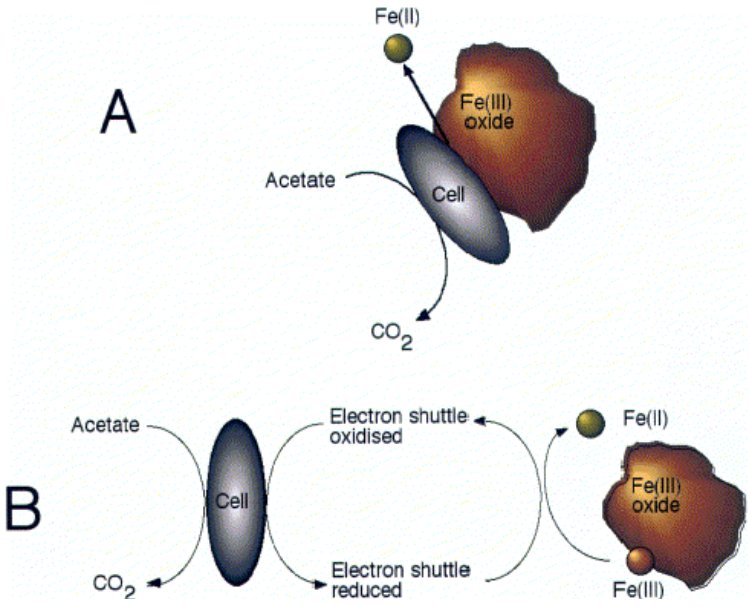

Figure 1.1 Mechanisms of reduction of insoluble Fe(III) oxides, via (A) direct contact with the surface of the cell or (B) an extracellular electron shuttle (Lloyd, 2003)**.**

Research on these organisms has been strongly stimulated through the availability of their genome sequences (http://www.tigr.org). Several studies demonstrated that electron transport to Fe(III) and Mn(IV) involved direct reduction by c-type cytochromes (Lloyd, 2003). At circum-neutral pH, Fe(III) oxides are highly insoluble, yet they are accessible to respiring metal reducing bacteria. A direct transfer was proposed because reduction activities of cytochromes were localized to the outer membrane or surface of the cell (Lloyd, 2003).

In addition, soluble extracellular quinones and humic acids were shown to function as 'electron shuttles' between these metal- reducing bacteria and the mineral surface (Lovley *et al.*, 1996). The reduced hydroquinone moieties abiotically transfer electrons to Fe(III) oxides. The oxidized humic acid is reduced by the microorganism, leading to further rounds of electron shuttling to the insoluble mineral (Nevin and Lovley, 2002). The release of a quinone-containing extracellular electron shuttle by *S. oneidensis* which may promote electron transfer to Fe(III) and Mn(IV) was also suggested (Newman and Kolter, 2000). An important new discovery was made recently when it was shown that *G. metallireducens* can access Fe(III) oxide by chemotaxis (Childers *et al.*, 2002). The strain synthesizes pili and flagella when grown on insoluble Fe(III) and Mn(IV) minerals, but not when grown on soluble forms of the metals. It was hypothesized that *Geobacter* species sense depletion of soluble electron acceptors and synthesize the appropriate appendages that allow movement and subsequent attachment to Fe(III) and Mn(IV) minerals.

In addition to the reduction of Fe(III) and Mn(IV), several Fe(III)-reducing bacteria have the capacity to reduce toxic metals and metalloids such as chromium, selenium, arsenic, uranium. However, the ability to enzymatically reduce these toxic metals is not restricted to Fe(III) reducers and in most cases, this metal reduction metabolism does not support growth (Lovley, 1993).

The reduction of highly toxic and mobile Cr(VI) to the less toxic, less mobile Cr(III) is likely to be a useful process for treatment of Cr(VI) contaminated waters and soils (Palmer and Wittbrodt, 1991). Reduction of Cr(VI) is widespread and found in *Escherichia coli*, *Pseudomonas* species, *S. oneidensis* and *Aeromonas* species, *Bacillus* species, *Streptomyces* species, etc. (Wang, 2000). Cr(VI)-reduction was demonstrated in membrane fractions of the dissimilatory Fe(III)-reducer *S. oneidensis* (Myers *et al.*, 2000). The reduction of Cr(VI) by the sulfate-reducing bacteria *Desulfovibrio desulfuricans* and *Desulfovibrio vulgaris* was shown to be catalyzed by cytochrome c_3 (Lloyd *et al.*, 2001; Lovley and Phillips, 1994). The involvement of cytochromes in Cr(VI) reduction was also demonstrated in *Enterobacter cloacae* (cytochrome *c*) (Wang *et al.*, 1989b) and in *E. coli* (cytochrome b and d) (Shen and Wang, 1993). Cr(VI) reductase activity in *Pseudomonas putida* and *Pseudomonas ambigua* is located in the soluble fraction of the cell. Although anaerobic conditions are generally required to induce maximum reduction activity against Cr(VI), analyses suggested that the enzyme systems of *P. putida* and *P. ambigua* can operate under aerobic conditions, with NAD(P)H serving as the electron donor (Park *et al.*, 2000; Suzuki *et al.*, 1992). Cr(VI) is initially reduced to Cr(V), which is subsequently reduced to Cr(III), the non-toxic form.

In the case of As(V), it should be noted that reduction results in the more mobile and toxic As(III) and as a consequence, this process has not been proven useful for bioremediation. However, dissimilatory arsenic reduction could be used to extract insoluble As(V) from contaminated soil (Lloyd and Lovley, 2001). Several organisms capable of growing through dissimilatory reduction of As(V) have been isolated, e.g. the dissimilatory Fe(III)-reducing bacterium *Sulfurospirillum barnesii* (Stolz *et al.*, 1997) and its close relative *Sulfurospirillum arsenophilum* (Stolz *et al.*, 1999), and *Chrysiogenes arsenatis*, of which a periplasmic arsenate reductase could be purified and characterized (Krafft and Macy, 1998; Macy *et al.*, 1996). The sulfate-reducing bacterium *Desulfosporosinus auripigmentum* reduces As(V) followed by sulfate, resulting in the formation of As_2S_3-precipitates (orpigment)

as an indirect resistance mechanism (Newman *et al.*, 1998). Recently, a bacterium was enriched from anoxic bottom water from Mono Lake, California, that could achieve growth with sulfide and arsenate (Hoeft *et al.*, 2004). In other microorganisms, the reduction of As(V) to As(III) is preceding the actual arsenic-resistance mechanism. This is the case for the *ars*-operon encoded resistance mechanism, where the As(V) reduction step is catalyzed by the ArsC reductase and precedes efflux of As(III) from the cell (Mukhopadhyay *et al.*, 2002). The reduction of As(V) to As(III) is necessary to avoid re-entry of As via the phosphate uptake system, which interacts with H_3AsO_4, mistaking it for H_3PO_4.

The dissimilatory Fe(III)-reducing bacteria *G. metallireducens* and *S .oneidensis* can conserve energy for anaerobic growth via uranium reduction. This reduction of the relatively mobile U(VI) to U(IV), which readily precipitates as the insoluble mineral uraninite, may be used to remove uranium from contaminated groundwater and soils (Gorby and Lovley, 1992). Other bacteria including a *Clostridium* sp. (Francis, 1994) and the sulfate- reducing bacteria *D. desulfuricans* (Lovley *et al.*, 1993b) and *D. vulgaris* (Lovley and Phillips, 1994) are also able to reduce U(VI) but no bacterial growth accompanied the reduction process. In *D. vulgaris*, hydrogenase transports electrons to cytochrome c_3, which functions as U(VI) reductase (Lovley and Phillips, 1994). Cytochrome c_3 is also involved in hydrogen- dependent U(VI) reduction in *D. desulfuricans*, but in combination with organic electron donors, additional pathways were suggested that bypass the cytochrome (Payne *et al.*, 2002).

In addition to the enzymatically direct reduction pathway, sulfate reducing bacteria are also able to reduce metals indirectly. This process is an outcome of their metabolism: during sulfate respiration, hydrogen sulfide is produced which can reduce soluble toxic metals, often to less toxic or less soluble forms (Tebo, 1995).

1.2.4. Intra- and extracellular complexation, sequestration and precipitation

The intrinsic properties of the bacterial cell including those related to the cell wall structure, the production of extracellular polymeric substances such as exopolysaccharides, and binding or precipitation of metals inside or outside the cell (Gadd, 1992) provide alternative possibilities to exclude toxic heavy metals from the cellular metabolism of microorganisms. These mechanisms also provide means to avoid reentry of toxic heavy metals into the cells after their active export by resistance mechanisms.

Extracellular sequestration is one strategy for bacteria to protect metal- sensitive, essential cellular components against metal toxicity and involves alterations in the cell wall, membrane or envelope of the microorganism (Bruins *et al.*, 2000). Extracellular sequestration of a wide range of cations was observed for *R. metallidurans* CH34. By cultures of this strain, zinc, cadmium, cobalt and nickel removal resulted in the immobilization of the metal cations as complexes with carbonates, bicarbonates and hydroxides. These processes are induced by carbon dioxide emitted by the metabolic activity of the cells as well as the alkalization of the extracellular environment resulting from the activity of the CBA transporters involved in the efflux of these metal cations. The metal complexes were shown to precipitate in the form of carbonate crystals at the level of the cell surface (Collard *et al.*, 1994; Diels *et al.*, 1993a; Diels *et al.*, 1993b; Taghavi *et al.*, 1997b). This extracellular sequestration of heavy metal cations at the cell surface is considered to be an important post-efflux mechanism which prevents re- entry of the heavy metals into the cell, particularly when the extracellular concentrations are high. It is estimated that extracellular polysaccharides and outer membrane proteins have important post-efflux functions as well (Diels *et al.*, 1995).

The storage of excess copper in the periplasmic space was also reported. Some strains of *E. coli* can survive in copper-rich environments that would normally overwhelm the chromosomally encoded copper homeostatic systems. Such strains

possess additional plasmid-encoded genes that confer copper resistance (Rensing and Grass, 2003). Copper-resistance operons have been characterized from *R. metallidurans* (Mergeay *et al.*, 2003), *Pseudomonas* sp. (Cooksey, 1994), *Xanthomonas* sp. (Lee *et al.*, 1994), and *E. coli* (Brown *et al.*, 1995). The genes from these copper-resistant determinants are highly homologous and probably have similar functions. Rensing and Grass (2003) proposed a mechanism for such plasmid-encoded copper detoxification, based on the Pco-system in *E. coli*. Recently, a plasmid-encoded copper resistance operon from *Lactococcus lactis*, *lco*, was described and represents a new combination of structural genes for copper resistance, which has not been identified in other bacteria (Liu *et al.*, 2002).

Pb(II)-resistant strains of *Citrobacter freundii* and *Staphylococcus aureus* have also been isolated that accumulated lead as an intracellular lead phosphate, though the molecular mechanism of detoxification remains to be elucidated (Levinson and Mahler, 1998). The lead-resistance operon in *R. metallidurans*, in addition to a lead efflux system, encodes for the PbrD-protein that may function as a chaperone for Pb(II) and might be involved in cytoplasmic Pb(II) sequestration (Borremans *et al.*, 2001). A second gene of this operon, *prbB*, seems to play a key role in the extracellular sequestration of Pb, probably as lead phosphates.

Some bacteria possess metal binding components that are involved in intracellular complexation processes. Such intracellular sequestration is defined as the accumulation of metals within the cytoplasm to prevent exposure to essential cellular components. Metals commonly sequestered are Cd^{2+}, Cu^{2+}, and Zn^{2+}. Recently, a strategy for tolerance to metals in prokaryotes was described which involves the production of metallothioneins (MTs), a family of cysteine-rich proteins that bind and sequester metal ions (Cavet *et al.*, 2003). MTs are thought to constitute the main mechanism by which eukaryotic cells regulate intracellular metal ion concentrations (Nordberg, 1998). Functionally homologous MTs are found in several bacteria (Robinson *et al.*, 2001), but SmtA of the cyanobacterium *Synechococcus*, which confers resistance to Zn^{2+} and Cd^{2+}, is the

best characterized (Blindauer *et al.*, 2001). The expression of *smtA* is induced in response to elevated concentrations of zinc or cadmium via the action of SmtB, a zinc- responsive negative regulator of *smtA* transcription (Huckle *et al.*, 1993). Recently, a metallothionein (BmtA) was identified and characterized from the cyanobacterium *Oscillatoria brevis* (Liu *et al.*, 2003). *Pseudomonas putida* also produces a cysteine-rich protein, which may be related to metallothioneins and confers to intracellular Cd^{2+} sequestration (Trevors *et al.*, 1986). However, the production of MTs as the main mechanism of heavy metal-tolerance is rather exceptional amongst bacteria.

Precipitation of metals as metal sulfides or phosphates is an alternative way of increasing the resistance of microorganisms to metals. Sulfate-reducing bacteria (SRB) couple the oxidation of organic compounds or molecular H_2 with the reduction of sulfate as an external electron acceptor under anaerobic conditions, a process known as dissimilatory sulfate reduction (Barton and Tomei, 1995). The end product of this reaction is hydrogen sulfide. When heavy metals are present in different environments above a certain threshold, they can be toxic to SRB and thus restrict SRB growth and sulfide production. However, once sulfide is formed, it readily reacts with the heavy metals to form metal sulfide (MeS) precipitates, resulting in a lower sulfide concentration and a reduced metal toxicity. These MeS precipitates exhibit extremely low solubility and are relatively stable in environments under low redox conditions (Hao, 2000). Moreover, hydrogen sulfide can reduce soluble toxic metals, often to less toxic or less soluble forms (Tebo, 1995). However, Bridge and coworkers (Bridge *et al.*, 1999) demonstrated that the sulfate-reducing bacterium *Desulfococcus multivorans* possess extracellular metal-binding capacity that is unrelated to sulfide- production. In addition, cells of *Desulfovibrio desulfuricans*, *Desulfovibrio vulgaris* and *Desulfovibrio* sp. 'Oz-7' were able to reduce Pd(II) to Pd(0) using hydrogen or formate as electron donor, with biocrystallization of Pd(0) on the cell surface. In this way, Pd(0) which is widely used as industrial reduction catalyst to trap H_2 as

H•, was recovered and recycled (Baxter- Plant *et al.*, 2003). The biologically deposited Pd(0) has also been demonstrated as a novel bioinorganic catalysts for the reduction of Cr(VI) (Mabbett *et al.*, 2004).

Phosphate, which is an essential nutrient, is generally not released in large quantities by living organisms (Lloyd and Lovley, 2001). However, *Citrobacter* sp. was demonstrated to couple biologically liberated phosphate to the formation of metal phosphate biominerals. In *Citrobacter* sp., a periplasmic acid-phosphatase (named PhoN) is associated with the outer membrane and exocellular lipopolysaccharide (LPS) and catalyzes cleavage of phosphate from glycerol 2-phosphate, resulting in the accumulation of high concentrations of phosphate at the cell surface (Lovley, 1993). Mineral formation with the uranyl ion (UO_2^{2+}) was initiated by nucleation at the phosphate groups of the LPS, with crystal growth driven by enzymatically generated phosphate. As a result, cells precipitated large quantities of uranium and cadmium phosphates.

1.3. Methods for studying microbial community composition and activity

Remediation technologies using microorganisms are feasible alternatives to cleaning of soils or concentrating heavy metals in polluted waters by physical or chemical means. Significant progress has been made in understanding the roles of microorganisms in mineral cycling and in the application of these processes to the bioremediation of metals and radionuclides (Lloyd, 2003). Imminent developments in the field include the further genetic improvement of strains and the adaptation of existing methodologies to large-scale and *in situ* decontamination processes. For the technical improvement of bacterial processes such as immobilization or biosorption of heavy metals, it is clear that the processes taking place in the cell microenvironment need to be better understood if the mineralization process needs to be improved (Valls and de Lorenzo, 2002). For example, the development and optimization of an efficient immobilization process for heavy metal as metal sulfides, which is mediated by sulfate-reducing

bacteria, implies an insight in the SRB community composition and activity, and in the way this community is affected by different operational parameters such as supply of nutrients, COD/ SO_4^{2-} ratio, the presence of alternative electron acceptors, and heavy metal-concentration. Moreover, the presence of non-sulfate reducers should be taken into account, e.g. methanogens, acetogens, fermenting bacteria, since these bacterial groups might influence the sulfate reduction process by competition or synthrophy (Colleran *et al.*, 1995; Laanbroek and Veldkamp, 1982; Oude-Elferink *et al.*, 1994). During the treatment process, one should be able to demonstrate and follow up the presence and activity of the metal transforming consortium, this in order to optimize the process conditions. Microbial and molecular tools have been developed that aim to reveal and understand the relationships between structural and functional diversity in such microbial ecosystems, and to monitor bacterial activity during bioremedial treatment processes (Plaza *et al.*, 2001). Such tools can be used for the follow-up of specific groups of bacteria, or to determine the microbial community composition. Here, as an example, we will focus on techniques used for the study of SRB-populations. Due to their great economic, environmental and biotechnological importance, populations of sulfate-reducing bacteria have been intensively studied during the last decades. Several methods exist to characterize and quantify SRB-populations.

1.3.1. Non-molecular identification and characterization

Before molecular techniques became available to the microbiologist, microbial identification methods were solely based on the isolation, cultivation, metabolic characteristics and morphology of strains. Culture methods for enumeration of SRB in the environment were generally based on most- probable- number (MPN) techniques (Beliaeff and Mary, 1993) with enumeration media that usually contain lactate as the main carbon and energy source and that indicate the presence of sulfate reducers by the formation of a black precipitate of ferrous

sulfide (FeS) (Jain, 1995; Postgate, 1984; Tanner, 1989). Although important for ecological studies, these techniques are of limited usefulness for quantification and characterization of environmental populations, as it is now well recognized that most strains do not grow in vitro, either because cultivation media poorly resemble natural growth conditions or because different strains of microorganisms are interdependent (Amann *et al.*, 1995; Gibson *et al.*, 1987; Ward *et al.*, 1990). Recently, a radiotracer MPN-technique (T-MPN) with natural media was developed which greatly improved the enumeration of sulfate reducers in environmental samples (Brandt *et al.*, 2001; Vester and Ingvorsen, 1998).
Microscope techniques, e.g. scanning electron microscopy (SEM), phase contrast microscopy, confocal laser scanning microscopy (CLSM), can be useful for a rough characterization of biofilms and sludge (Labrenz *et al.*, 2000; Silva *et al.*, 2002). Nevertheless, complementary characterization studies are essential because this way of identifying microbes is based on cell morphology only, which is for most sulfate reducing bacteria not very distinctive (Castro *et al.*, 2000).

In order to avoid problems associated with traditional microbiological techniques, culture- independent methods for detection and enumeration of bacterial species, including the sulfate reducers, were developed. Immunodetection methods such as an immunoassay for measuring the enzyme adenosine-5'-phosposulfate (APS) reductase (EC 1.8.99.2) (Odom *et al.*, 1991), which catalyzes the reduction of APS to sulfite and AMP (adenosine- monophosphate), a key step in the reduction of sulfate to sulfite by all SRB, are promising. Also, SRB-specific antibodies were successfully used for the detection of SRB in oil field waters and coastal sediments (Christensen *et al.*, 1992; Lillebaek, 1995). Because the production of antibodies requires the availability of a pure culture of microorganisms, these methods can't detect microorganisms that have not yet been obtained in pure culture (Raskin *et al.*, 1995) and which display a different immunological behavior. Furthermore, one should be aware that antibodies can cross-react with other non-related strains (Smith, 1982). However, in the last few years, very

efficient techniques have been developed to build and design large libraries of antibody fragments, such as phage display libraries, and ingenious selection procedures have been established to derive antibodies with the desired characteristics (Benhar, 2001; Hoogenboom *et al.*, 1998).

Another culture-independent approach is the analysis of phospholipid ester linked fatty acids, known as (PL) FAME- patterns or PLFA- profiles. A large database of PLFA profiles has been developed from pure culture studies (Dowling *et al.*, 1986; Kohring *et al.*, 1994; Taylor and Parkes, 1983; Vainshtein *et al.*, 1992). Although this database has been successfully used to identify and quantify SRB in sediments (Kleikemper *et al.*, 2002b; Taylor and Parkes, 1985) and granular sludge (Kaksonen *et al.*, 2003; Oude- Elferink *et al.*, 1998), it should be taken into account that yet undiscovered SRB-species and -genera may inhabit anaerobic environments which might impede an accurate insight in their community structure if only PLFA is used.

1.3.2. Molecular-biological identification and characterization methods

To describe the composition and activity of microbial communities, over the last decade, important advances in molecular biology led to the development of culture-independent molecular ecological methods, based on the analysis of DNA and RNA. Hybridization techniques and Polymerase Chain Reaction (PCR)-fragment cloning, followed by restriction and/ or sequencing analysis, enable assessment of the diversity of the microbial community in terms of the number of different species and, to a lesser extent, the relative abundance of these species. Genetic fingerprinting techniques provide a global picture of the genetic structure of the bacterial community (Ranjard *et al.*, 2000). However, none of these techniques is all- encompassing or perfect (Muyzer, 1998). Problems and biases occur due to the PCR- amplification step (i.e. choice of primers, inhibition of the enzyme by humic compounds, formation of chimeric PCR products, preferential amplification) (Winzingerode *et al.*, 1997). In addition, the

individual techniques have their specific limitations. Despite these problems and limitations, a combination of these techniques can reveal a great deal about the microbial community (Torsvik and Ovreas, 2002). Nowadays, these molecular tools are routinely used for studying the diversity and distribution of individual bacterial species, including SRB, in complex microbial communities such as sediments (Dhillon *et al.*, 2003; Joulian *et al.*, 2001; Kleikemper *et al.*, 2002a; Orphan *et al.*, 2001), granular sludge (Kaksonen *et al.*, 2003; Santegoedts *et al.*, 1999), biofilms (Santegoedts *et al.*, 1998) and microbial mats (Nakagawa *et al.*, 2002; Teske *et al.*, 1998). Molecular studies of bacterial populations using a single gene can be based on either core housekeeping genes or on functional genes. Analyzing the microbial community genome, the "metagenome", will result in huge amounts of genomic, evolutionary and functional information which can stimulate further biotechnological research (Lorenz and Schleper, 2002; Rodriguez- Valera, 2004; Torsvik and Ovreas, 2002). By applying molecular phylogenetic analysis, DNA microarrays, functional genomics and *in situ* activity measurements, the metabolic potential and activity of microbial communities can be revealed, as well as the interactions that occur within them.

1.3.2.1. Analyses based on a single gene

Molecular methods based on the sequence of PCR- amplified genes, especially 16S rRNA genes, became very important to reveal the genetic and functional diversity among microbial communities. These methods include PCR fragment cloning, followed by restriction and/ or sequence analysis, genetic fingerprinting techniques such as Denaturing Gradient Gel Electrophoresis (DGGE), Single-Strand- Conformation Polymorphism (SSCP), Restriction Fragment Length Polymorphism (RFLP), and hybridization techniques such as slot-blot hybridization and Fluorescence In Situ Hybridization (FISH).

Cloning and sequencing of PCR- amplified fragments is frequently used to assess community diversity in terms of the number of different species and, to a lesser extent, their relative abundance. The cloning step allows separation of the amplified sequences so that they can be characterized individually using RFLP and/ or sequencing-analysis. Sequencing allows the identification of uncultured bacteria as well as estimation of their relatedness to known culturable species (Ranjard *et al.*, 2000) by comparing the clone sequences to sequences in the GenBank database using the BLAST program (Basic Local Alignment Search Tool, http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul *et al.*, 1990) of the Ribosomal Database Project (Maidak *et al.*, 1994). Although successful in the exploitation of microbial diversity, the cloning approach is neither suitable for studying many different microbial communities simultaneously, nor for quantifying the species abundance and spatial distribution or behavior of microbial communities over time; the technique is time-consuming, labor-intensive and thus impractical for the high-throughput analysis of multiple samples (Muyzer, 1998). Moreover, these methods are biased due to the PCR and the cloning strategy used, causing an inaccurate image of the diversity of the bacterial populations (Rayney *et al.*, 1994; Winzingerode *et al.*, 1997).

Genetic fingerprinting techniques are also based on PCR and thus suffer from the same biases, but because they are less time-consuming and relatively easier to perform they allow the simultaneous comparison of bacterial communities (Ranjard *et al.*, 2000). Genetic fingerprinting is based on the principle of resolving the diversity of the amplified sequences simply by differential electrophoretic migration on agarose or polyacrylamide gels, which depend on their size or sequence.

Denaturing Gradient Gel Electrophoresis (DGGE), which is routinely used in many laboratories for the characterization of microbial communities, especially between different populations, is an example of a fingerprinting technique. Mixtures of PCR products are separated in polyacrylamide gels containing a linear gradient of

DNA denaturants (urea and formamide). Sequence variation among the different DNA molecules, such as their G/C content, influences their melting behavior, and therefore molecules with different sequences will stop migrating at different positions in the gel (Muyzer *et al.*, 1993). Another technique based on the same principle is Temperature Gradient Gel Electrophoresis (TGGE) (Riesner *et al.*, 1991). The DGGE- TGGE approach can be used for studying the diversity, dynamics, and gene expression of microbial communities (Muyzer, 1998). Furthermore, individual bands may be excised, reamplified, and sequenced for the taxonomic identification of the bacterial species within a community (Ranjard *et al.*, 2000). This is especially useful when analyzing changes in microbial community composition, e.g. due to changes in environmental parameters. One of the limitations of the technique is the separation of only relatively small fragments, up to 500 base pairs, which restricts the information for phylogenetic identification well as for probe or primer design. Another problem is the misinterpretation of fingerprints, e.g. when degenerated PCR-primers are used in the preceeding PCR amplification step, double bands may appear in the DNA pattern. Furthermore, the formation of heteroduplex molecules during PCR might contribute to interpretation difficulties (Ferris and Ward, 1997). Also, some bacteria have multiple operons with sequence heterogeneity for genes such as the 16S rRNA gene, which may lead to overestimation of the number of bacteria (Nübel *et al.*, 1996). Finally, some studies demonstrated that fragments of different sequences might migrate at the same position (Buchholz- Cleven *et al.*, 1997; Vallaeys *et al.*, 1997).

Another fingerprinting technique for studying microbial diversity is Single-Strand-Conformation Polymorphism (SSCP). Denatured PCR products are separated on a non-denaturing gel, based on differences in the folding conformation of single-stranded DNA. In order to simplify the SSCP pattern, one of the PCR primers is fluorescently labeled or biotinylated. SSCP electrophoresis can be used to reveal the presence of bacterial species in microbial populations, and is also used to study population activity and dynamics (Delbès *et al.*, 2001; Muyzer,

1998). One limitation of the method is that only very short (ca. 150 bp) can be optimally separated.

Restriction Fragment Length Polymorphism (RFLP), otherwise known as Amplified Ribosomal DNA Restriction Analysis (ARDRA), and Terminal-RFLP (T-RFLP) are also frequently used for the characterization of microbial communities. RFLP methods involve the digestion of PCR-amplified genes by restriction enzymes, followed by separation of the restricted fragments on agarose or polyacrylamide gels (Ranjard *et al.*, 2000). A problem for estimating the microbial diversity by using ARDRA is that the number of fragments is not related to the number of different amplified DNA fragments, nor to the number of community members, but by the distribution of the restriction sites for the fragmenting enzymes within the amplified fragment. T-RFLP solves this problem: the use of fluorescently end-labeled PCR primers makes it possible to detect only the terminal fragments, and as a consequence the obtained profiles are simpler in terms of the number of bands. A databank has been produced of 16S rRNA T-RFLP patterns of a large set of bacterial species, which makes it possible to identify the bands present in a community by a profile to profile comparison (Marsh, 1999). However, this technique is not very suitable when analyzing microbial communities which consist of many different species.

One of the techniques for quantifying the abundance of a particular species in a bacterial population is slot-blot hybridization. In this approach, community DNA is spotted on nylon membranes and hybridized with labeled group- or species-specific probes. The relative abundance of the taxon can be estimated by comparing the signal obtained with the specific hybridization probe to that of a universal probe (Muyzer, 1998). A derived method is *in situ* hybridization or whole- cell hybridization, generally using fluorescent labeled probes (FISH). Because whole cells are hybridized, artifacts arising from biases in DNA-extraction, PCR amplification and cloning are avoided (Hill *et al.*, 2000). To be detected, soil microbes must be metabolically active and possess cell walls

sufficiently permeable to allow penetration of the probe. Progress is being made to overcome problems associated with penetration of probes in cells (Macnaughton *et al.*, 1994). FISH provides a more accurate quantification of cells as compared to slot- blot assays (Amann *et al.*, 1995). However, they both have the limitation that only bacteria for which probes exist can be studied (Muyzer, 1998), and sample autofluorescence is a major problem that can generate quantification artifacts (Amann and Ludwig, 2000).

Recently, two quantitative techniques have been developed based on the amplification of specific microbial sequences by means of PCR, namely competitive PCR (cPCR) and real-time PCR. In the cPCR-approach, a fixed amount of a competitor DNA, which is amplified by the same primer set as the target DNA, is added in a known concentration to a serial dilution of a template DNA of unknown concentration (Wang *et al.*, 1989a). The target is distinguished from the competitor in post- PCR analysis, either by a difference in size or by the presence of a unique restriction site; its concentration is determined by comparison of the intensity of a stained amplicon (Becker *et al.*, 2000). This technique has the advantage that contaminants co- extracted with nucleic acids (e.g. humic acids, organic contaminants, metals, chelators...) have similar effects on the amplification of the target and the competitor sequences (Berthe *et al.*, 1999). However, quantitative competitive PCR requires time- and resource-consuming post- PCR analyses. Real-time PCR is an alternative, single-step quantification technique, in which *Taq* polymerase activity or the accumulation of the amplicon is directly monitored during or after the PCR. Accumulation of PCR products can be monitored either by using a fluorescent dye, such as SYBRGreen™ (Higuchi *et al.*, 1992; Wittwer *et al.*, 1997), or by TaqMan probe hydrolysis. The latter approach makes use of the 5'→3' exonuclease activity of *Taq* DNA polymerase for the cleavage of an oligonucleotide, the TaqMan probe, that hybridizes to the PCR template (Holland *et al.*, 1991). This probe is fluorescently labeled at both ends (Lee *et al.*, 1993). The fluorescent dye at the 5' end serves as a reporter, and its fluorescence is quenched by the dye at the 3' end of the probe. During the

elongation step of each PCR cycle, the *Taq* DNA polymerase hydrolyzes the annealed probe with its 5′ nuclease activity, thereby releasing the reporter's fluorescence. The fluorescence increases logarithmically as the PCR-reaction proceeds, until a reagent becomes limiting. Quantification is based on predefined threshold fluorescence: the higher the amount of initial template DNA, the earlier the fluorescence will cross this threshold. Copy number of the initial target DNA is determined by comparing the threshold cycle $C\tau$ with a standard curve (Gruntzig *et al.*, 2001). Real-time PCR quantification is highly specific because in addition to requiring two consensus sequences for the design of a PCR primer set, it requires a specific conserved internal sequence for the specific hybridization of the internal labeled probe. However, fluorescence quenching and autofluorescence can significantly affect TaqMan PCR enumeration accuracy (Stults *et al.*, 2001).

Nucleic acid microarrays, or DNA chips are a recent advance in molecular technology. They provide a powerful tool for multiplexed detection of nucleic acids and thus offer a tremendous potential for microbial community analysis and process monitoring in both basic and applied environmental science. DNA microarrays also demonstrate the potential to directly investigate microbial community dynamics and metabolic activity in environmental samples, even without using PCR (Small *et al.*, 2001). Various microarray formats have been developed for bacterial detection and microbial community analysis of environmental samples, such as phylogenetic oligonucleotide arrays, functional gene arrays, community genome arrays, and whole- genome open reading frame arrays, and were recently discussed in detail by Zhou (2003). Common approaches for microarray fabrication and analyses include cDNA and oligonucleotide probes affixed on planar, channel glass, gel element, and microbial surfaces. The targets are fluorescent or radioactive- labeled total mRNA, DNA derived from mRNA by reverse transcription (RT)-PCR, or genomic DNA (Ball and Trevors, 2002). The most common method for introducing the microarray detection label includes PCR

or (RT)-PCR amplification of a target sequence with a labeled primer or by chemical labeling. After hybridization and washing, the microarray is scanned and the hybridization patterns can be qualitatively and quantitatively analyzed (Cho and Tiedje, 2002).

a) Phylogenetic identifying genes

The development of techniques to analyze nucleic acids made it possible to study the microbial diversity at the genetic level. Microorganisms are detected, identified and enumerated by the analysis of genes. The 16S ribosomal RNA gene is an excellent molecular marker for this purpose, because (i) it is present in all prokaryotes, (ii) the nucleotide sequence of 16S rRNA- genes contain highly conserved as well as highly variable regions, which makes it possible to design general and specific primers and probes, (iii) it has sufficient sequence information for phylogenetic inference, and (iv) it is an important cellular compound, which facilitates detection (Muyzer, 1998). The application of molecular biological techniques to detect and identify microorganisms by their 16S rRNA or its encoding gene is well established and is widely used to determine the microbial diversity and to analyze the structure of microbial communities. Depending on the objectives of the analysis, the molecular methods can be applied to the community's DNA or either its RNA. Whereas rDNA only reflects the presence of organisms, the amount of rRNA per cell is roughly proportional to its metabolic activity (Wagner, 1994). Thus, in order to demonstrate the presence of specific bacterial groups and to analyze and follow up the composition of bacterial populations as well as their spatial and temporal changes, these molecular methods are applied to the community's DNA, whereas the bacterial activity will be studied using the community's RNA.

In order to obtain insight in a specific group of bacteria, such as SRB, the 16S rRNA-gene based molecular methods can be refined by using group-, genus-, or

strain specific hybridization probes or PCR- primers (Amann *et al.*, 1992; Daly *et al.*, 2000; Devereux *et al.*, 1992; Fukui *et al.*, 1999; Hines *et al.*, 1999; Hristova *et al.*, 2000; Loy *et al.*, 2002; Manz *et al.*, 1998; Ravenschlag *et al.*, 2000; Stubner, 2002). Loy & Wagner plus coworkers successfully developed and used a microarray consisting of of 132 16S rRNA gene-targeted oligonucleotide probes covering all recognized lineages of sulfate reducing prokaryotes (SRP) for high-resolution screening of clinical and environmental samples (Loy *et al.*, 2002). The microarray, named SRP-PhyloChip, has great potential for rapid screening of SRP diversity in complex samples and microarray SRP diversity fingerprints allow identification of relevant probes for further characterization of a sample by PCR or quantitative hybridization. This is a valuable option if large numbers of samples are to be analyzed for temporal or spatial variations in SRP diversity. Recently, real-time PCR assays were developed for the quantitative detection of the 16S rDNA of Gram- positive sulfate-reducing bacteria (Stubner, 2002), of the "main" SRB- groups *Desulfovibrionaceae*, *Desulfobacteraceae* and *Desulfobulbus* sp. and of subgroups within the first two main groups (Stubner, 2004).

Serial analysis of ribosomal sequence tags (SARST) is a novel, powerful approach for high-throughput profiling of microbial diversity in both medical, industrial or environmental samples (Neufeld *et al.*, 2004). Ribosomal sequence tags (RSTs) from bacterial small subunit rRNA gene V1-regions were amplified and ligated into concatamers in a series of enzymatic reactions. Subsequently, concatamers are cloned and sequenced, resulting in up to 20 RSTs per sequencing reaction, hence offering a significant increase in throughput over traditional rDNA clone libraries. The SARST procedure is based on SAGE and on the approach to generate genomic sequence tags (GSTs) from bacterial genomic DNA (Dunn *et al.*, 2002). The protocol was optimized as such to minimize PCR and cloning biases. RST libraries were generated from a defined mixture of pure cultures and from duplicate arctic soil DNA samples. The results showed that SARST is functional, reproducible and that the distribution of RSTs in the defined community library

reflects the composition of the original sample. Specific primers could be designed based on sequence data from abundant soil RSTs for further phylogenetic analysis. However, the distribution of sequences in RST libraries is biased by multiple *rrn* operons and similarities between 16S rDNA sequences of different species, thereby frustrating accurate quantitative inferences. Nevertheless, the SARST approach is accurate enough to reflect population trends and make comparisons between samples or treatments based on increase or decrease in specific sequence representation.

Molecular identification methods based on the comparison of the variable regions in the 16S rRNA gene are considered as one of the most accurate. However, in some cases, 16S ribosomal DNA sequences are not suitable for identification and differentiation between bacterial strains of the same genus because of high similarity percentages. For example, the 16S rDNA sequences of *Lactobacillus plantarum* and *Lactobacillus pentosus* share an identity value of >99% (Collins *et al.*, 1991; Quere *et al.*, 1997), and within the *Mycobacterium* genus, the interspecies percent similarity of the 16S rRNA gene sequences of *M. kansasii* and *M. gastri* is 100% (Rogall *et al.*, 1990). Also, genetic relationships within a genus may be disturbed by recombination events, and in order to reflect the evolution of the genome as a whole, it is necessary to complete the interpretation of single- gene phylogenies, such as those based on 16S rRNA, by studying more loci (Gaunt *et al.*, 2001). The *recA* gene, among others, has been proposed to be used as a phylogenetic marker. RecA is a small protein which takes part homologous DNA recombination, SOS induction, and DNA damage- induced mutagenesis. Due to its fundamental role, the *recA* gene is ubiquitous. Some regions of RecA are conserved between species and other regions are highly variable, thus allowing comparison between both close and distant relatives, and large- scale phylogenies of *recA* in bacteria are consistent with the corresponding rRNA phylogenies. These criteria make it a useful marker for phylogenetic analyses (Eisen, 1995; Lloyd and Sharp, 1993). The *recA* gene was successfully

used as an alternative sequencing target in several studies to complement identification methods based on 16S rRNA gene sequencing (Blackwood *et al.*, 2000; Gaunt *et al.*, 2001; Torriani *et al.*, 2001).

b) Functional genes

Although the 16S rRNA approach provides the most general framework for studies of SRB-communities in the environment and has the potential to reveal interactions between sulfate reducers and other microorganisms such as methanogens or acetogens (Oude- Elferink *et al.*, 1998), it has its limitations. Retrieved sequences frequently are related to uncultivated organisms, which makes it impossible to unambiguously predict the physiology or metabolic capabilities of the organism from which the 16S rRNA-gene was derived. This concerns in particular sulfate reducers: indeed, sulfate reducing bacteria constitute a paraphyletic group with members among the *δ- proteobacteria*, gram-positive bacteria and even *Archaea* (Castro *et al.*, 2000) and some lineages of sulfate reducers are closely related to organisms that are unable to carry out dissimilatory sulfate reduction. Thus, novel lineages of sulfate reducers cannot be identified by their 16S rRNA sequence alone. Therefore, in addition to 16S rRNA-gene based community analysis, the functional gene approach has been used to identify bacteria responsible for specific biogeochemical processes in the environment. This means that physiology is inferred from functional gene sequences coding for enzymes that are essential to the target organism (Dhillon *et al.*, 2003; Joulian *et al.*, 2001; Minz *et al.*, 1999).

First attempts to study SRB-populations via a functional gene approach were based on hydrogenase-genes in *Desulfovibrio* species. Hydrogenases play an important role in the hydrogen metabolism of SRB (Voordouw, 1990). Distribution analysis of three types of hydrogenases among 22 members of the genus *Desulfovibrio* revealed that a [NiFe] hydrogenase was present in all *Desulfovibrio* species, while the genes for the [Fe] hydrogenase and [NiFeSe] hydrogenase had

a more limited distribution (Voordouw *et al.*, 1990). The [NiFe] hydrogenase is also thought to be essential in dissimilatory metal reduction by SRB (Lovley *et al.*, 1993a). In order to understand the importance of metal reduction by SRB and to reveal the genetic diversity of *Desulfovibrio* species in environmental samples, PCR primers were designed for amplification of ~0.45 kb fragment of the [NiFe] hydrogenase genes of *Desulfovibrio* species and used in combination with DGGE (Wawer and Muyzer, 1995). In this way, the number of different *Desulfovibrio* species could be determined. Later, these primers were used for direct assessment of *Desulfovibrio* species in groundwater of an uranium mill tailing site (Chang *et al.*, 2001).

The best studied example for analysis of SRB communities using the functional gene approach is the use of the *dsr*-gene, which encodes for the dissimilatory sulfite reductase (DSR, EC 1.8.99.1). This enzyme catalyzes the final step on sulfate respiration, the reduction of sulfite to sulfide, and hence is required by all sulfate reducers. Consequently, this enzyme has been found in all dissimilatory sulfate-reducing prokaryotes investigated so far (Klein *et al.*, 2001). Because of the remarkable conservation of the *dsr*-sequence, a general PCR primer set dsr-1F/ dsr-4R, could be developed which allowed PCR- amplification of a 1,9 kb DNA fragment encoding most of the α and β subunits of the *dsrAB*-genes from all recognized lineages of sulfate reducing prokaryotes (Karkhoff- Schweizer *et al.*, 1995; Wagner *et al.*, 1998). Recent studies of the environmental diversity of uncultured SRB populations indicate that sequence analysis of *dsr* genes is effective for the detection of SRB within a complicated microbial community structure such as sediments (Dhillon *et al.*, 2003; Joulian *et al.*, 2001; Thomsen *et al.*, 2001), microbial mats (Minz *et al.*, 1999; Nakagawa *et al.*, 2002), on the back of deep- sea worms (Cotrell and Cary, 1999), uranium tailing sites (Chang *et al.*, 2001) and hydrocarbon- degrading consortia (Pérez- Jiménez *et al.*, 2001). Moreover, sequence analysis of PCR-amplified environmental *dsr* genes often demonstrate the presence of unknown sulfate- reducing prokaryotes that could not be identified by 16S rRNA-sequencing analysis (Dhillon *et al.*, 2003; Joulian

et al., 2001; Minz *et al.*, 1999; Nakagawa *et al.*, 2002; Thomsen *et al.*, 2001). Recently, a competitive PCR method was developed based on the amplification of the *dsrAB* genes for assessing the abundance of sulfate-reducing microorganisms in estuarine sediments (Leloup *et al.*, 2004). A 50-mer functional gene microarray (FGA), containing 50-base-pair probes for genes involved in nitrogen cycling (*nir*S, *nir*K, *nif*H, *amo*A and *pmo*A) and sulfate reduction (*dsr*A and *dsrB*), was tested on 2 µg of bulk community DNA from marine sediments (S. Tiquia *et al.*, unpublished data). Very strong hybridizations were obtained and resolution was at the species-level, indicating that the 50- mer FGA has potential for use as a specific, sensitive and possibly quantitative tool for characterizing the composition, structure, activity and dynamics of microbial communities in natural environments, in parallel with other molecular monitoring methods (Zhou, 2003).

Although the phylogenetic trees derived from the topology of *dsr* sequences of reference strains are overall congruent with those inferred from the 16S rRNA, there are some contradictions caused by multiple lateral gene transfer events of the *dsrAB* genes (Klein *et al.*, 2001; Wagner *et al.*, 1998). It was discovered that *dsrAB* genes of several *Desulfotomaculum* spp. and *Thermodesulfobacterium* spp. had been laterally transferred from unidentified ancestors of sulfate reducers within the *δ- proteobacteria* (Figure 1.2). Consequently, this complicates the interpretation of phylogenetic *dsr* trees in environmental diversity studies of SRB. The *dsr*- sequence of *Desulfobacula toluolica* was first thought to be a xenolog as well (Figure 1.2), but it was found that the sequence was incorrect and originated from a contamination (M. Wagner, personal communication).

In addition to the dissimilatory sulfite reductase, sulfate respiring prokaryotes possess the adenosine-5'-phosphosulfate (APS) reductase, which is highly conserved (Fritz *et al.*, 2000) and is generally found in sulfate reducing prokaryotes (Rabus *et al.*, 1999); therefore, it appears to be an ideal candidate for phylogenetic analysis (Hipp *et al.*, 1997). Recently, the diversity and distribution of SRB communities in gastrointestinal tracts were studied via PCR-amplification and DGGE of a ~0,4 kb fragment of the APS reductase subunit A

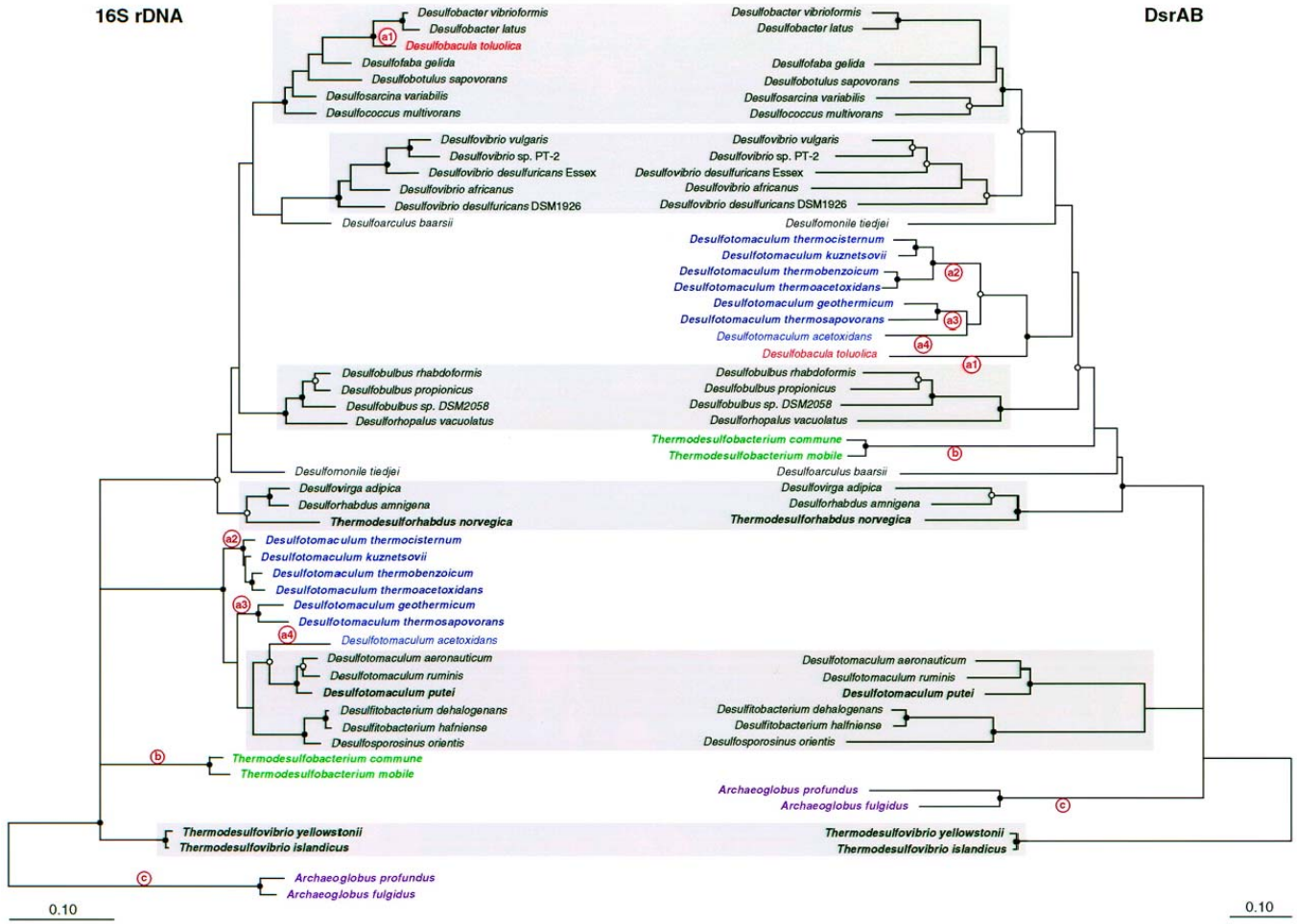
16S rDNA
DsrAB
Desulfobacter vibrioformis
Desulfobacter latus
Desulfobacula toluolica
Desulfofaba gelida
Desulfobotulus sapovorans
Desulfosarcina variabilis
Desulfococcus multivorans
Desulfovibrio vulgaris
Desulfovibrio sp. PT-2
Desulfovibrio desulfuricans Essex
Desulfovibrio africanus
Desulfovibrio desulfuricans DSM1926
Desulfoarculus baarsii
Desulfomonile tiedjei
Desulfotomaculum thermocisternum
Desulfotomaculum kuznetsovii
Desulfotomaculum thermobenzoicum
Desulfotomaculum thermoacetoxidans
Desulfotomaculum geothermicum
Desulfotomaculum thermosapovorans
Desulfotomaculum acetoxidans
Desulfobulbus rhabdoformis
Desulfobulbus propionicus
Desulfobulbus sp. DSM2058
Desulforhopalus vacuolatus
Thermodesulfobacterium commune
Thermodesulfobacterium mobile
Desulfovirga adipica
Desulforhabdus amnigena
Thermodesulforhabdus norvegica
Desulfotomaculum aeronauticum
Desulfotomaculum ruminis
Desulfotomaculum putei
Desulfitobacterium dehalogenans
Desulfitobacterium hafniense
Desulfosporosinus orientis
Archaeoglobus profundus
Archaeoglobus fulgidus
Thermodesulfovibrio yellowstonii
Thermodesulfovibrio islandicus
a1
a2
a3
a4
b
c
0.10
0.10

Figure 1.2 Comparison of 16S rRNA- and DsrAB phylogenetic trees of sulfate- and sulfite-reducing prokaryotes. Consistent monophyletic groups between both trees are shaded gray. *DsrAB* recipient or donor lineages are indicated by circled letters (a to c) located above or below the branching point, respectively (Klein *et al.*, 2001)**.**

(*apsA*) gene (Deplancke *et al.*, 2000). Up to date, no environmental SRB-population studies based on APS as a functional marker gene have been reported. This is probably due to the fact that a thorough phylogenetic framework of APS reductase from cultivated sulfate reducers has become available only recently. Nevertheless, a comparison of the *apsA*- and 16S rRNA-based phylogenetic trees revealed major differences in the relative branching order for *Thermodesulfovibrio islandicus* and *Thermodesulfobacterium* spp., *δ- proteobacteria* belonging to the "*Syntrophobacteraceae*", the "*Nitrospinaceae*", and the desulfobacteraceal *Desulfocella halophila*. These discrepancies in tree topologies can be explained by lateral transfer of *apsA*-genes across bacterial divisions. Hence, as is the case for the *dsr* gene as a genetic marker, environmental APS reductase gene sequences now can be at least linked to most of the recognized lineages of sulfate reducing prokaryotes but phylogenetic inferences in environmental diversity studies should be interpreted cautiously (Friedrich, 2002).

1.3.2.2.Community genome analysis

Bacterial population genetics and genomics make the value of information based on just one gene sequence questionable (Rodriguez- Valera, 2002). For example, molecular ecological surveys of soils frequently indicate the presence of sequences affiliating with *Acidobacteria*. The phylum *Acidobacteria* is comprised of three described species as well as some recently identified isolates, but the majority of this phylum consists of uncultivable species. Although 16S rRNA studies can provide insight into the huge extent of diversity found among the *Acidobacteria*, most often there is no way to functionally or physiologically characterize the corresponding microorganisms (Quaiser *et al.*, 2003). In addition, bacteria of the same species with virtually indistinguishable 16S rRNA

genes can display tremendous genomic variation (Béjà *et al.*, 2002; DeLong, 2002). Despite these shortcomings, the functional gene- approach will be further explored and exploited and will undoubtedly provide very interesting information. For functional gene analysis it is very important to keep in mind that protein coding genes are much less conserved at the DNA sequence level because of species-specific codon usage, which not only complicates the experimental work but also increases the risk of biasing or missing entirely important groups (Rodriguez- Valera, 2004). Thus, there remains a need for fast and accurate methods for the quantitative and qualitative assessment of environmental microbial diversity (Greene and Voordouw, 2003). Novel genome-based tools are rapidly being developed and significantly advance the current knowledge of genome content, diversity, population biology and evolution in microbial populations (DeLong, 2002). Cultivation-independent, environmental 'metagenomic' analyses has recently been used to improve the understanding of the ecological and biotechnological potential of microorganisms. The term 'metagenome' stands for the total chromosomal DNA that can be extracted from an environmental microbial community (Rondon *et al.*, 2000). To characterize the soil metagenome, DNA libraries were constructed by direct cloning of large genomic fragments from environmental samples into Cosmid, Fosmid or bacterial artificial chromosome (BAC) vectors (Ball and Trevors, 2002; DeLong, 2002). Clones were found to express Dnase, antibacterial protein, lipase and amylase activities (Rondon *et al.*, 2000). Quaiser and coworkers applied the environmental genomic approach to characterize uncultivated *Archeae* from soil, and extended the approach to the characterization, genome diversity and evolution of *Acidobacteria* (Quaiser *et al.*, 2002). The metagenome sequencing approach was also used to identify DNA and protein sequences linked to pathogenic traits found in biofilms in drinking-water networks (Schmeisser *et al.*, 2003). Venter and coworkers (Venter *et al.*, 2004) applied metagenomic shotgun sequencing to microbial populations collected en masse from seawater samples from the Sargasso Sea; they demonstrated its efficiency to obtain a relatively unbiased and

quantitative picture of the diversity of >1800 genomic species and identified >1.2 million previously unknown genes. Metagenomic analysis provides functional information through genomic sequence and expression of traits, but other methods are required to link the specific functions to the bacterial group from which they are derived, and concomitant quantitative and comparative analysis of expressed rRNA genes and genes for key enzymes in relation to environmental factors are required to obtain information about the phylogeny and ecology of functional bacterial groups (Torsvik and Ovreas, 2002). The limiting step here is the library screening to select clones containing the genes of interest (Rodriguez-Valera, 2004). Diversa corporation (http://www.diversa.com) has patented several technologies and tools to address these limitations, such as the DiverseLibraries^{SM} collection, which is an assembly of genomic DNA representing the complete genomic diversity present in many, complex microbial samples, each containing as many as 10 000 distinct genomes, or the GigaMatrix™ platform which is an ultra high-throughput screening platform that utilizes plates with a 100 000-well density. Nevertheless, metagenomic libraries remain a powerful tool for exploring soil microbial diversity, providing access to the genetic information of uncultured soil microorganisms. Eventually, this will lead to a more realistic understanding of prokaryotic biodiversity and functionally complex microbial communities, and will provide biotechnology with new tools (Rodriguez- Valera, 2002). Wu *et al.* (2002) reported the potential of community genome arrays (CGAs) for the species- and strain-level differentiation of microorganisms that are closely related to each other on the basis of their rRNA. The microarray contained genomic DNA isolated from representative microorganisms from the environment and hence, the main disadvantage of the CGA is that only cultured components of a community can be monitored. BAC clones could be used to fabricate CGAs, thereby allowing the assessment of uncultivated community components (Zhou, 2003). This would be one of the main advantages of the CGA- approach in comparison with the conceptually analogous membrane- based reverse sample genome probing (RSGP) method. In RSGP, labeled total community DNA is

hybridized to macroarrays on which denatured genomes of cultured microorganisms are spotted (Greene and Voordouw, 2003).

Recently, much attention is given to the generation of 'genomic signatures' that can discriminate sequences from different prokaryotes (Karlin, 1998). Comparative genome sequencing of strains within the same species has revealed as much as 20% gene variation (Boucher *et al.*, 2001). This significant amount of genomic variation in the same 16S rDNA- defined population implies that unique strain-specific biomarkers can be obtained to characterize and monitor the dynamics of genomic variants. The challenge is to develop methods that are sensitive enough to capture these genetic differences. Zabarovska *et al.* (2003) proposed to create *NotI* passports, i.e. databases containing *NotI* tags. These restriction site tagged sequences (RSTS) comprised 19 bp of sequence information, and a comparison of 1312 tags from available sequenced *E. coli* genomes revealed only 219 tags that were not unique. NotI passports allowed discrimination between closely related bacterial species and even strains. The limitation is that certain species have only very few or no *NotI* sites. Hence, the procedure has the potential to assess the diversity of complex microbial communities and to quantitatively and qualitatively identify particular bacterial strains. Dunn *et al.* (2002) developed a method to generate Genomic Signature Tags (GSTs), which are short (e.g. 21 bp) sequence fragments sampled more or less at random from microbial genomes. The tag sequences and abundances are used to create a high-resolution GST sequence profile of the genomic DNA and can be used to design PCR primers for additional genetic information from the source DNA. The method has been modified and is currently being evaluated as a quantitative monitoring tool for complex microbial communities (D. van der Lelie, personal communication).

However, the genetic information encoded in such genomic tags is often unique and shows no homology to known sequences, which obscures their phylogenetic positioning (Wei *et al.*, 2004). Sandberg *et al.* (2001) investigated the identification of bacterial genomic sequences using *k*- mer distributions instead

of sequence matching and it was found that genome sequences as short as 400 bp could be correctly classified with an accuracy of 85% by using a simple Bayesian classifier. This work was recently extended *in silico* to recognize and cluster contiguous sequence fragments of 35 bp from both individual genomic sequences and genomes of mixed bacterial populations (Papamichail *et al.*, unpublished data).

Wei and coworkers (Wei *et al.*, 2004) described a new strategy to simultaneously compare sequence- based structural features of microbial communities, based on Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR, which previously has been widely used for bacterial strain- specific fingerprint typing (de Bruijn, 1992) and for fingerprinting of rhizosphere microbial communities (Di Giovanni *et al.*, 1999). ERIC-PCR profiles of intestinal microflora were transferred onto a nylon film to form an array-like organization consisting of amplified genomic DNA fragments distributed to reflect differences in community diversity. Next, ERIC-PCR amplicons from another community sample were DIG (dioxygenine)-11-dUTP-labeled to hybridize with the community DNA arrays. In this way, the investigators were able to identify commonly shared sequences without prior knowledge of the population composition or clone sequences to prepare the DNA array. Thus, the method combines the large-scale processing capacity of DNA array hybridization with the visualization power of community fingerprinting techniques. Moreover, DNA fragments of shared bands can be eluted, cloned and sequenced to provide a basis for genome- specific biomarker design. This strategy to couple DNA fingerprinting with molecular hybridization cannot be applied in 16S rDNA-based PCR-TGGE/ DGGE fingerprinting techniques due to the high homology between 16S rDNA of different bacterial species (Wei *et al.*, 2004).

1.4. Bioremediation processes based on microbial heavy metal detoxification mechanisms

Biological approaches for the remediation of heavy metal contamination offer the potential for a highly selective removal of toxic metals coupled with a considerable operational flexibility, as these approaches can be used both *in situ* or *ex situ*. Also, biological approaches have the potential for being improved by biostimulation, bioaugmentation and natural gene transfer (Dong *et al.*, 1998). Moreover, the availability of molecular monitoring tools for the detection and follow- up of the biological process, as was discussed before, allows optimization of the treatment process at a lab-scale, and adjustment of the on site application. Molecular techniques for investigating environmental microbial populations have developed considerably lately and allow in-depth investigations that are of significant scientific and ecological interest. However, it should be kept in mind that these methods often require sophisticated equipment or software and hence are expensive; moreover, time-consuming and laborious molecular studies are not suitable as decision tools for on site process adjustments when time presses. Therefore, a critical appraisal of the level of detail and information that the molecular analyzes should provide, needs to be undertaken. If one aims to follow biological processes mediated by a specific bacterial population such as SRB, FISH and DGGE fingerprinting based on the 16S rRNA-gene or a functional gene are generally used since they allow a rapid and adequate insight in community structures.

1.4.1. Bioremediation of heavy metal-contaminated waters

Biological remediation processes for metal polluted ground- or wastewater can be applied either *ex situ* or *in situ* (Figure 1.3).

The *ex situ* strategies are based on the principle of pump-and-treat: water is pumped up and treated in an above-ground reactor by mechanisms of biosorption

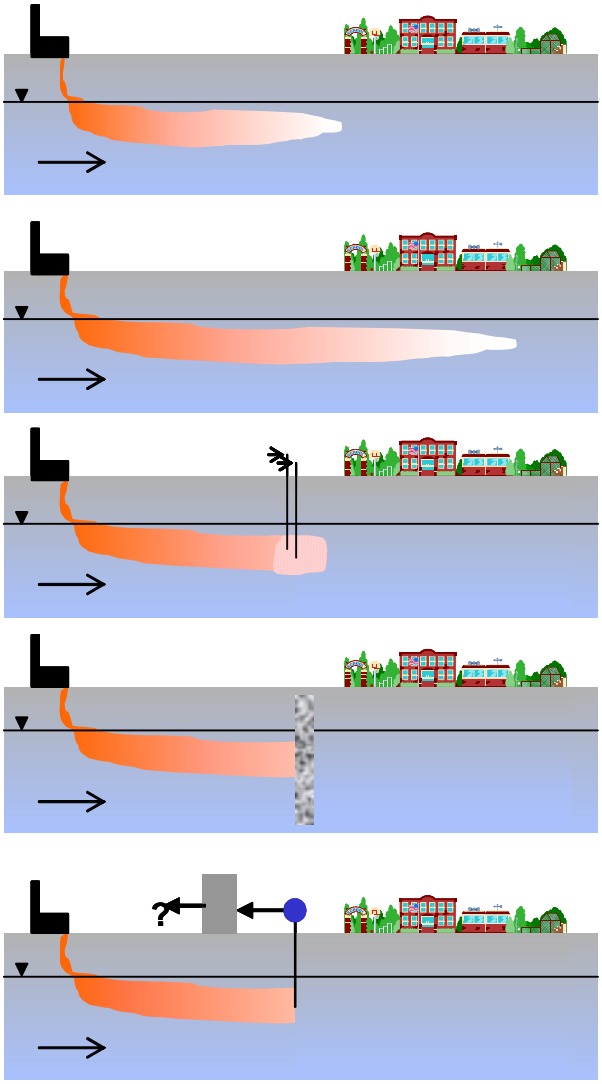

Figure 1.3 Different treatment technologies for heavy metal-contaminated groundwater plumes.

or bioprecipitation. Various reactor designs exist, such as continuously stirred tank reactor, upflow anaerobic sludge blanket reactor, and fluidized-bed reactor, packed-bed reactor. Although pump-and-treat processes have demonstrated to be

efficient in heavy metal removal, installation costs and retention time are high, especially when large volumes of water have to be treated, which is often the case when treating polluted groundwater at industrial sites. However, the advantage is that the contaminating metals are collected and can easily be disposed of. Low-costs alternatives are *in situ* processes, such as the installation of a subsurface permeable barrier or the creation of a reactive zone or biobarrier. Permeable reactive barriers are receiving a great deal of attention as an innovative, cost-effective technology for *in situ* clean up of groundwater contamination. A wide variety of materials are being proposed for use in subsurface barriers, including zero-valent metals (e.g. iron metal), humic materials, oxides, surfactant-modified zeolites, and oxygen- and nitrate releasing compounds. These materials remove dissolved metals by immobilization within the barrier or reduction to less harmful forms. The primary removal processes include sorption and precipitation, chemical reaction, and biologically mediated reactions. These processes were reviewed in detail by Scherer and coworkers (Scherer *et al.*, 2000).

Another *in situ* approach is the creation of a reactive zone or biobarrier. This is a subsurface zone where the activity of metal transforming microorganisms is stimulated by injection of nutrients. As the groundwater passes through this zone, bacterial activity will result in metal retention, resulting in clean groundwater, avoiding further dispersion of the contaminating metals to the surrounding environments. The principle of a reactive zone is generally based on precipitation of metals as metal sulfides by SRB. A major concern with the *in situ* methods is that changes in pH or redox can easily result in the mobilization of the precipitated contaminants. Therefore, these processes require that the contaminants are either removed at the end of the remediation process, or the continuous monitoring of the site, even after completion of the remediation.

1.4.1.1. Biosorption

Biosorption is an emerging technology that uses biological materials to remove metals or radionuclides from solution through adsorption. Biosorption can be defined as the ability of biological materials to accumulate heavy metals from wastewater through metabolically mediated or physico- chemical pathways of uptake or binding (Vieira and Volesky, 2000). Metal accumulative bioprocesses involve biosorptive (passive) uptake by using non- living biomass or bioaccumulation by applying living cells (Veglio and Beolcini, 1997). Many studies have demonstrated the efficiency of metal removal by microbial biomass in a range of reactor formats and under a range of physical and chemical conditions (Gadd, 2000a). The main drawback in the use of pure biosorptive materials is that existing ion-exchange synthetic resins provide a similar performance and their use is already well established. Nevertheless, biosorption methods seem to be more effective than their physicochemical counterparts in removing dissolved metals at low concentrations (below 2- 10 mg l^{-1}) (Bunke *et al.*, 1999) and demonstrate higher specificity, which avoids overloading of binding sites by alkaline- earth metals. On the other hand, application of active and growing cells offers the potential for optimization through development of resistant species and cell surface modification, as was used so far for enhancing the biosorption characteristics of fungi (Malik, 2004).

1.4.1.2. Bioprecipitation

Where reduction of a metal to a lower redox state occurs, its mobility and toxicity can be reduced, thus offering potential bioremediation applications for ground- and wastewater. Indirect precipitation by the formation of metal sulfides and phosphates is the strategy that has received much interest to develop biotechnological processes for metal precipitation (Valls and de Lorenzo, 2002). Remediation strategies by means of reducing metal toxicity and mobility of

contaminating metals in waters can be applied either *ex situ* in pump- and- treat reactor systems, or *in situ*, via permeable reactive barriers or by creating an *in situ* reactive zone or biobarrier.

Dissimilatory iron- reducing bacteria have an important role in oxidizing organic contaminants (e.g. aromatic hydrocarbons) in the subsurface but are also capable of immobilizing contaminant metals by reduction to less soluble forms. For example, reduction of U(VI) to the less soluble U(IV) by *G. metallireducens* can be the basis of U(VI) removal from contaminated waters and leachates (Gorby and Lovley, 1992; Lovley and Coates, 1997) and this method has been proposed as a remediation strategy for a uranium- contaminated site in New Mexico (Finneran *et al.,* 2002; Holmes *et al.*, 2002). Anderson et al. (2003) demonstrated the feasibility of *in situ* bioremediation of uranium- contaminated groundwater by the activity of *Geobacter* species in a field-scale pilot test by combining analytical analyzes with PLFA and 16S rDNA sequencing. North and coworkers (North *et al.*, 2004) used quantitative PCR of *Geobacter*-type 16S rRNA- genes together with 16S rRNA gene clone libraries to analyze changes in metal- reducing microbial communities in uranium-contaminated subsurface sediments during an *in situ* bioremediation experiment. The potential of microbiological reduction of Cr(VI) by the dissimilatory metal reducing bacterium *S. alga* was studied in laboratory batch experiments and on field- scale by Friedly and coworkers (Friedly *et al.*, 1995). It was demonstrated that *S. algae* BrY is able to repeatedly generate surface reactive Fe(II) for the chemical reduction of Cr(VI) to the less toxic, less mobile Cr(III) in the presence of iron- chromium precipitates. Thus, besides its role in direct biological Cr(VI) reduction, the *Shewanella* strain also biologically regenerates surface associated ferric iron (Fe(III)) to ferrous iron (Fe(II)) for the chemical reduction of Cr(VI) by surface associated ferrous iron. Hence it follows that dissimilatory iron- reducing bacteria can be used to establish permeable reactive barriers (Nyman *et al.*, 2002).

Sulfate-reducing bacteria have received much attention because of their reductive precipitation capacity of toxic metals such as Cr(VI) and U(VI), which is mediated by cytochrome c proteins. Biofilms of SRB enzymatically reduced and precipitated Cr(VI) (Smith and Gadd, 2000). Cloning and expression of the cytochrome c_7–gene from *D. acetoxidans* in *D. desulfuricans* lead to an enhanced expression of metal reductase activity in the recombinant strain. Such overproduction of active cytochrome c_7 could be important in fixed- enzyme reactors or in the production of organisms with enhanced metal reductase activities for bioremediation (Aubert *et al.*, 1998).

Besides dissimilatory reduction, sulfate reducers are important for their indirect reductive metal precipitation mechanisms, for example, reduction of Cr(VI) can be a result of bacterial respiration or indirect reduction by Fe^{2+} and the produced sulfide, or reduction by the produced H_2S (Lloyd *et al.*, 2000). Sulfate-reducing bacteria are anaerobic heterotrophs utilizing a wide range of organic substrates and SO_4^{2-} as electron acceptor. Immobilization of metals as metal sulfide complexes due to the sulfide production activities of SRB is one of the best-known natural precipitation systems. Bioprecipitation of metals by SRB requires an anaerobic environment, low redox conditions (below -100 mV) and an electron donor such as lactate or methanol (Mulligan *et al.*, 2001). Mixed sulfate-reducing bacterial consortia are more effective than pure cultures in the removal of heavy metals from solution (White and Gadd, 1998; White and Gadd, 2000).

SRB have been successfully used in the treatment of waters and leachates in large-scale bioreactors and in pilot laboratory surveys (Gadd, 2000b; Gadd and White, 1993). Sulfate-reducing bacterial biofilm reactors may offer a means of process intensification and entrap or precipitate metals such as Cd or Cu at the biofilm surface (White and Gadd, 1998; White and Gadd, 2000). U(VI) was immobilized from solution in a biofilm of *D. desulfuricans* G20 as a result of combined enzymatically and chemically (by means of microbially generated H_2S) processes (Beyenal *et al.*, 2004). For the treatment of contaminated waters such as acid mine drainage (AMD), biotechnological applications of SRB might be a

promising alternative for chemical treatment, which generally involves the addition of lime (Chang *et al.*, 2000; Drury, 1999; Dvorak *et al.*, 1992; Jong and Parry, 2003). SRB-activity in porous and permeable reactive walls, installed in the path of contaminated waste, may provide a promising alternative for remediating metal-contaminated wastes (Benner *et al.*, 2002; Waybrant *et al.*, 1998). Recently, the long-term viability of a full-scale permeable reactive barrier, utilizing bacterially mediated SO_4^{2-} reduction to promote metal sulfide precipitation and alkalinity generation, was demonstrated by Benner and coworkers (Benner *et al.*, 2002). By injection of nutrients, an *in situ* anaerobic reactive zone is created in the path of migrating groundwater, where the activity of the indigenous SRB- population and the concomitant metal sulfide precipitation is stimulated. The feasibility of such an *in situ* metal precipitation (ISMP) process to treat abandoned underground mines or waste dumps (Lee and Saunders, 2003; Saunders *et al.*, 2001) and a zinc-contaminated, aerobic sandy aquifer (Janssen and Temminghoff, 2004) has been demonstrated on field-scale. Several patents were granted for *in situ* systems that precipitate dissolved metals (Blowes and Ptacek, 1994; Geraghty and Miller, 1996; NATO/CCMS and 1999, 1999).

High concentrations of different heavy metals inhibit SRB (Hao, 2000). However, some strains of SRB can produce exopolymers that complex metals; the resultant metal complexes are less toxic to SRB. Also, the microbes can adapt to adverse environments including metal-contaminated systems (Beech and Cheung, 1995).

Metal precipitation can also be mediated by the liberation of inorganic phosphate from organic donor molecules. A *Citrobacter* sp. which was isolated from metal-polluted soil was capable of accumulating high levels of uranium, zirconium and nickel through the formation of highly insoluble metal phosphates (Basnakova and Macaskie, 1999; Macaskie *et al.*, 2000). Hence, the phosphate precipitation process by *Citrobacter* sp. has a clear potential for bioremediation and has already been applied to the removal of uranium from mine water and to the accumulation of radiotoxic elements (Macaskie *et al.*, 1994; Macaskie *et al.*, 1997).

1.4.2. Treatment of heavy metal contaminated soil

Metals in soil, sludge or solid waste need to be removed from the matrix by solubilization in a liquid phase. Afterwards, these water- solubilized metals can be concentrated in a second step, the desolubilization phase. The solubilization of heavy metals can be done via autotrophic or heterotrophic leaching, the use of metallophores, or by chemical leaching followed by microbial treatments. Metals displaced in this way into the water phase can be desolublized via biologically induced adsorption, precipitation, and transformation or complexation processes (Diels *et al.*, 1999).

Bioleaching is a simple and effective technology for metal-extraction of mineral ores, based on the activity of acidophilic iron- and sulfur-oxidizing bacteria, mainly *Thiobacillus ferrooxidans*, *Thiobacillus thiooxidans*, and *Leptospirillum ferrooxidans* (Olson et al., 2003). This technology was initially developed for the leaching of gold from pyrosulfite minerals and today, seven plants have been commissioned for the biomining of gold using this process (Brierley and Brierley, 2001). Other commercial applications of biohydrometallurgy are copper, cobalt and uranium recovery. Bacterial leaching is carried out in an aerobic and acid environment (pH 4) and can be performed by direct or indirect means. In direct leaching, the bacteria need to make physical contact with the mineral sulfide surface. Metalsulfides such as CuS, ZnS, NiS, are oxidized to produce sulfuric acid, which can desorb the metals from the soil by substitution of protons (Mulligan *et al.*, 2001). In indirect bioleaching, the bacteria generate a lixiviant which chemically oxidizes the sulfide mineral. In acid solution, this lixiviant is Fe(III): the bacteria convert Fe(II) to Fe(III) which in turn oxidizes sulfur minerals (Bosecker, 1997). Although most interest arises from a hydrometallurgical perspective, leaching of contaminating metals from soils and other matrices is also possible (White *et al.*, 1998). In fact, bioleaching of heavy metals from contaminated aquatic sediments using indigenous sulfur-oxidizing bacteria can be better than sulfuric-acid treatment for metal solubilization from contaminated

aquatic sediments (Seidel *et al.*, 1998). Chen and Lin (2004) investigated the feasibility of bioleaching processes for metal-contaminated sediments in an air-lift reactor and defined the optimal sulfur concentration by linking the acidification of sediment slurry and metal solubilization to FISH analysis with *Thiobacillus*- species specific probes. Bioleaching was also demonstrated to be an efficient and cost- effective means of removing heavy metals from sewage sludge without seriously affecting its soil conditioning and fertilizing properties (Chan *et al.*, 2003; Couillard and Mercier, 1991; Wong and Henry, 1984). As an alternative, the microbial production of organic acids or heterotrophic leaching is of developing interest in pollution treatment and metal recovery. Heterotrophic leaching may be particularly appropriate for wastes of high to neutral pH, as most *Thiobacilli* cannot solubilize effectively above pH 5.5 (Gadd, 2000a). Members of the genus *Bacillus* are most effective in heterotrophic metal solubilization (Bosecker, 1997). Heterotrophic bacteria contribute to metal leaching presumably by the enzymatic reduction of highly oxidized metal compounds (Ehrlich, 1980) or by the production of organic acids and hydrophilic compounds such as phenol derivatives (Bosecker, 1988).

SRB- containing reactors have been used in the removal of heavy metals from soil in a microbial integrated decontamination process: sulfur-oxidizing bacteria were used to leach metals from the contaminated soils, and the released metals were subsequently removed using bacterial sulfate reduction in an internal sedimentation reactor (White *et al.*, 1998). This combination of bioleaching followed by separate bioprecipitation of leached metals by SRB proved to be effective in removing and concentrating a range of metals, including Zn, Cu and Cd, from contaminated soil. The bioleaching process can be applied *ex situ*, but where soil and water conditions are appropriate, the soil leaching component might be carried out *in situ*.

The patented system Bio Metal Sludge Reactor (BMSR) (Diels *et al.*, 1992) relies on the siderophore- mediated metal solubilization and biocrystallization by *Ralstonia metallidurans* CH34 to treat sandy soils contaminated with heavy metals. Siderophores are Fe(III) ligands which are excreted to aid iron assimilation (Gadd, 2001), although they also bind other metals such as Mn, Cr(III) and radionuclides (Birch and Bachofen, 1990). The bacterium solubilizes the metals via the production of metal complexing siderophores, and adsorbs the metals in their biomass, on metal-induced outer membrane proteins and by bioprecipitation. The BMSR system consists of a continuous stirred tank reactor that is fed with contaminated soil to which water and nutrients are added. A unique feature of the process is that, due to biological factors produced by the bacterium, the soil treated with CH34 will sedimentate while the bacteria stay in suspension, which allows the subsequent separation of the metal- loaded bacteria and process water in a decantation device. The bacteria can be recovered by either a flotation or a flocculation process. With this soft treatment system, a large decrease in the bioavailable fraction of Cd, Zn and Pb was obtained (Diels *et al.*, 1999). This is in contrast to chemical leaching by acids or complexing agents, which removes most ions, but often leaves a small fraction of highly bioavailable and thus toxic metals.

Another patented technology is the MEtal REmoval by Sand Filter INoculation (MERESAFIN) system, which is based on the inoculation of a sand filter with metal bioprecipitating (*Ralstonia metallidurans* CH34) and metal biosorbing (*Pseudomonas mendocina* AS302 and *Arthrobacter* sp. BP7/26) bacteria (Diels *et al.*, 2003). During contact with HM-containing wastewater, metals are adsorbed by the biofilm. Subsequently, the metal loaded biomass is removed from the supporting material, while the residual, resting biomass on the substratum can be reused for a next treatment cycle. The efficiency and reliability of the concept for HM removal was shown by combining analytical results with biofilm microscopy techniques and molecular tools such as 16S rDNA- DGGE and PCR-detection of the

cnr- and *czc*- operons in CH34, encoding Ni- and Co-resistance and Cd, Zn, and Co-resistance respectively (Collard *et al.*, 1994; Pumpel *et al.*, 2001).

Recently, a novel approach for removal of heavy metals from polluted soils was described by Valls *et al* (2000). A mouse metallothionein was expressed on the surface of *Ralstonia metallidurans* to promote biosorption. The recombinant strain was found to have an enhanced ability for immobilizing Cd^{2+} ions. Furthermore, inoculation of the recombinant strain in Cd^{2+} polluted soil resulted in a significant decrease of the toxic effects of the heavy metal on the growth of tobacco plants. Thus, microbial metal immobilization and the resulting decrease in metal bioavailability can be used in moderately polluted fields, allowing their use in agriculture (Valls *et al.*, 2000). It has also been shown that inoculation of metal-resistant bacteria into soils protected the indigenous bacterial community from the effects of heavy metals (Stephen *et al.*, 1999). Such bioaugmentation strategies can be applied for the *in situ* protection of micro-and macrobiota from metal toxicity.

1.5. Conclusions

Toxicity of heavy metals and metalloids in both natural and antropogenetically impacted environments is an important issue of current public health policies and ecosystem restoration. Microorganisms are able to adapt to the constraints of heavy metal toxicity by developing or acquiring specific resistance mechanisms, or by changing metal speciation, mobility and bioavailability using processes that are linked to their general metabolism. These microorganisms play a crucial role in the environmental fate of toxic heavy metals, and their activities have the potential to be applied for both *in situ* and *ex situ* treatment of polluted soils, waste and groundwater. The molecular and genetic elucidation of the metal-microbe interactions, together with the ability to monitor microbial community composition and activity during the treatment processes represents a tremendous

progress in the further development and optimization of different bioremediation strategies for the detoxification of heavy metal pollution.

During the last decades, major advances have been made in understanding the mechanisms of interactions between microorganisms and heavy metals, and in the application of specialized microorganisms for the *in situ* and ex situ treatment of heavy metal and radionuclide contaminated soils, wastes and groundwater. The efficiency of heavy metal bioremediation depends on the presence and the activities of the microorganisms involved, which is in turn affected by environmental conditions, operational parameters and the local composition of the overall microbial community composition. Hence, when opting for a biological remediation strategy, important questions to be answered as part of the overall remediation strategy include: (i) are microorganisms with the desired characteristics and activities present at the contaminated site, (ii) what is their activity, (iii) and how is the microbial community composition and functioning influenced by environmental parameters and process conditions? Recently, molecular and non- molecular methods for the identification and characterization of bacteria and their specific properties have been used to assess the composition and activity of microbial communities found at heavy metal-contaminated sites. For the future, these techniques contain the promise to be applied as complementary tools to classical chemical and physiological analytical methods (heavy metal concentrations and speciation, redox potential, etc.) to monitor the spatial and temporary changes in microbial community composition and functioning.

Molecular and microbial monitoring tools, when combined with batch, column and pilot studies, enable the optimization and follow- up of the biological processes during the developmental phase, as well as during the actual bioremediation treatment. At the lab-scale, they will demonstrate the presence of active microbial communities and identify the "key players" whose activities are crucial for a successful remediation strategy. They will also become important

management tools to follow up the efficiency of bioremediation processes, especially when applying *in situ* remediation. After completion of such an *in situ* remediation, molecular methods will add to information on the longevity of bacterial growth, activity and metal immobilization. When combined with batch, column and pilot studies, they can be used as part of the remediation startup phase to define optimal process conditions: comparison of the bacterial community composition and activity for different process conditions with their respective metal removal efficiency will predict the success or failure of the final remediation, and will facilitate the selection of optimal process conditions. Once the bioremediation strategy is being applied, the monitoring methods can be used for the follow up, and as a decision tool for necessary process adjustments.

The only limitation for large scale, high throughput application of microbial monitoring as part of a management strategy for the remediation of contaminated sites is the cost price of the analysis as well as the need for specialized labor and equipment. However, due to the recent developments of tests for the detection of pathogens, it can be envisaged that cheap tests for the detection of specific groups of environmentally important microorganisms or their functions, such as SRB, will become available in the near future. As a result of this development, monitoring tools will become an integrated part of the management decision system for the remediation of contaminated sites when efficient, cost-effective and reliable bioremediation technologies are applied.

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CHAPTER 2

Aim and Outline of the Thesis

Environmental contamination by toxic heavy metals and metalloids (HMM) is of increasing economic, public health and environmental significance. In contrast to most organic contaminants, HMM are never degraded. For the treatment of metal contaminated wastewater and groundwater, a growing interest exists for bioprecipitation technologies based on the activity of sulfate-reducing bacteria (SRB) as an alternative over chemical methods.

Sulfate-reducing bacteria are pivotal in the global carbon and sulfur cycling, and are found in many reduced environments such as marine sediments, anoxic waters, biofilms, anaerobic sludge and (contaminated) aquifers. Characteristically, SRB couple the oxidation of hydrogen or organic carbon to the reduction of sulfate to hydrogen sulfide, which readily precipitates metals as metal sulfides or acts as a strong reducing reagent. In addition to this indirect H_2S-mediated metal reduction process, some SRB species are capable of direct, enzymatical reduction of HMM, thereby reducing the HMM mobility and / or its toxicity. Sulfate reduction can be applied for metal removal both *ex situ* ("pump and treat") as *in situ* ("*in situ* metal precipitation"). The effectiveness or failure of these metal precipitation processes is closely linked to the presence and activity of SRB communities, and the way these communities are affected by process parameters, environmental conditions or other, non sulfate-reducing microorganisms. Hence, information on the identity, diversity and specific activity of SRB communities is essential, not only during lab-scale or pilot-scale validation experiments, but especially during on site application of SRB activity as part of a remediation strategy.

Direct information on SRB community composition and activity may be obtained by using molecular ecological methods such as the construction and analysis of phylogenetically relevant gene libraries, denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP), fluorescence *in situ* hybridization (FISH) and microarray studies. These methods have been used previously to investigate SRB communities in marine sediments, seawater, and anaerobic bioreactors. Most of the molecular studies on SRB-diversity are based on 16S rRNA gene analysis, although the functional gene approach, based on the *dsr* (dissimilatory sulfite reductase)-gene, is gaining importance as it provides a direct link to physiology and frequently reveals a greater SRB diversity than the 16S rRNA gene based approaches.

The objectives of the present study were: (1) to develop molecular tools for monitoring the diversity and changes in indigenous SRB communities found in heavy metal contaminated environmental samples; (2) to apply these tools for the follow-up and optimization of *in situ* metal precipitation (ISMP) processes in microcosm and column experiments; and (3) to evaluate whether these molecular monitoring methods can be used in a reliable way for the follow up and as decision-making tools for necessary process adjustments in a pilot scale studies where ISMP is being evaluated as an effective and reliable remediation strategy for the treatment and containment of a heavy metal contaminated groundwater plume.

Chapter 3 describes the development of SRB-specific molecular monitoring tools, based on either the 16S rRNA-or the *dsr*-gene. SRB-subgroup or –genus specific PCR primers for the 16S rRNA-gene were adapted from previous studies and improved in their specificity, or new primers sets were designed. A DGGE method was developed to rapidly assess the diversity of SRB-populations based on their *dsrB* (dissimilatory sulfite reductase β-subunit) gene. These molecular methods

were successfully applied to DNA extracted from HMM contaminated aquifer samples.

In **Chapter 4**, the feasibility of ISMP as a groundwater remediation technology for a heavy metal polluted groundwater plume present at an industrial site was demonstrated using batch experiments. The developed molecular tools allowed us to link the results of sulfate-and heavy metal-removal to the response of the indigenous SRB population under different batch conditions (i.e. amendment of different types of carbon-source/ electron donor, yeast extract, VitB12).
Building on the knowledge gathered with the batch experiments, column experiments were conducted in **Chapter 5** to investigate the ISMP process in a more realistic aquifer system. The efficiency, reliability and sustainability of the ISMP process was explored by amending different types of carbon-sources, adding N/P nutrients, changing COD/ SO_4^{2-}-ratio's and testing the effect of intentional disruption of amendment supply. The sulfate reduction and metal precipitation was evaluated by both analytical and molecular tools. During the cause of these experiments we were confronted with temporal process failures, which caused release of heavy metals. This metal leaching problem was also observed when substrate supply was terminated, making the sustainability of the ISMP process under certain circumstances questionable.

Finally, **Chapter 6** provides a summarizing discussion and perspectives of the work.

CHAPTER 3

Analysis of Sulfate-Reducing Bacterial Communities by *dsrB* Gene Based DGGE

Abstract

Sulfate-reducing bacteria (SRB) mediate the direct and indirect reduction of heavy metals and metalloids (HMM), and consequently play an important role in bioremediation technologies for HMM contaminated groundwaters, e.g. via the *in situ* metal precipitation (ISMP) of metal sulfides. To realize an effective metal bioremediation process based on the activity of SRB, it is necessary to monitor both their physico-chemical and biological characteristics, both of which are closely linked to the composition of the sulfate-reducing microbial community. In this study, a DGGE method based on the *dsrB* (dissimilatory sulfite reductase β-subunit)-gene sequence was developed to rapidly assess SRB community dynamics over time and space. Together with SRB subgroup and genus specific 16S rDNA-PCR and 16S rDNA-DGGE analysis of the total microbial community, *dsrB*-DGGE was applied to different aquifer samples derived from industrial sites with HMM contaminated groundwater plumes, where the presence and activity of SRB was demonstrated by the analytical follow-up of microcosm or pilot experiments for ISMP. Although the 16S rDNA-PCR and DGGE approach allowed a first characterization of the SRB community-composition, the *dsrB* gene based DGGE approach revealed considerable more genetic diversity between different environmental and experimental conditions.

3.1. Introduction

Due to their dissimilatory metal reduction capacities and for their indirect reductive metal precipitation mechanisms (Hao, 2000), sulfate-reducing bacteria (SRB) have received much attention in the field of remediation technologies for heavy metals and metalloids (HMM) decontamination. SRB have been successfully used in the treatment of heavy metal contaminated water and leachates in large-scale bioreactors and in pilot laboratory surveys (Gadd, 2000; Gadd and White, 1993) and have the potential to be applied in an *in situ* bioreactive zone for immobilizing heavy metals in groundwater, a process called *in situ* metal precipitation (ISMP) (Hao, 2000).

The efficiency of the HMM bioremediation strategy depends on the activity of SRB which, in turn, is affected by environmental conditions, operational parameters and the local composition of the overall microbial community. Hence, when opting for a remediation strategy based on the activity of SRB there is, complementary to classical chemical and physiological analytical methods (heavy metal concentrations and speciation, redox potential, etc.), a need for tools to monitor the spatial and temporal changes in SRB community composition and functioning. These tools comprise culture-independent methods specifically targeting SRB, such as cloning and sequencing of PCR-amplified genes (Orphan *et al.*, 2001), PCR with SRB-subgroup specific primers (Daly *et al.*, 2000; Hill *et al.*, 2000), DGGE (denaturing gradient gel electrophoresis) (Kleikemper *et al.*, 2002b; Santegoedts *et al.*, 1998), FISH (fluorescence *in situ* hybridization) (Kleikemper *et al.*, 2002a; Tonolla *et al.*, 2000), Real-Time PCR using SRB-group specific primers (Stubner, 2004), and nucleic acid microarrays (Loy *et al.*, 2002). The 16S rRNA gene sequences of SRB form the general framework for these techniques. Although phylogenetic analysis based on 16S rRNA genes may support speculation about biological functions, an understanding of the effectiveness of SRB-mediated bioremediation processes will significantly increase when this information is complemented by analysis of genes encoding enzymes of

physiological or metabolic relevance. For sulfate-reducing microbial communities, the *dsr*-gene which encodes the dissimilatory sulfite reductase (DSR, EC 1.8.99.1), a key enzyme in sulfate reduction that catalyzes the reduction of sulfite to sulfide and hence is required by all sulfate reducers (Klein *et al.*, 2001), is the best studied example of such a functional marker (Karkhoff-Schweizer *et al.*, 1995; Klein *et al.*, 2001; Wagner *et al.*, 1998).

This work describes the development of a PCR-DGGE method for rapidly exploring the diversity of SRB-populations based on their *dsrB* (dissimilatory sulfite reductase β-subunit)-gene. For this purpose, a new forward primer GC-DSRp2060F was designed, which was combined with the DSR4R reverse primer (Wagner *et al.*, 1998) for the PCR amplification of a ~ 350 bp fragment of the *dsrB* gene. The *dsrB*-DGGE method was applied to examine the composition and dynamics of SRB-populations in HMM contaminated aquifer samples of microcosm experiments and an on site pilot test for ISMP. The specificity and reliability of the *dsrB*-DGGE assays were verified by cloning and sequencing of excised DNA bands, SRB-subgroup and –genus specific PCR detection of the 16S rRNA-gene and DGGE analysis of whole community 16S rDNA.

3.2. Materials and methods

3.2.1. Environmental samples

Aquifer and groundwater samples were derived from three different heavy metal contaminated industrial sites in Belgium, and were designated as Pb112 and Pb118, A410 and B305, MF02, MF03, MF04, MF05 and MF06. The groundwater composition of the contaminated sites is summarized in Table 3.1.

Aquifer and groundwater samples Pb112 and Pb118 were collected from different sampling points at the site of a steel plant in Antwerp (Belgium). Samples were used in microcosm experiments, which were setup for exploring the feasibility of ISMP as a groundwater remediation strategy at the sampling sites. During these

microcosm experiments, the response of the indigenous SRB-population to amendment with different C-sources and electron-donor types (i.e. acetate, molasses, Hydrogen Release Compound®) was investigated. Additional microcosm series were used as controls: one received no extra carbon source to account for consumption of indigenous carbon sources, and in one the microbial population was poisoned with formaldehyde to account for abiotic processes.

Table 3.1 Analysis of the groundwater composition at the study sites [a]

	Sample					
Parameters	Pb112	Pb118	A410	B305	MF	Norm
pH	6.8	4.6	10.1	9.6	4.1	
SO_4^{2-} (mg l^{-1})	200	200	200	110	1460	
Zn (mg l^{-1})	7.67	19.3			444	0.5
Ni (mg l^{-1})		0.44			144	0.04
Cd (mg l^{-1})		34.0	5.56	60.1	53.5	0.005
Co (mg l^{-1})					31.2	0.1
As (mg l^{-1})			0.7	0.06		0.02
Fe (mg l^{-1})	2.0	34.0	5.56	60.1	34.5	

[a] Blank fields indicate that concentrations were below legalized concentrations

Aquifer and groundwater samples A410 and B305 originated from an arsenic-contaminated harbor site in Antwerp (Belgium). In microcosm experiments with aquifer and groundwater samples A410 and B305, the effect of amending lactate, molasses or HRC® as a C-source/ electron donor was investigated in addition to the controls. Aquifer samples MF02, MF03, MF04, MF05 and MF06 were derived from a heavy metal-contaminated site of a nonferrous industry in the Kempen (Belgium) where an ISMP field-scale pilot test was installed. The test field consisted of 1 injection well and 6 monitoring wells. Molasses was injected through the injection well IF02 and was spread via migration with the contaminated groundwater plume in North Eastern direction. Monitoring wells MF05 and MF06 were considered as blank controls, since no molasses was expected to pass through. However, due to groundwater pumping activities, there

was also a groundwater flow in South Western direction. Therefore, stimulated ISMP activity was also observed at monitoring well MF05. Overall, sulfate removal and heavy metal-precipitation took place in the order MF04 > MF03 > MF02> MF05> MF06. Aquifer material was collected by pumping groundwater from the wells at 10 m below soil surface over a 0.45 µm EZ-Pak™ Membrane filter (Millipore).

A detailed description of the setup and outcome of the ISMP experiments was beyond the scope of this study and will not be discussed here. During the pilot test and the microcosm experiments, aquifer samples were collected for extraction of community DNA and for further molecular analyses, in order to link differences in the efficiency of the ISMP process (as it was demonstrated by analytical follow-up of HMM-and SO_4^{2-}concentration) to the composition and activity of the SRB-population.

3.2.2. Bacterial strains

The bacterial strains *Desulfovibrio termitidis* (DSM 5308), *Desulfovibrio desulfuricans* (DSM 642), *Desulfovibrio gigas* (DSM 1382), *Desulfovibrio vulgaris (*DSM 1744), *Desulfomicrobium norvegicum* (DSM 1741), *Desulfomicrobium escambiense* (DSM 10707), *Desulfotomaculum acetoxidans* (DSM 771), *Desulfotomaculum aeronauticum* (DSM 10349), *Desulfotomaculum ruminis* (DSM 2154), *Desulfosporosinus orientis* (DSM 7439), *Desulfosporosinus auripigmentus* (DSM 13351), *Desulfobacter latus* (DSM 3381), *Desulfococcus biacutus* (DSM 5651), *Desulfobulbus rhabdoformis* (DSM 8777), *Desulfobacterium autotrophicum* (DSM 3382), *Desulfonema magnum* (DSM 2077) and *Desulfosarcina variabilis* (DSM 2060) were used for testing the specificity of the PCR assays and for the evaluation of the reliability of the *dsrB* based DGGE approach. Bacterial strains were grown in the media described by the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany).

3.2.3. Extraction and purification of DNA

Total genomic DNA was extracted from 5 ml of pure SRB cultures as described before (Leys *et al.*, 2004). DNA extracts were obtained from 2 g of aquifer samples using a protocol modified from El-Frantoussi *et al.* (1999). After overnight precipitation with ethanol (100%) at -80°C, DNA was resuspended in 400 µl H_2O and purified over a Wizard® Minicolumn DNA purification kit (Promega). DNA was resuspended in 50 µl Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8). For PCR purposes, the DNA concentration was measured spectrophotometrically (NanoDrop® ND-1000 spectrophotometer, NanoDrop Technologies Inc.) and adjusted to a concentration of 100 ng μl^{-1}.

3.2.4. DGGE analysis of dsrB gene fragments

In order to enable a DGGE-analysis which is specific for the SRB-community, a new degenerate forward PCR primer was designed, based on an internal *dsr* sequencing primer described by Pérez-Jiménez *et al.* (2001), i.e. DSRp2060F (5'-CAACATCGTYCAYACCCAGGG-3'), to which a 40 bp GC-clamp was attached to the 5' end (Table 3.2).

The selectivity of the primer was evaluated using the program BLASTN (Basic Logical Alignment Tool, http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul *et al.*, 1990) available from GenBank. Important negative controls are Dsr-like proteins, which have significant homology with Dsr proteins but are found in non sulfate-reducers such as the hyperthermophilic archaeon *Pyrobaculum islandicum* (U75249) and the photosynthetic sulfur bacterium *Allochromatium vinosum* (U84760), where they catalyze the oxidation of sulfurous components during growth (Molitor *et al.*, 1998, Schedel *et al.*, 1979). By combining this forward primer with the DSR4R reverse primer (Wagner *et al.*, 1998), an approximately 350 bp *dsrB* gene fragment was obtained for DGGE-analysis. PCR amplification was performed in a total volume of 100 µl in a Biometra T3 Thermocycler.

Table 3.2 DNA sequence homology between the DSRp2060F primer and *dsrB* gene sequences of SRB and non-SRB strains

Organism	*dsrB* gene Genbank sequence Accession no.	Primer [a]
		DSRp2060F (bp 2060-2081) 5′ CAA CAT CGT YCA YAC CCA GGG 3′
Desulfobacter vibrioformis	AJ250472	––– T–– ––– ––– ––– ––– –––
Desulfofaba gelida	AF334593	––– ––– ––– ––– ––– ––– –––
Desulfobotulus sapovorans	U58120	––– T–– T–– ––– ––– ––– –––
Desulfosarcina variabilis	AF191907	––– ––– ––– ––– ––– ––– –––
Desulfococcus multivorans	AJ277107	––– T–– T–– ––– ––– ––– –––
Desulfovibrio vulgaris	U16723	––– ––– ––– ––– ––– ––– –––
Desulfovibrio desulfuricans	AF273034	––– ––– G –– ––– G –– T–– –––
Desulfomicrobium escambiense	AB061531	––– ––– ––– ––– ––– ––– –––
Desulfomonile tiedjei	AF334595	––– ––– ––– ––– ––– ––– –––
Desulfobulbus propionicus	AF218452	––– ––– ––– ––– ––– ––– –––
Desulforhopalus vacuolatus	AF334594	––– ––– ––– ––– ––– ––– –––
Desulfovirga adipica	AF334591	––– ––– ––– ––– ––– ––– –––
Desulforhabdus amnigena	AF337901	––– T–– ––– G–– ––– ––– –––
Desulfotomaculum acetoxidans	AY015580	––– ––– T–– ––– ––– A–– –––
Desulfotomaculum aeronauticum	AY015578	––– ––– ––– ––– ––– ––– A––
Desulfitobacterium dehalogenans	AY337903	––– ––– ––– ––– ––– – T– A––
Desulfosporosinus orientis	AF271767	––– T–– ––– ––– ––– ––– A––
Archaeoglobus fulgidus	M95624	––– ––– T–– ––– ––– – T– –––
Solar Lake Mat Clone 934	AF179345	––– ––– ––– ––– ––– ––– –––
Uncultured prokaryote clone dsrSbII-88	AY167475	––– ––– A –– ––– ––– ––– –––
Uncultured SRB clone UMTRAdsr624-8	AY015596	––– – G – ––– G –– ––– ––– –––
Pyrobaculum islandicum	U75249	GGG ––––AA – AC ATG TAC T–C
Allochromatium vinosum	U84760	– G – GC– GCC – G – ––– – GT –AT

[a] Dashes indicate nucleotides identical to the sequence of the DSRp2060F primer against the *dsrB* gene.

Each PCR mixture contained 1 µl template DNA, 10 µl 10× Ex Taq™ reaction buffer, 100 µM of each dNTP, 2.5 U of Ex Taq™ DNA polymerase and 1 µM of each primer. The *Taq* polymerase, dNTP's and PCR buffer were purchased from TaKaRa (TaKaRa Schuzo Co., Ltd., Biomedical Group, Japan). All primers were synthesized by Qiagen (Qiagen Operon GmbH, Germany). Thermal cycling was carried out by using an initial denaturation step of 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and elongation at 72°C for 1 min. Cycling was completed by a final elongation step of 72°C for 10 min. Positive controls containing purified DNA from SRB reference organisms were included in all of the PCR amplification experiments along with negative controls (no DNA added). The PCR products were examined on 1.5% ethidium bromide-stained agarose gels.

Community patterns based on *dsr* genes were generated using DGGE analysis of PCR-amplified *dsrB* genes. Optimal denaturing conditions were defined based on the theoretical melting temperatures of amplification fragments that were calculated with the Melt95 program (Melt Analysis Software, Version 1.0.1, INGENY International BV, The Netherlands). A 1-mm-thick 8% polyacrylamide gel with a denaturing gradient containing 40 to 70% urea-formamide was used for the DGGE-apparatus. Electrophoresis was performed with the INGENYphorU-2 system (INGENY International BV, The Netherlands) in 1× TAE (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA at pH 8.0) running buffer at 60°C, with a constant voltage of 120 V for 15h. After electrophoresis, gels were stained in a 1× TAE buffer containing 1× SYBR Gold nucleic acid gel stain (Molecular Probes Europe BV, The Netherlands) and photographed under UV light using a Pharmacia digital camera system with Liscap Capture software (ImageMaster VDS; Liscap Image Capture, Version 1.0, Pharmacia Biotech, England).

The detection limit of the (GC-) DSRp2060F/ DSR4R PCR assay was determined by applying PCR on 10-fold dilution series of genomic DNA extracts of *Desulfovibrio desulfuricans* (DSM 642) with an initial concentration of 33.1 ng μl^{-1}.

3.2.5. Cloning and sequencing of dsrB gene fragments

Bands from the *dsrB* based DGGE fingerprints were excised, dissolved in 50 µl of H_2O (deionized) and incubated overnight at 4°C in order to elute the DNA from the gel. 1 µl of the solution was used in a PCR amplification reaction with the corresponding GC-clamped primers. The PCR product was cloned into the pCR®2.1-TOPO® plasmid vector and *Escherichia coli* TOP10 cells using TOPO-TA cloning vector kit according to manufacturer's instructions (Invitrogen). Clones containing recombinant plasmids were examined for the presence of the appropriate insert by PCR using the GC-DSRp2060F/ DSR4R primers. Cloned fragments were compared in DGGE with the community fingerprint of the parent aquifer to identify which signals from the community fingerprint were cloned. Clone inserts with different DGGE-patterns were selected for sequencing of an expected 350 bp fragment (Westburg Genomics, Westburg, The Netherlands).

3.2.6. SRB-subgroup and – genus specific PCR amplification of 16S rRNA-genes

A series of SRB-specific primers sets previously described by Daly *et al.* (2000) and Loy *et al.* (2002) was used. However, as reported by Mills and coworkers (Mills *et al.*, 2003), we observed cross-reactivity of two primer sets, namely DCC305/ DCC1165 and DSV230/ DSV838 with the *Firmicutes* phylum and the class of γ-*proteobacteria*. This prompted us to check the specificity of all primers described by Daly *et al.* (2000) within an alignment of 16S rRNA-genes of SRB and non-sulfate reducing strains that are frequently detected in sulfate-or metal-reducing environments or are close relatives of SRB genera such as *Clostridium*, *Bacillus*, *Shewanella*, *Thiobacillus*, *Methanosarcina*, and modify primer sequences or develop new primers if necessary. This alignment was constructed with the Bionumerics software (Bionumerics Version 1.01., Applied Maths, Belgium).

Table3.3 SRB-subgroup or –genus specific 16S rRNA-gene targeting PCR primers

Primer [a]	Annealing temp (°C)	Sequence 5'-3'	Specificity	Reference
DSVII 230F [b]	63	GAGYCCGCGTYYCATTAGC	*Desulfovibrio* sp., *Desulfomicrobium* sp.	(Daly *et al.*, 2000)
DSVII 838R [b]	63	CCGACAYCTARYATCCATC	*Desulfovibrio* sp., *Desulfomicrobium* sp.	(Daly *et al.*, 2000)
DSM172F	64	AATACCGGATAGTCTGGCT	*Desulfomicrobium* sp.	(Loy *et al.*, 2002)
DSM1469R	64	CAATTACCAGCCCTACCG	*Desulfomicrobium* sp.	(Loy *et al.*, 2002)
DFM140 F	58	TAGMCYGGGATAACRSYKG	*Desulfotomaculum* sp., *Desulfosporosinus* sp.	(Daly *et al.*, 2000)
DFM842 R	58	ATACCCSCWWCWCCTAGCAC	*Desulfotomaculum* sp., *Desulfosporosinus* sp.	(Daly *et al.*, 2000)
DSP140F	60	AAAKCCGGGACAACCCTTG	*Desulfosporosinus* sp.	This study
DSP1107R [c]	60	CTAAAYACAGGGGTTGCG	*Desulfosporosinus* sp.	(Loy *et al.*, 2002)
DSB127F	62	GATAATCTGCCTTCAAGCCTGG	*Desulfobacter* sp.	(Daly *et al.*, 2000)
DSB II 1273R [b]	62	CYYTTTGCRRAGTCGCTGCCCT	*Desulfobacter* sp.	(Daly *et al.*, 2000)
DBM169F	64	CTAATRCCGGATRAAGTCAG	*Desulfobacterium* sp.	(Daly *et al.*, 2000)
DBM1006R	64	ATTCTCARGATGTCAAGTCTG	*Desulfobacterium* sp.	(Daly *et al.*, 2000)
DBBII 121F [b]	66	CGCGTAGATAACCTGTCTTCATG	*Desulfobulbus* sp.	(Daly *et al.*, 2000)
DBBII 1237R [b]	66	GTAGTACGTGTGTAGCCCTGGTC	*Desulfobulbus* sp.	(Daly *et al.*, 2000)
DCC140F	65	CTRCCCYYGGATYSGGGATAAC	*Desulfococcus* sp., *Desulfonema* sp., and *Desulfosarcina* sp.	This study
DCC1273R	65	CTYRCTCTCGCGAGYTCGCTACCCT	*Desulfococcus* sp., *Desulfonema* sp., and *Desulfosarcina* sp.	This study

[a] Primer's short name used in the reference or in this study; numbers refer to the annealing position on the 16S rDNA according to Brosius *et al.* (Brosius *et al.*, 1978)
[b] Modified in position or sequence from the reference
[c] Based on probe DFM1107 from the reference

In addition, primer selectivity was evaluated by the BLASTN program (Altschul *et al.*, 1990), and by application of the primer sets in PCR reactions on the DNA of SRB target strains and negative controls. The nucleotide sequence of primer DSP1107R was based on microarray oligonucleotide probes as designed by Loy *et al.* (2002). For the *Desulfococcus* sp. -*Desulfonema* sp. –*Desulfosarcina* sp.- subgroup, the PCR-primer set DCC 140F/ 1273R was newly developed. PCR amplification products were of the expected size (data not shown) and were only obtained with DNA from target SRB strains. All primers, their target groups or genera, and relevant references are described in Table 3.3. PCR was performed as described above, with annealing temperatures ranging from 55 to 64°C (depending on the 16S rDNA-specific primer pair – see Table 3.3). The PCR products were examined on 1.5% ethidium bromide-stained agarose gels.

3.2.7. DGGE analysis of total microbial community 16S rDNA PCR amplicons

PCR amplifications were performed with the eubacterial primers GC40-63F (5'-GC clamp-CAGGCCTAACACATGCAAGTC-3') and 518R (5'-ATTACCGCGGCTGCTGG-3') as described by El-Frantoussi *et al.* (1999). The PCR products were used for DGGE analysis using the same denaturing and electrophoresis conditions as described above.

3.2.8. Cloning and sequencing of 16S rRNA-gene fragments

DNA bands were excised, reamplified with the corresponding GC-clamped primers and cloned into the pCR®2.1-TOPO® plasmid vector and *Escherichia coli* TOP10 cells using TOPO-TA cloning vector kit. Clones containing recombinant plasmids were examined for the presence of the appropriate insert by PCR using the vector primers M13F/ M13R, followed by PCR with the GC-63F/ 518R primer set. Cloned fragments were compared in DGGE with the community fingerprint of the parent aquifer to identify which signals from the community fingerprint were cloned.

Clone inserts with different DGGE-patterns were selected for sequencing of a ~450 bp long fragment.

3.2.9. DsrB-and 16S rRNA-gene sequence data analysis

DNA-sequences from clones were submitted to GenBank for preliminary similarity analysis using the Advanced BLASTN Search program (Altschul *et al.*, 1990) of the GenBank (http://www.ncbi.nlm.nih.gov/BLAST). In order to translate *dsrB* sequences into protein sequences, they were submitted to the 'transeq' algorithm of the EMBOSS program (version 1.9.1., BEN, Belgium). The obtained protein sequences were imported in an alignment of DsrB protein sequences of SRB strains of the δ-*proteobacteria*, low G+C Gram positive bacteria and sulfate-reducing *Archaea*. Deduced sequences were edited manually to remove regions of ambiguous positional homology. Phylogenetic analyses were performed with the Bionumerics software (version 2.50, Applied Maths, Belgium). Distance-based evolutionary trees were constructed using the neighbor joining algorithm of Saitou and Nei (1987). Distances were conducted using the Kimura 2 parameter. The topography of the branching order within the dendrogram was evaluated by applying the Maximum Parsimony character based algorithm in parallel combined with bootstrap analysis with a round of 1000 samplings. The DsrB sequences of *Thermodesulfovibrio yellowstonii* (GenBank accession no. AAC24112) and *Thermodesulfovibrio islandicus* (GenBank accession no. AAK83214) were used as the outgroup to root the tree, according to Klein and coworkers (Klein *et al.*, 2001), who suggested that the dissimilatory sulfite reductases of the *Archaeaglobales* have a bacterial origin, since paralogous outgrouping of the DsrA and DsrB subunits suggests that the root of the Dsr tree is along the *Thermodesulfovibrio* line of descent.

3.2.10. Nucleotide accession numbers

The *dsrB* gene fragment and 16S rRNA-gene nucleotide sequences of the clones obtained in this study were deposited in the GenBank database under the accession nos. AY731444 to AY731457, and AY731426 to AY731443 respectively.

3.3. Results

3.3.1. Differentiation of PCR-amplified dsrB-genes by DGGE

Wagner and coworkers (Wagner *et al.*, 1998) designed a PCR primer set, DSR1F/ DSR4R, which amplifies most of the α- plus β-subunit (1.9 Kb) of the *dsr*-gene. However, since this amplicon's size is too large for separation by DGGE, a novel primer, (GC-) DSRp2060F, was designed. This primer's sequence was based on an internal sequencing primer described by Pérez-Jiménez (2001), to which a 40 bp GC-clamp was attached 5'. By combining this forward primer with the DSR4R reverse primer, a ~350 bp fragment of the *dsrB* gene was amplified.

The theoretical assumptions concerning primer selectivity were evaluated by performing PCR on DNA extracted from SRB strains belonging to genera of the low G+C Gram positive bacteria and *δ-proteobacteria*. All obtained PCR products had the expected size of 350 bp and no aspecific bands were observed. The detection limit of the GC-DSRp2060F/ DSR4R PCR assay was determined in triplicate by applying PCR on 10-fold dilution series of genomic DNA extracts of *Desulfovibrio desulfuricans* (DSM 642) with an initial concentration of 33.1 ng μl^{-1}. Overall, the GC-DSRp2060F/ DSR4R PCR assay enabled detection down to 3.3 pg of pure genomic DNA; without GC-clamp, an increase in the detection limit was observed leading to a detection limit of 33.3 fg of pure genomic DNA (data not shown). However, in environmental samples this detection limit is expected to be less sensitive, as it is not only dependent on the primer sensitivity but also on the efficiency of the DNA-extraction procedure.

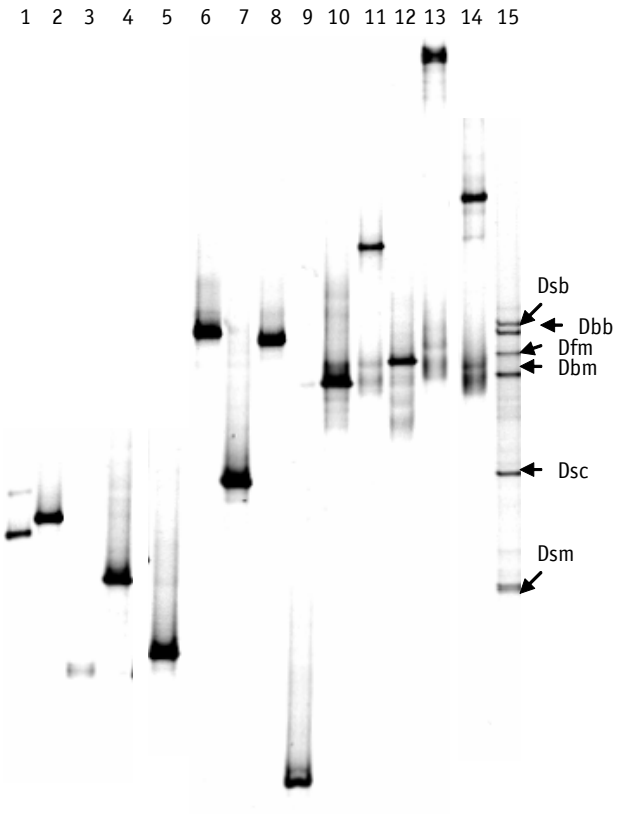

Figure 3.1 DGGE analysis of *dsrB* gene fragments amplified with primer pair GC-DSRp2060F/ DSR4R from pure strains and a DNA mixture. Lanes: 1, *Desulfovibrio desulfuricans* DSM 642; 2, *Desulfovibrio termitidis* DSM 5308; 3, *Desulfovibrio gigas* DSM 1382; 4, *Desulfomicrobium norvegicum* DSM 1741; 5, *Desulfomicrobium escambiense* DSM 10707; 6, *Desulfobacter latus* DSM 3381; 7, *Desulfosarcina variabilis* DSM 2060; 8, *Desulfobulbus rhabdoformis* DSM 8777; 9, *Desulfococcus biacutus* DSM 5651; 10, *Desulfobacterium autotrophicum* DSM 3382; 11, *Desulfotomaculum ruminis* DSM 2154; 12 *Desulfotomaculum aeronauticum* DSM 10349; 13, *Desulfosporosinus auripigmentus* DSM 13351; 14, *Desulfotomaculum acetoxidans* DSM 771; 15, mixture of DNA-extracts of *Desulfobacter latus* DSM 3381 (Dsb), *Desulfobulbus rhabdoformis* DSM 8777 (Dbb), *Desulfotomaculum aeronauticum* DSM 10349 (Dfm), *Desulfobacterium autotrophicum* DSM 3382 (Dbm), *Desulfosarcina variabilis* DSM 2060 (Dsc), and *Desulfomicrobium norvegicum* DSM 1741 (Dsm).

The potential of the PCR-DGGE method to differentiate between different *dsrB* gene sequences from different sulfate-reducing bacteria was investigated by comparing pure strain DGGE-patterns of PCR amplicons of SRB strains of different phyla, families, and genera (Figure 3.1). The majority of the GC-DSRp2060F/ DSR4R amplicons showed single DGGE bands that migrated at different positions.

Different species of the same genus showed different fingerprints, e.g. the PCR-products obtained for *Desulfovibrio desulfuricans* (lane 1), *Desulfovibrio termitidis* (lane 2) and *Desulfovibrio gigas* (lane 3), or *Desulfomicrobium norvegicum* (lane 4) and *Desulfomicrobium escambiense* (lane 5) clearly migrated differently. This was also observed for strains belonging to different genera and phyla. However, some strains revealed extra bands of low intensity, e.g. *Desulfovibrio desulfuricans* (lane 1) and *Desulfotomaculum acetoxidans* (lane 14). DNA bands of *Desulfovibrio desulfuricans* and *Desulfobacterium autotrophicum* were excised, cloned and sequenced, and their *dsrB* gene sequences affiliated for > 99% to the *dsrB* gene of the respective SRB strain in the GenBank database. A DNA-mixture of a set of six sulfate-reducing strains (*Desulfomicrobium norvegicum* (DSM 1741), *Desulfotomaculum aeronauticum* (DSM 10349), *Desulfobacter latus* (DSM 3381), *Desulfobulbus rhabdoformis* (DSM 8777), *Desulfobacterium autotrophicum* (DSM 3382), and *Desulfosarcina variabilis* (DSM 2060), made by mixing equal amounts of equal DNA concentrations (100 ng μl^{-1}), was PCR amplified with the GC-DSRp2060F/ DSR4R primer set and separated by DGGE (lane 15). The obtained DNA-profile showed six bands of equal intensity at the same position as the pure strains, indicating the efficiency of the *dsrB*-DGGE concept.

3.3.2. PCR detection and DGGE profiling of dsrB gene sequences in heavy metal contaminated aquifer samples from ISMP microcosm and pilot experiments

The diversity of *dsrB* genes in SRB-populations was examined in aquifer samples A410 and B305, Pb112 and Pb118, which were used in ISMP microcosm studies. In addition, the *dsrB* DGGE approach was applied to aquifer samples derived from the site of an ongoing *in situ* metal precipitation pilot test. The *dsrB* gene sequences were amplified from the total community DNA using the GC-DSRp2060F/DSR4R approach and the resulting amplicons were subjected to DGGE analysis. Dominant bands were cloned and sequenced (Table 3.4).

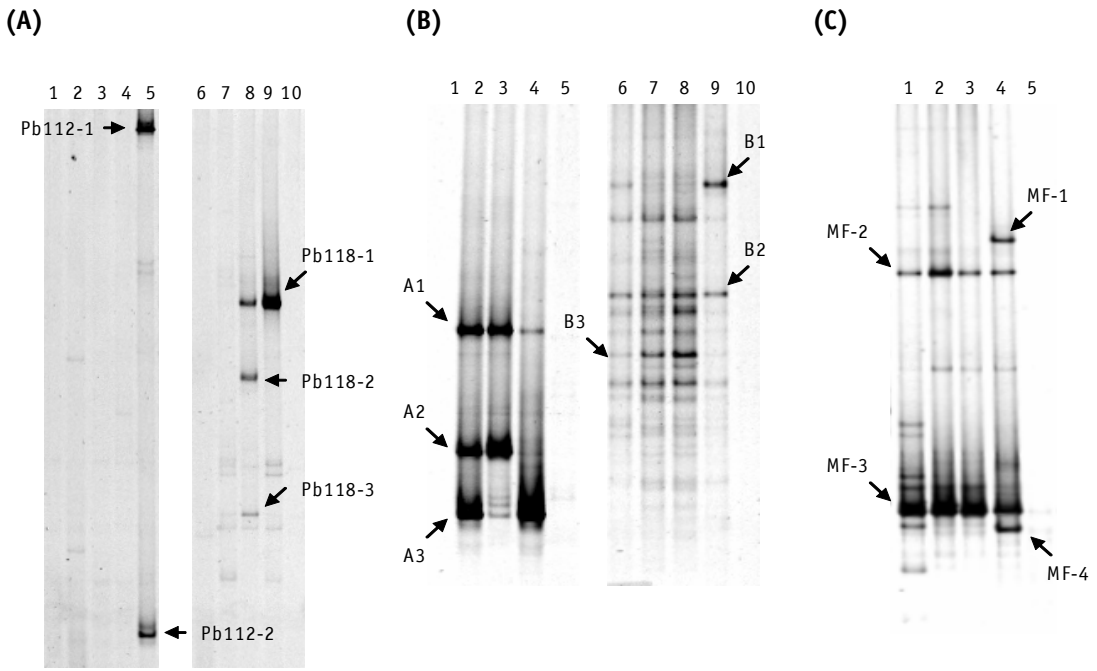

Figure 3.2 (A) *dsrB*-based DGGE fingerprint of aquifer samples of Pb112 and Pb118 microcosm experiments. Legend: A, aquifer; G, groundwater; Lanes: 1, Pb112 A + G + formaldehyde; 2, Pb112 A + G; 3, Pb112 A + G + HRC®; 4, Pb112 A + G + molasses; 5, Pb112 A + G + acetate; 6, Pb112 A + G + formaldehyde; 7, Pb118 A + G; 8, Pb118 A + G + HRC®; 9, Pb118 A + G + molasses; 10, Pb118 A + G + acetate. **(B) *dsrB*-based DGGE fingerprint of aquifer samples of A410 and B305 microcosm experiments.** Lanes: 1, A410 A + G; 2, A410 A + G + HRC®; 3, A410 A + G + molasses; 4, A410 A + G + lactate; 5, A410 A + G + formaldehyde; 6, B305 A + G; 7, B305 A + G + HRC®; 8, B305 A + G + molasses; 9, B305 A + G + lactate; 10, B305 A + G + formaldehyde. **(C) *dsrB*-based DGGE fingerprint of aquifer samples MF02, MF03, MF04, MF05, MF06 of an ISMP pilot experiment.** Lanes: 1, MF02; 2, MF03; 3, MF04; 4, MF05; 5, MF06. Excised and cloned bands are indicated within the aquifer fingerprints based on the comparison of migration profiles of pure clones and aquifer profiles.

a) Samples Pb112 and Pb118

For the setups with Pb112, only the addition of acetate resulted in sulfate-and Zn removal after 25 weeks: the SO_4^{2-}-concentration decreased 23%, and the Zn-concentration decreased 81%. Moreover, only for the acetate-amended setup a *dsrB* gene PCR fragment could be obtained.

The *dsrB* based DGGE community profile (Figure 3.2A) showed little diversity: only two DNA bands were observed, which were cloned and sequenced. The deduced protein sequences were related for 94% to the DsrB protein sequence of *Desulforhopalus singaporensis* (band Pb112-1), clustering within the family of *Desulfobulbaceae* (Figure 3.3), while the protein sequence corresponding to band Pb112-2 exhibited 75% sequence similarity with the DsrB of *Desulfoarculus baarsii*.

For the setups with Pb118, sulfate-and metal removal from the groundwater was most significant when molasses or HRC® were supplied as a C-source/ electron donor: after 25 weeks, up to 85% of the initial sulfate was consumed, whereas Zn and Ni-concentrations in the water phase decreased with more than 99% efficiency. Also, 35% to 40% of Fe was removed from the solution. For the setup without the addition of nutrients, low metal precipitation activities (> 15%) were obtained, indicating some residual activity. When acetate was added, no sulfate-consumption or metal-removal could be observed. PCR with the GC-DSRp2060F/ DSR4R primer set resulted in a *dsrB* gene amplicon for the condition without extra C-source and for the molasses and HRC®-amended set-ups as was expected based on the metal removal data. Only one intense DNA-band, Pb118-1, was revealed in the DGGE profile for the condition with molasses, which was also present in the profile of the setup with HRC® (Figure 3.2A) and consequently, the corresponding SRB strain is assumed to be responsible for an efficient ISMP process. Both *dsrB*

Table 3.4 Cloned *dsrB* gene sequences retrieved from HMM-contaminated aquifer samples.

Origin	DGGE band designation	Genebank Accession no.	Nearest match in BLASTX analysis		
			Protein (Accession no.[a])	Host	Protein identities
Pb112	Pb112-1	AY731444	*DsrB* (AAL57466)	*Desulforophalus singaporensis*	94%
	Pb112-2	AY731445	*DsrB* (AAK83216)	*Desulfoarculus baarsii*	75%
Pb118	Pb118-1	AY731446	*DsrB* (AAK82967)	*Desulfotomaculum acetoxidans*	92%
	Pb118-2	AY731447	*DsrB* (AAK58405)	*Desulfotomaculum putei*	84%
	Pb118-3	AY731448	*DsrB* (AAG28586)	*Desulfobulbus propionicus*	87%
A410	A1	AY731449	*DsrB* (AAC24110)	*Desulfobotulus sapovorans*	97%
	A2	AY731450	*DsrB* (AAL99083)	*Desulfovibrio aespoeensis*	96%
	A3	AY731451	*DsrB* (AAL57452)	*Desulfonatronum lacustre*	89%
B305	B2	AY731452	*DsrB* (BAB55558)	*Desulfomicrobium norvegicum*	95%
	B3	AY731453	*DsrB* (AAC24104)	*Desulfotomaculum aeronauticum*	85%
MF	MF-1	AY731454	*DsrB* (AAK13056)	*Desulfotomaculum acetoxidans*	88%
	MF-2	AY731455	*DsrB* (AAK13056)	*Desulfotomaculum acetoxidans*	89%
	MF-3	AY731456	*DsrB* (AAK58407)	*Desulfotomaculum aeronauticum*	79%
	MF-4	AY731457	*DsrB* (AAK13056)	*Desulfotomaculum geothermicum*	86%

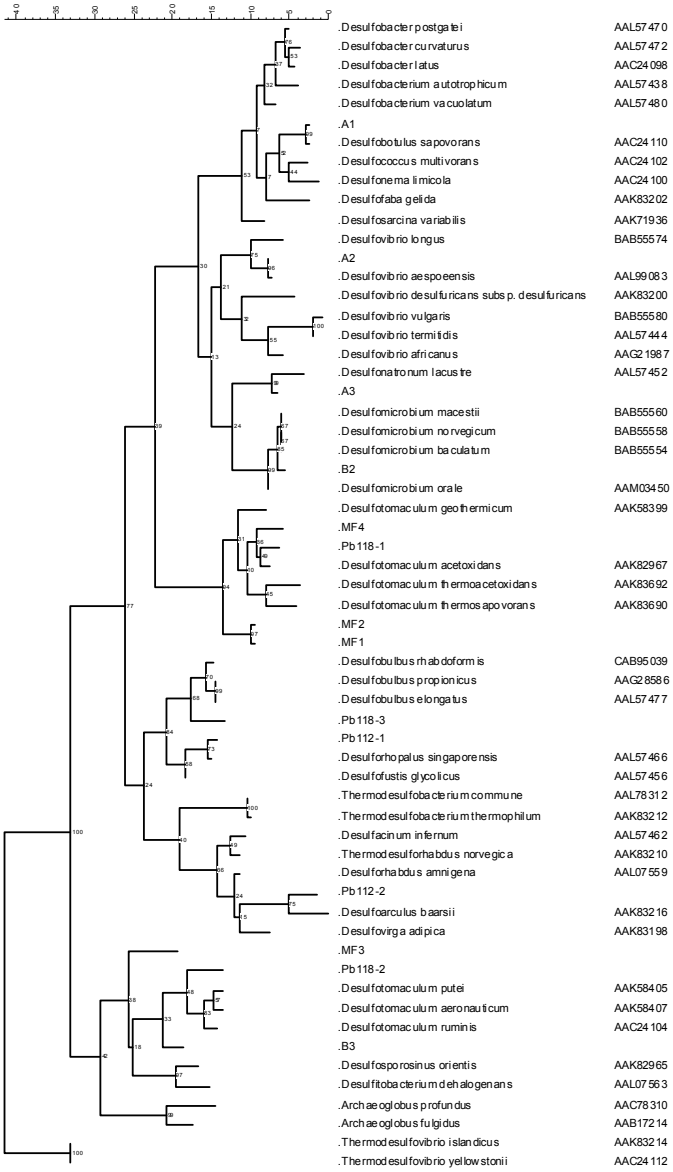

Figure 3.3 Phylogenetic analysis of DsrB protein sequences detected in aquifers Pb112 and Pb118, A410 and B305, MF02, MF03, MF04, MF05 and MF06. The evolutionary tree was generated by the neighbor joining method; evolutionary distances were generated using the Kimura 2 parameter. The Maximum Parsimony algorithm was used to evaluate branching orders. Bootstrap resampling (1000 replicates) of the tree was performed to provide confidence estimates for the inferred topologies. Percentages of bootstrap support are indicated at the branch points. An out-group of the DsrB proteins of *Thermodesulfovibrio yellowstonii* and *Thermodesulfovibrio islandicus* was included to root the tree. The bar at the top indicates the estimated evolutionary distance.

sequences were found to be identical. The deduced protein sequences affiliated for 92% with the DsrB protein of *Desulfotomaculum acetoxidans*, clustering within the DsrB protein lineage of the *Desulfotomaculum* species that is monophyletic with δ-proteobacterial Dsr sequences (Klein *et al.*, 2001) (Figure 3.3).
HRC® amendments resulted in two additional DNA-bands, Pb118-2 and Pb118-3, of which the deduced DsrB protein sequences were related to the DsrB proteins of *Desulfotomaculum putei* (84%) and *Desulfobulbus propionicus* (87%), respectively. The DGGE fingerprint of the condition where no extra nutrients were added was more complex and the DNA bands were fainter, indicating that little SRB-strain selection had occurred and suggesting that there was low sulfate-reduction activity, as was confirmed after groundwater analysis.

b) Samples A410 and B305

For aquifer and groundwater A410, amendment with HRC® and lactate resulted after 8 weeks in 99% removal of initial SO_4^{2-}-concentration, a decrease of 61% of Fe-concentration, and a reduction of 43% of the As(V)-concentration. The molasses amended set-up showed a SO_4^{2-}-consumption of 82% and a decrease in Fe and As(V) concentrations of 52% and 41% respectively. Since pH conditions were rather high (pH 10), As(V) removal is thought to be mainly due to coprecipitation with iron sulfides (Moore *et al.*, 1989) or by adsorption by metal sulfides (Davis, 1984), instead of direct reduction to As(III) and subsequent precipitation as orpigment (As_3S_2). Only for the experimental setups amended with molasses, lactate, and HRC®, a *dsrB*-PCR amplicon was obtained. DGGE analysis of the amplicons (Figure 3.2B) showed that one DNA band, designated A1, was commonly found. The DNA-bands were excised for cloning and sequencing purposes, and it was found that their *dsrB* fragment sequences were identical. The deduced protein sequence of A1 affiliated for 97% to that of the DsrB protein of *Desulfobotulus sapovorans*, a member of the *Desulfobacteraceae* family (Figure 3.3). The setups with molasses and HRC® had an additional DNA

band in common, named A2, of which the deduced protein sequence clustered for 96% to the DsrB protein sequence of *Desulfovibrio aespoeensis*. The DGGE banding patterns of the conditions with HRC® and lactate both show a very intense DNA band, A3. Its corresponding protein sequence exhibited 89% sequence similarity with *Desulfonatronum lacustre,* forming a divergent cluster with the DsrB sequences of other members of the order of *Desulfovibrionales*, such as *Desulfovibrio gigas* and *Desulfomicrobium escambiense* (Figure 3.3). This DNA-band was also present in the profile of the setup with molasses but with a much lower intensity. Linking these results to the results of analytical analyses, it might be concluded that the sulfate-reduction and metal precipitation activity of this *Desulfonatronum*-related SRB strain results in a slightly more effective ISMP process.

For the microcosm experiments with aquifer and groundwater B305, the initial As(V) concentration (0.06 mg l^{-1}) was about 10× lower compared to A410 (0.7 mg l^{-1}), contained less sulfate (110 mg l^{-1} instead of 200 mg l^{-1} SO_4^{2-}), but its Fe concentration were 10x higher (5.56 mg l^{-1} in A410, 60.1 mg l^{-1} in B305). Although the initial pH was 9.6, addition of molasses or HRC® resulted in a significant decrease in pH (pH < 7.5). Together with a redox value E_0 < -400 mV, these conditions could result in direct reduction of As(V) to As(III) by SRB, and the subsequent formation of As_3S_2 (Jong and Parry, 2003). After 10 weeks, sulfate was completely consumed in the setups with lactate, molasses and HRC® but the decrease in As(V) concentration was most significant for HRC® (83%) and molasses (71%). For lactate, As(V) removal was no more than 63%. Also for the condition without additional C-source/ electron donor, a decrease in As(V) was observed (40%) together with a SO_4^{2-}-consumption of 32%. These differences in analytical results were reflected in the *dsrB* based DGGE profile of the DNA extracts (Figure 3.2B). Only for the abiotic control, no PCR fragment could be obtained. For the molasses and HRC® amended setups and for the setup without added substrates, DGGE-community profiles were very similar to each other and showed greater diversity than the profile of the lactate-amended setup. One DNA

band (B2) was commonly found in these conditions but was less intense for the setup with lactate; the corresponding DsrB protein sequence affiliated most closely (95%) to that of *Desulfomicrobium norvegicum*. DNA band B3 was revealed in the DGGE pattern of the molasses and HRC® -amended conditions, but with much lower intensity in the setup with lactate, which suggests that this SRB strain might be a key player in an effective metal bioprecipitation process in these microcosms. Its deduced DsrB protein sequence showed 85% identity to the DsrB protein of *Desulfotomaculum aeronauticum*, forming a divergent cluster with the genera *Desulfosporosinus* and *Desulfitobacterium* (Figure 3.3). One DNA band could only be detected in the condition with lactate (B1), but cloning of the excised band was not successful.

c) Samples MF02, MF03, MF04, MF05 and MF06

The *dsrB* based DGGE approach was also applied to samples MF02, MF03, MF04, MF05 and the control MF06, which were derived from an field-scale pilot test for ISMP. Despite the fact that results of *on site* physico-chemical analysis showed that sulfate-and heavy metal removal occurred in the order MF04 > MF03 > MF02 > MF05, the *dsrB* based DGGE community profiles were very similar for all samples (Figure 3.2C), and no clear link could be established between the observed differences in ISMP activity and the DGGE community patterns. The community profile of MF02, MF03 and MF04 were dominated by 2 intense DNA-bands, designated as MF-2 and MF-3. Both DNA-bands were also present in the *dsrB* fingerprint of sample MF05. The protein sequence of band MF-2 exhibited 89% sequence similarity with the DsrB protein sequence of *Desulfotomaculum acetoxidans*, whereas the MF-3 deduced protein sequence clustered with *Desulfotomaculum aeronauticum* (79% protein similarity). Additional DNA-bands MF-1 and MF-4 were observed in the *dsrB* based DGGE pattern of MF05. Their deduced protein sequence clustered with the DrsB proteins of *Desulfotomaculum*

acetoxidans (88% protein similarity) and *Desulfotomaculum geothermicum* (86% protein similarity), respectively.

3.3.3. 16S rRNA-gene based analysis of the SRB-community composition and structure

In order to verify the reliability of the *dsrB* based DGGE fingerprinting method for community characterization, the results were compared to results based on the 16S rRNA gene. First, the outcome of *dsrB* based DGGE was compared to results of PCR amplification with SRB subgroup and genus specific 16S rDNA primers (Table 3.3). In addition, the outcome of *dsrB* DGGE was compared to results of eubacterial 16S rDNA DGGE analysis (Figures 3.4, 3.5, 3.6). Only for the DNA extracts where *dsrB* genes were detected, dominant DNA bands in the DGGE profiles were excised, cloned and sequenced (Table 3.5).

a) Samples Pb112 and Pb118

For the Pb112 microcosm experiments, only with the DSVII 230F/ DSVII 838R primer set that is specific for the detection of the *Desulfovibrio-Desulfomicrobium*-like SRB group, a 500 bp PCR amplicon was obtained, and this only in the acetate-amended setup (data not shown). Since no PCR product was obtained with the *Desulfomicrobium*-genus specific primer set DSM172F/ DSM1469R, it was concluded that the DSVII 230F/ DSVII 838R amplicon originates from SRB-strains of the genus *Desulfovibrio*. This correlates with the similarity of the protein sequence derived from *dsrB*-DGGE band Pb112-2 to the DsrB protein of *Desulfoarculus baarsii*. However, using the 16S rDNA primers we found no indication for the presence of strains related to the genus *Desulforhopalus* (*dsrB*-DGGE band Pb112-1). 16S rDNA DGGE bands Pb112-S1 and Pb112-S2 were uniquely observed in the profile of the acetate-amended setup with aquifer and groundwater Pb112 (Figure 3.4).

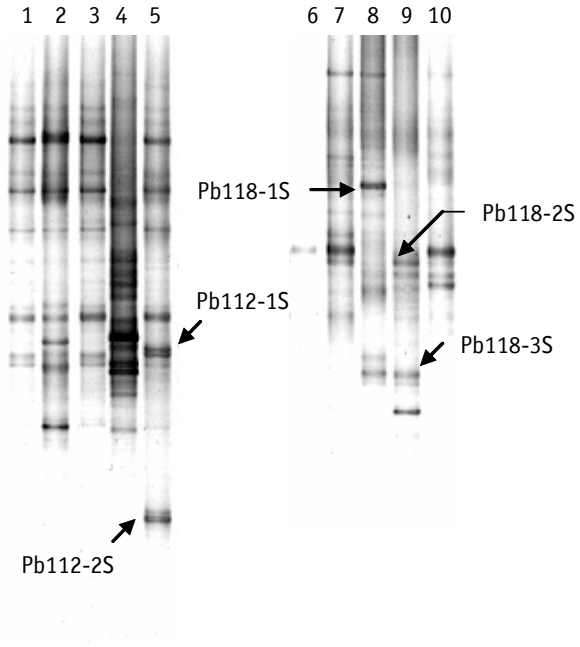

Figure 3.4 16S rRNA-based DGGE fingerprint of aquifer samples of Pb112 and Pb118 microcosm experiments. Legend: A, aquifer; G, groundwater; Lanes: 1, Pb112 A + G + formaldehyde; 2, Pb112 A + G; 3, Pb112 A + G + HRC®; 4, Pb112 A + G + molasses; 5, Pb112 A + G + acetate; 6, Pb112 A + G + formaldehyde; 7, Pb118 A + G; 8, Pb118 A + G + HRC®; 9, Pb118 A + G + molasses; 10, Pb118 A + G + acetate. Excised and cloned bands are indicated within the aquifer fingerprints based on the comparison of migration profiles of pure clones and aquifer profile.

Since acetate was the only experimental condition were SRB-activity and *dsrB* gene sequences were detected, we assumed that these DNA-bands might originate from the SRB strains. Indeed, the sequence of DNA band Pb112-1S exhibited 98% sequence similarity with *Desulfoarculus baarsii*, thereby confirming the sequencing result of the *dsrB*-DGGE band Pb112-2, whereas the sequence of Pb112-1S affiliated closely to the 16 rRNA-gene of the uncultured sulfate-reducing bacterium strain 141 (94% sequence similarity) whose closest cultured relative was *Desulforhopalus singaporensis* (92% sequence similarity).

In the Pb118 microcosm setups with molasses and HRC®, *Desulfotomaculum*-and *Desulfosporosinus*-like 16S rRNA-genes were detected (data not shown). No amplicon was obtained with DSP140F/ DSP 1107R primer set, indicating that the DFM140F/ DFM842R amplicon originates from *Desulfotomaculum* strains conform

to the results of *dsrB* gene sequence analysis. In the HRC® set-up a *Desulfobulbus*-like 16S rRNA-gene was detected with the DBB 121F/ DBB 1237R primer set, conform to the identification of *Desulfobulbus propionicus* with the *dsrB* based DGGE approach. The 16S rDNA DGGE community profiles of the molasses-and HRC®-amended setups had DNA band Pb118-1S in common (Figure 3.4). As expected from the *dsrB* gene sequencing results, this DNA-band corresponded to a SRB strain related to *Desulfotomaculum acetoxidans* (95% sequence similarity). The remaining common DNA-bands in the molasses-and HRC® DGGE-fingerprints did not show any relationship with sulfate-reducers but affiliated with 16S rRNA-gene sequences of members of the order *Actynomycetales* and the genus *Clostridium*, which might play a role in the conversion of the carbon source for the consortium.

b) Samples A410 and B305

For the A410 microcosm set-ups with molasses, lactate, and HRC®, an amplicon was obtained only with primers specifically detecting the *Desulfovibrio-Desulfomicrobium*-subgroup (data not shown). Since no PCR product was obtained with the *Desulfomicrobium*-genus specific primer set, it can be concluded that SRB-strains of the genus *Desulfovibrio* are predominantly present in the A410 microcosm setups. This corresponds with the cloning and sequencing results of the *dsrB*-DGGE band A2 (related to DsrB of *Desulfovibrio aespoeensis*) and A3 (related to *DsrB* of *Desulfonatronum lacustre*). However, there is no indication for the presence of strains related to the genus *Desulfobotulus* (*dsrB*-DGGE band A1). In the 16S rDNA-DGGE patterns of the A410 microcosms (Figure 3.5), two DNA bands were found in common for the setups with HRC, molasses and lactate, namely A-1S and A-2S. Since the *dsrB* based DGGE patterns also had two DNA bands in common for these setups, namely A1 and A3, we expected their sequences to affiliate with the 16S rRNA-genes of *Desulfobotulus sapovorans* and *Desulfonatronum lacustre*. The clone sequence of DNA band A-1S exhibited 97%

sequences similarity with *Desulfonatronum lacustre*, according to the *dsrB*-DGGE band A3, but the clone sequence of A-2S was most closely (96%) related to *Desulfovibrio aespoeensis*, conform to *dsrB* based DGGE band A2. No other cloned and sequenced DNA bands corresponded with the 16S rRNA-gene of a sulfate-reducer. When the SRB-specific primer sets were applied to DNA extracts of the B305 microcosm experiments, amplicons were obtained with the primer sets DFM140F/ DFM842R, DSVII 230F/ DSVII 838R and DSM172F/ DSM1469R (data not shown).

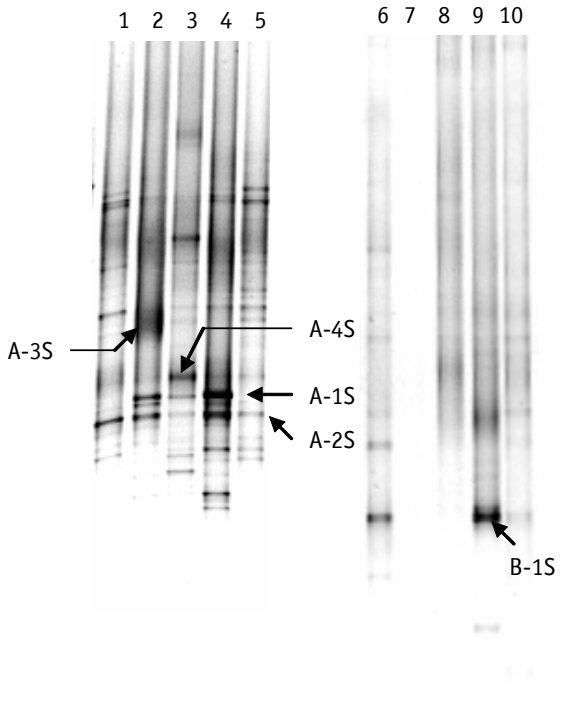

Figure 3.5 16S rRNA-based DGGE fingerprint of aquifer samples of A410 and B305 microcosm experiments. Legend: A, aquifer; G, groundwater. Lanes: 1, A410 A + G; 2, A410 A + G + HRC®; 3, A410 A + G + molasses; 4, A410 A + G + lactate; 5, A410 A + G + formaldehyde; 6, B305 A + G; 7, B305 A + G + HRC®; 8, B305 A + G + molasses; 9, B305 A + G + lactate; 10, B305 A + G + formaldehyde. Excised and cloned bands are indicated within the aquifer fingerprints based on the comparison of migration profiles of pure clones and aquifer profile.

Table 3.5 Cloned 16S rRNA-gene sequences retrieved from HMM-contaminated aquifer samples

Origin	DGGE band designation	GenBank Accession no.	Nearest match in BLASTN analysis	Genebank Accession no.	Sequence identities
Pb112	Pb112-1S	AY731426	*Desulfoarculus baarsii*	M34403	98%
	Pb112-2S	AY731427	*Desulforhopalus singaporensis*	AF118453	92%
Pb118	Pb118-1S	AY731428	*Clostridium straminisolvens*	AB125279	87%
	Pb118-2S	AY731429	*Clostridiales bacterium oral clone*	AF481208	94%
	Pb118-3S	AY731430	*Desulfotomaculum acetoxidans*	Y11566	95%
	Pb118-4S	AY731431	*Micropruina glycogenica*	AB012607	93%
A410	A-1S	AY731432	*Desulfonatronum lacustre*	AF418171	97%
	A-2S	AY731433	*Desulfovibrio aespoeensis*	X95230	96%
	A-3S	AY731434	*Clostridium quinii*	X75745	97%
	A-4S	AY731435	*Alkaliphilus auruminator*	AB037677	97%
B305	B-1S	AY731436	*Thiobacillus* Q strain LMD81.11	AJ289884	98%
MF	MF-1S	AY731437	unidentified *ε-proteobacterium* BD4-8	AJ619043	93%
	MF-2S	AY731438	*Comamonadaceae* bacterium FJS31	AY315178	99%
	MF-3S	AY731439	*Sphingomonas* sp. B28161	AJ001052	97%
	MF-4S	AY731440	*Sphingobium yanoikuyae* isolate 4.9	AJ627397	92%
	MF-5S	AY731441	uncultured β-*proteobacterium* clone Spb283	AJ422174	95%
	MF-6S	AY731442	*Desulfotomaculum reducens*	U95951	91%
	MF-7S	AY731443	*Desulfotomaculum geothermicum*	AF113543	96%

These results confirm cloning and sequencing results of *dsrB*-DGGE bands B2 and B3, of which the protein sequences affiliated respectively with DsrB sequences of the genera *Desulfomicrobium* and *Desulfotomaculum*. The 16S rDNA-DGGE profiles of the B305 experiments showed very few DNA-bands compared to the *dsrB*-DGGE profiles. DNA-band B-1S was found to be very intense in the profile of the molasses-amended setup (Figure 3.5). However, its clone sequence was most similar (98%) to *Thiobacillus* Q strain LMD81.11.

c) Samples MF02, MF03, MF04, MF05, MF06

The presence of *Desulfotomaculum*-and *Desulfosporosinus* like 16S rRNA-genes in all MF samples, with the exception of the MF06 control sample (data not shown), was demonstrated using the DFM 140F/ DFM 842R primer set. No amplicon was obtained with DSP140F/ DSP 1107R primer set, showing that the DFM140F/ DFM842R amplicon originates from *Desulfotomaculum* strains, which is conform to the results of *dsrB* gene sequence analysis. Again, no information about the diversity of the *Desulfotomaculum*-population is obtained.
When the 16S rDNA-DGGE was applied to the MF samples (Figure 3.6), one intense DNA-band (MF-7S) appeared in the fingerprints of MF03, MF04 and MF05. Comparing the fingerprints with those obtained with the *dsrB*-based DGGE approach, it was observed that the presence of MF-7S coincided with the presence of the *dsrB*-derived band MF-4. As anticipated, its DNA sequence affiliated most closely (96%) to *Desulfotomaculum geothermicum*. One 16S rDNA-band appeared only in the MF05 sample; this band (MF-6S) was expected to correspond to the same SRB-strain that possessed the *DsrB* protein MF-3; this assumption was correct, since its 16S rDNA sequence was most related to *Desulfotomaculum reducens* (91% sequence similarity) and *Desulfotomaculum aeronauticum* (90% sequence similarity).

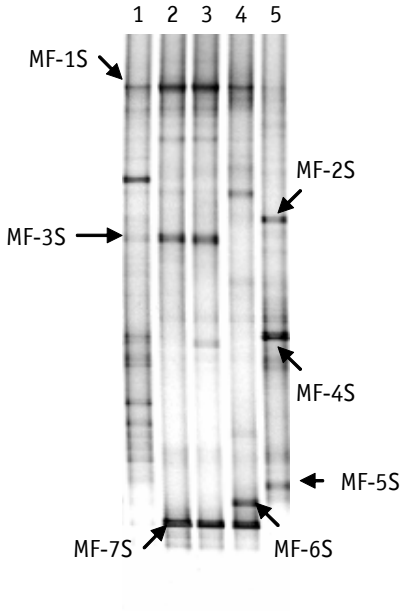

Figure 3.6 16S rRNA-based DGGE fingerprint of aquifer samples MF02, MF03, MF04, MF05, MF06 of an ISMP pilot experiment. Lanes: 1, MF02; 2, MF03; 3, MF04; 4, MF05; 5, MF06. Excised and cloned bands are indicated within the aquifer fingerprints based on the comparison of migration profiles of pure clones and aquifer profile.

3.4. Discussion

In this study we describe the development of a specific PCR-DGGE method to rapidly assess the diversity of sulfate-reducing populations in environmental samples based on their *dsrB* gene. The specificity of the GC-DSRp2060F/ DSR4R DGGE-primer set was proven by PCR on DNA from pure SRB cultures, which resulted in PCR products of the expected 350 bp, and DNA sequences of DGGE bands showing > 99% similarities with the *dsrB* gene of their corresponding SRB strains. The detection limit of the primer set without GC-clamp was 33.1 fg μl^{-1}, but attachment of a 40 bp GC-clamp for DGGE analysis resulted in a 100-fold decrease of this detection limit. Leys *et al.* (submitted for publication) and Vanbroekhoven *et al.* (2004) found that the attached GC-clamp plays a crucial role in processes controlling PCR-sensitivities of respectively *Mycobacterium* and *Acinetobacter* specific PCR systems. DGGE-analysis of amplified *dsrB* gene

fragments could differentiate between SRB at the genus and species level, showing that the *dsrB*-based DGGE can be used for diversity analysis of SRB-populations. However, some strains (e.g. *Desulfovibrio desulfuricans* and *Desulfotomaculum acetoxidans*) showed multiple-band DGGE patterns. Although additional *dsr*-gene homologs may exist in SRB strains, it was demonstrated that *Desulfotomaculum* species do not contain multiple *dsr* copies (Klein *et al.*, 2001). Since DGGE-analysis of the 16S rRNA-gene of SRB-cultures showed single-band DGGE-patterns, it excluded the possibility of culture impurities (data not shown). Probable, the presence of multiple bands for one strain is a consequence of the degeneracy of the DSRp2060F-primer, resulting in two or more copies of the same *dsrB* gene that display minor sequence variation, possibly followed by the formation of heteroduplexes that are less stable and melt under weaker denaturing conditions. Although the possible appearance of multiple-band DGGE fingerprints for single SRB strains might cause overestimation of the SRB-population diversity, it should be noted that these extra bands had minor intensities and that they were not found in the DGGE fingerprints of environmental samples.

Although the *dsr*-based phylogenetic tree is largely consistent with conventional 16S rDNA-based phylogenetic tree topologies, seven thermophilic *Desulfotomaculum* species and two *Thermodesulfobacterium* species were reported to possess non-orthologous *dsr*-genes (Klein *et al.*, 2001). However, recent studies of the diversity of uncultured SRB populations by cloning and sequencing of PCR-amplified *dsr* genes shows that the method is effective to estimate the diversity and distribution of SRB within a complex microbial communities in ecosystems such as sediments (Dhillon *et al.*, 2003; Joulian *et al.*, 2001; Liu *et al.*, 2003; Thomsen *et al.*, 2001), microbial mats (Minz *et al.*, 1999; Nakagawa *et al.*, 2002), anaerobic digesters (Tang *et al.*, 2004), deep-sea worms (Cotrell and Cary, 1999), uranium tailings (Chang *et al.*, 2001) and within enriched hydrocarbon-degrading consortia (Pérez-Jiménez *et al.*, 2001). Moreover,

sequence analysis of PCR-amplified environmentally recovered *dsr* genes often identified species as sulfate-reducers, whereas 16S rRNA-gene sequencing analysis could not relate them to any cultivated organism, also because some lineages of sulfate-reducers are closely related to organisms that are unable to carry out dissimilatory sulfate reduction (Dhillon *et al.*, 2003; Joulian *et al.*, 2001; Minz *et al.*, 1999; Nakagawa *et al.*, 2002). Most molecular investigations of sulfate reducers in the environment used the DSR1F/ DSR4R primer set of Wagner *et al.* (1998), which encompasses 1,9 Kb of the *dsr* gene, instead of the 350 bp *dsrB* fragment used here. Liu *et al.* (2003) compared *dsrAB* phylogenies with *dsrA* and *dsrB* phylogenies and found that comparison of longer sequence tracks improved the reproducibility and low-order branch resolution (*dsrAB* > *dsrB* > *dsrA*) but demonstrated that these fine-scale differences did not alter the outcome of genetic diversity studies of SRB-populations in continental margin sedimentary habitats. Others also demonstrated that the use of 400 bp sequences of Dsr subunits was sufficient for phylogenetic analysis of the SRB-community colonizing the back of the deep-sea worm *Alvinella pompejana* (Cotrell and Cary, 1999), in deep-sea cold sediment (Fukuba *et al.*, 2003) and in an anaerobic digester (Tang *et al.*, 2004).

Here, the *dsrB*-based DGGE method was successfully used to establish the diversity within the SRB-communities in different HMM contaminated environmental samples. Sequence analysis of the *dsrB*-based DGGE fingerprints revealed the presence of *dsrB* genes closely related to those of *Desulforhopalus singaporensis*, *Desulfoarculus baarsii*, *Desulfovibrio aespoeensis*, *Desulfonatronum lacustre*, *Desulfomicrobium norvegicum*, *Desulfobulbus propionicus*, *Desulfobotulus sapovorans*, *Desulfotomaculum aeronauticum*, *Desulfotomaculum acetoxidans*, and *Desulfotomaculum putei*. Protein similarities varied from 75% to 97%. Fukuba and coworkers (Fukuba *et al.*, 2003) calculated that the deduced amino acid sequences of DsrA and DsrB proteins of the same genus show high homologies, e.g. amino acid homology of 82% was shown between the DsrB of sulfate-reducing bacteria *Desulfovibrio vulgaris* and *Desulfovibrio gigas*, and a DsrB

homology of 75% was seen between the sulfate-reducing archaea *Archaeaglobus fulgidus* and *Archaeaglobus profundus*. They suggested that two sequences displaying a DsrA or DsrB-protein homology greater than 75% would belong to a coherent group (namely the same genus), to loosely related genera, or to the same family. Thus, sequencing results of *dsrB* derived fragments allowed characterization of the composition of the SRB population. Although it was expected that phylogenetic interpretation of DGGE fingerprints of distinct lineages might be complicated due to lateral gene transfer of *dsr* genes (Klein *et al.*, 2001; Stahl *et al.*, 2002), the only effect of lateral acquisition of *dsr* genes is that the position of DNA bands on the DGGE-gel will not reflect the G+C content of the host genome but of the *dsr* gene donor. For example, *Desulfotomaculum dsrB* gene sequences which received the xenologous *dsr* genes from δ-*proteobacteria*, melt under higher denaturing conditions than orthologous *Desulfotomaculum dsrB* gene sequences.

The outcome of the *dsrB*-based DGGE method was compared to results of PCR amplification of SRB subgroup and genus specific primers and DGGE analysis of 16S rDNA. Although the use of SRB subgroup and genus specific PCR primers allowed a coarse insight in the SRB community composition in the different aquifer samples, they have the limitation that, obviously, only bacteria which are targeted by the primer sets can be detected. For example, no primer set is available for detection of the genus *Desulfobotulus*. Another shortcoming of PCR amplification with the primer sets is that they don't give information about the diversity within a particular SRB-population. For example, a PCR amplicon was obtained with the primer set DFM140F/ DFM842R for the Pb118 DNA, but this does not allow discrimination between different *Desulfotomaculum* species. In order to elucidate the members of the same genus within the SRB-population, a 16S rDNA-gene library can be constructed with the PCR amplicons, but this is very time-consuming and laborious, especially if one aims to monitor SRB-populations over space and time. An alternative would be to attach a GC-clamp to one of the primers and separate the PCR amplicons by DGGE.

With regard to 16S rDNA-DGGE profiling of the whole bacterial community, it can be concluded in general that 16S rDNA-based profiles did not show the same diversity in the SRB-communities as the *dsrB* based profiles. Usually, PCR-DGGE profiles obtained with 16S rRNA-gene based PCR primers yield complex patterns that reflect the composition of populations that constitute >10% of the microbial community (Gelsomino *et al.*, 1999). On the other hand, PCR biases can occur (Lapara *et al.*, 2000; Muyzer and Smalla, 1998; Ogino *et al.*, 2001). Our results suggest that preferential amplification of 16S rRNA genes may bias the interpretation of fingerprinting results with regards to the SRB-community. Thus, presumably a major advantage of the *dsrB*-DGGE fingerprinting technique is that minor populations of SRB in a highly diverse microbial community are not overlooked. However, combined use of the *dsr* and 16S rRNA genes will give more realistic and deeper insight into the function and structure (species composition) of polluted environmental habitats, especially when the SRB-activity is affected by other microorganisms such as methanogens, fermenting bacteria, acetogens, etc. In addition, this approach allows to estimate to what extent the SRB populations predominate in the whole microbial community.

In conclusion, the developed *dsrB*-based DGGE method is an effective tool to monitor the abundance, diversity and dynamics of sulfate-reducers in heavy metal-polluted environmental samples. In the context of optimization of bioremediation strategies such as ISMP, the profiling of SRB communities based on *dsr* genes should focus more on metabolically active microorganisms.

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CHAPTER 4

Molecular Monitoring of SRB Community Structure and Dynamics in Batch Experiments to Examine the Applicability of *in situ* Precipitation of Heavy Metals for Groundwater Remediation

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Abstract

Sulfate-reducing bacteria (SRB) are known for their capacity to reduce and precipitate heavy metals (HM) as metal sulfides, offering the opportunity to create an *in situ* reactive zone for the treatment of heavy metal-contaminated groundwater. Batch experiments were conducted in order to investigate the feasibility of such an *in situ* metal precipitation (ISMP) as a groundwater remediation strategy for an industrial site contaminated with elevated levels of Zn, Cd, Co and Ni. The potential of different types of carbon source/ electron donor (lactate, acetate, methanol, ethanol, Hydrogen Release Compound®, molasses) to stimulate the sulfate-reduction and metal-precipitation activity of the indigenous SRB community was explored. In addition, the effect of amending vitamin B12 and yeast extract was evaluated. The ISMP process was monitored by combining analytical analyzes of process parameters (SO_4^{2-}-concentration, HM-concentration, pH, E_h) with molecular tools such as SRB subgroup and genus specific PCR, denaturing gradient gel electrophoresis (DGGE), and phylogenetic analysis of clone sequences, based on either the 16S rRNA or the *dsr* (dissimilatory sulfite reductase) gene. The efficiency of different carbon sources to stimulate the ISMP process followed the order HRC > molasses > methanol >

lactate > ethanol > acetate. Within 10 weeks, the highest sulfate- and metal removal efficiencies ranged from 85% to 99%. Addition of yeast extract boosted the ISMP process, whereas vitamin B12 negligibly affected SRB-activity. Analysis of the sulfate-reducing population by SRB subgoup and genus specific PCR demonstrated that members of the genus *Desulfosporosinus* dominated in all batch tests, while 16S rDNA DGGE profiles additionally revealed the presence in the microbial communities of non-sulfate reducing bacteria within the family *Clostridium* and the ε-*proteobacteria*. The *dsrB*-based DGGE profiles allowed to assess the diversity and dynamics of the sulfate-reducing community and added to a better understanding of the effects of different batch conditions on the ISMP process.

4.1. Introduction

Contamination of soils and water bodies with heavy metals and metalloids is a widespread problem. Due to the extend of this problem, a need exists for low cost, non-invasive, *in situ* remediation technologies and this has resulted in a growing interest in heavy metal bioprecipitation via the formation of metal sulfides, a process mediated by sulfate-reducing bacteria.

Sulfate-reducing bacteria (SRB) couple the oxidation of organic compounds or molecular H_2 with the reduction of sulfate as an external electron acceptor under anaerobic conditions, a process known as dissimilatory sulfate reduction (Barton and Tomei, 1995). Reduction of sulfate by SRB is an ubiquitous process in water-saturated, reducing environments where the redox-potential (E_h) is below about -100 mV and sulfate is available (Postgate, 1984). The end product of this reaction is hydrogen sulfide, which sequesters heavy metals (HM) as stable metal sulfide precipitates. The ability of SRB to generate metal sulfides has been exploited in a range of applications for the treatment of HM-contaminated groundwater, such as bioreactors (Hammack *et al.*, 1994; Hulshoff *et al.*, 2001), reactive barrier walls (Benner *et al.*, 2002), and constructed wetlands (Lloyd *et*

al., 2004; McIntire *et al.*, 1990). By the formation of an *in situ* anaerobic reactive zone or biobarrier in the path of the groundwater plume, dissolved HM will precipitate as metal sulfides, thus preventing further spreading of the contamination to neighboring areas (Hao, 2000). This process is designated *in situ* metal precipitation (ISMP).

Before the application of the ISMP technology, two major questions have to be answered: (i) is there an indigenous SRB-community whose sulfate-reduction and metal-precipitation activity can be stimulated to obtain an optimal ISMP process that decreases the contamination levels of HM in the groundwater below the legislative levels; and (ii) what are the optimal process conditions (type of C-source/ electron donor, pH, COD/ SO_4^{2-}-ratio,...) for promoting and maintaining this activity? Hence, the applicability of the ISMP technology first has to be investigated by means of batch experiments, and in a next step, the process should be optimized in column experiments (Janssen and Temminghoff, 2004). The conclusions of such preliminary studies will eventually lead to a go-no go decision for an on site application. The evaluation and optimization of the ISMP process is facilitated when physical/ chemical analysis techniques are combined with molecular tools that specifically monitor the abundance, diversity and dynamics of the indigenous sulfate-reducing community.

This paper details the results of batch-scale feasibility tests for ISMP, using aquifer and groundwater of the site of a nonferrous company with a severe heavy metal (Zn, Cd, Ni, Co) contamination, low pH, high sulfate-concentration and a naturally high Fe-concentration. During these experiments, the response of SRB to different process conditions (amendments of different types of C-source/ electron donor, vitamin B12, yeast extract) was examined by means of molecular methods (SRB subgroup and species specific PCR, DGGE fingerprinting, and phylogenetic analysis of both 16S rDNA and *dsrB* genes) as complementary tools to classical analytical methods (measurement of HM-concentration, SO_4^{2-}-concentration, pH, E_h).

4.2. Materials and Methods

4.2.1. Aquifer and groundwater samples

Samples were collected at the site of a nonferrous company in the Kempen (Belgium). Its aquifer and groundwater are contaminated with Zn, Cd, Ni and Co, and have a naturally high Fe-concentration. Groundwater has low pH and high sulfate-concentrations. The composition of the aquifer and groundwater is given in Table 4.1. Groundwater samples were collected from a network of small-diameter temporary wells at 10 m below soil surface. Water was pumped using a peristaltic pump and stored in glass bottles under N_2. Aquifer material was sampled within a few meters of the water sampling wells using a macro-core soil sampler, from a depth of 10 m. Glass bottles and cores were stored at 4°C prior to use.

4.2.2. Batch experiments

For the construction of batch tests, 80 g of saturated aquifer material was transferred to 250-ml Pyrex serum bottles under N_2 atmosphere in an anaerobic glove box (Coy Laboratory Products, Inc.). 186 ml of groundwater was dispensed into serum bottles after amendment of sterile, anoxic solutions that contained either acetate, lactate, methanol, ethanol, molasses or Hydrogen Release Compound® (Regenesis) to final carbon-concentration of 0.02% (w/v).
HRC® is a biodegradable poly-lactate ester, which hydrolyzes upon contact with water and is readily broken down by microorganisms. This process results in the release of lactate, which either can be used directly as electron donor, or can be metabolized into H_2 by fermenting bacteria. The produced H_2 in turn can serve as electron donor. Molasses contains approximately 30 to 50% (w/w) sucrose, which is easily biodegradable and its fermentation products, such as short-chain acids

(lactate, acetate, propionate,...), which can be used as carbon source by SRB (Lebel *et al.*, 1985).

Table 4.1 Composition of aquifer and groundwater used in batch experiments

Parameters	Analysis aquifer	
Dry matter	80%	
Clay fraction (<2 µm)	23.3%	
Sand fraction 50-2000 µm	73.2%	
Loam fraction 2-50 µm	3.5%	
Zn (mg kg^{-1})	0.618	
Co (mg kg^{-1})	0.117	
Cd (mg kg^{-1})	0.066	
Ni (mg kg^{-1})	0.236	
Fe (mg kg^{-1})	38.8	
TOC %C	0.12	
TIC %C	<0.01	
TC %C	0.12	
Total N (mg kg^{-1} dm)	<50	
Total S (mg kg^{-1})	1360	
Buffer capacity		
pH 4.3 mmol/l	0.04	
pH 8.3 mmol/l	0.50	
Parameters	**Analysis groundwater**	**Norm (mg l^{-1})**
E_h	-90 mV	
pH	5.6	
Zn (mg l^{-1})	160	0.5
Cd (mg l^{-1})	27.7	0.005
Ni (mg l^{-1})	67.6	0.04
Fe (mg l^{-1})	13.7	
Co (mg l^{-1})	28.8	0.1
Calcium (mg l^{-1})	140	
SO_4^{2-} (mg l^{-1})	1400	
PO_4^{3} -P (mg l^{-1})	<0.15	
Total P (mg l^{-1})	<0.075	
NO_3-N (mg l^{-1})	<1	
DOC mg l^{-1} C	4.1	
DIC mg l^{-1} C	37.6	
DC mg l^{-1} C	41.5	

Additional batch series were used as controls: one to which no carbon-source was added to account for consumption of indigenous carbon sources, and one in which the microbial population was poisoned with $HgCl_2$ to account for abiotic processes. A freshly prepared, anoxic, filter-sterilized vitamin B12 (cobalamine) solution was added in addition to the carbon amendments, because vitamin B12 was thought to stimulate SRB activity. In order to ensure that sulfate reduction would be dramatically promoted for at least one batch experiment, due to the presence of yeast extract, two conditions (with lactate and acetate, respectively) were amended with Postgate C medium (Postgate, 1984), from which additional C-sources or sulfate were omitted. Finally, two conditions were setup with a lower groundwater/ aquifer ratio (80 g aquifer + 100 ml groundwater, resulting in a aquifer/ groundwater ratio of 1.3 instead of 2.3) in order to create a different rate of inhibition and toxicity: on one hand, toxicity might decrease because less contaminated groundwater is added to the same amount of aquifer (plus microorganisms); on the other hand, the heavy metal contamination of the aquifer is less diluted and thus toxicity to microorganisms might be higher. Serum bottles were gas-tightly closed using butyl rubber stoppers and incubated statically at room temperature. All treatments were performed in duplicates.

4.2.3. Most Probable Number (MPN) cell counts

Aquifer material was analyzed for most probable numbers of SRB. 2 g of aquifer was shaken for 1h at 150 rpm in 10 ml of anoxic $MgSO_4$ (10 mM), and 1 ml was used for 10-fold dilution series with anoxic Postgate B medium (Postgate, 1984) as growth substrate, with lactate as carbon source. Growth was monitored by the formation of black amorphous iron sulfide (FeS) precipitates. Dilution series were prepared in triplicate under N_2 atmosphere in an anaerobic glove box (Coy Laboratory Products, Inc.) and incubated at room temperature in anaerobic jars containing Anaerocult® A catalyst (Merck).

4.2.4. Analytical methods

Groundwater samples of the batch experiments were collected periodically and without preceding filtration analyzed for sulfate concentration, soluble Zn, Ni, Cd, Co and Fe, redox potential and pH. Sulfate concentrations were determined turbidimetrically by means of barium sulfate precipitation analogous to EPA375.4 and US Standard Methods 4500-SO_4^{2-}E, using the Spectroquant Sulfate Cell Test (Merck) and measuring turbidity in a photometer at 525 nm. Zn, Co, Cd and Fe concentrations were measured by the use of ICP-AES (Induction Coupled Plasma Atomic Emission Mass Spectrometry) with an ATOMCOMP model 750 Mass Spectrometer (Jarrell Ash, Europe), while the Ni concentration was determined by AAS (Atomic Absorption Spectrometry) with a Perkin Elmer 51000 ZL. The pH was measured with a Liq Glass pH electrode (Hamilton). Redox potential of the samples was measured with an Oxytrode Platinum Redox electrode (Hamilton).

4.2.5. Isolation of SRB strains from batch aquifer

2 g of batch aquifer was shaken for 1h at 150 rpm in 10 ml of anoxic $MgSO_4$ (10 mM), and 100 µl was plated on solid Postgate B medium (Postgate, 1984) plus lactate as growth medium, and incubated at room temperature in anaerobic jars containing Anaerocult® A catalyst.

4.2.6. Extraction and purification of pure strain and community DNA

Total genomic DNA was extracted from pure SRB colonies as described before (Leys *et al.*, 2004). DNA was extracted from the different batch aquifers as described previously (see Chapter 3). For PCR purposes, the DNA concentration was measured spectrophotometrically (NanoDrop® ND-1000 spectrophotometer, NanoDrop Technologies Inc.) and adjusted to a concentration of 100 ng μl^{-1}.

4.2.7. Polymerase Chain Reaction

PCR on the extracted DNA was performed in a 100-µl volume. Eubacterial 16S rRNA-gene fragments of ~ 450 bp were PCR-amplified with primers GC40-63F (5'-GC clamp-CAGGCCTAACACATGCAAGTC-3') and 518R (5'-ATTACCGCGGCTGCTGG-3') (El-Fantroussi *et al.*, 1999). The primer sets for SRB subgroup and genus specific detection of the 16S rRNA-gene are reported in Table 4.2 and were applied in PCR as described previously (Chapter 3). The *dsrB* genes of SRB were PCR-amplified with primers GC-DSRp2060F (5'-GC clamp-CAACATCGTYCAYACCCAGGG-3') and DSR4R (5'-GTGTAGCAGTTACCGCA-3') (Wagner *et al.*, 1998) as described in Chapter 3. PCR amplification was performed in a Biometra T3 Thermocycler. Positive controls containing purified DNA from suitable reference organisms were included in all of the PCR amplification experiments along with negative controls (no DNA added). The PCR products were examined on 1.5% ethidium bromide-stained agarose gels.

4.2.8. Denaturing gradient gel electrophoresis

Community patterns based on 16S rRNA- and *dsrB* genes were generated using DGGE analysis (Muyzer and Smalla, 1998). The applied method has been described in Chapter 3. A DGGE marker was included on the gels for normalization of banding patterns.

4.2.9. Cloning and sequencing of 16S rRNA- and dsr-gene fragments

DFM 140F/ 842R amplified 16S rDNA fragments were cloned into the pCR®2.1-TOPO® plasmid vector and *Escherichia coli* TOP10 cells using TOPO-TA cloning vector kit (Invitrogen). Library screening was conducted by restriction fragment length polymorphism (RFLP) using *TaqI* and *AluI* (GibcoBRL).

Table 4.2 SRB subgroup or genus specific 16S rRNA gene targeting PCR primers

Primer [a]	Annealing temp (°C)	Sequence 5'-3'	Specificity	Reference
DSVII 230F [b]	63	GAGYCCGCGTYYCATTAGC	*Desulfovibrio* sp., *Desulfomicrobium* sp.	(Daly *et al.*, 2000)
DSVII 838R [b]	63	CCGACAYCTARYATCCATC	*Desulfovibrio* sp., *Desulfomicrobium* sp.	(Daly *et al.*, 2000)
DSM172F	64	AATACCGGATAGTCTGGCT	*Desulfomicrobium* sp.	(Loy *et al.*, 2002)
DSM1469R	64	CAATTACCAGCCCTACCG	*Desulfomicrobium* sp.	(Loy *et al.*, 2002)
DFM140 F	58	TAGMCYGGGATAACRSYKG	*Desulfotomaculum* sp., *Desulfosporosinus* sp.	(Daly *et al.*, 2000)
DFM842 R	58	ATACCCSCWWCWCCTAGCAC	*Desulfotomaculum* sp., *Desulfosporosinus* sp.	(Daly *et al.*, 2000)
DSP140F	60	AAAKCCGGGACAACCCTTG	*Desulfosporosinus* sp.	Chapter 3
DSP1107R [c]	60	CTAAAYACAGGGGTTGCG	*Desulfosporosinus* sp.	(Loy *et al.*, 2002)
DSB127F	62	GATAATCTGCCTTCAAGCCTGG	*Desulfobacter* sp.	(Daly *et al.*, 2000)
DSB II 1273R [b]	62	CYYTTTGCRRAGTCGCTGCCCT	*Desulfobacter* sp.	(Daly *et al.*, 2000)
DBM169F	64	CTAATRCCGGATRAAGTCAG	*Desulfobacterium* sp.	(Daly *et al.*, 2000)
DBM1006R	64	ATTCTCARGATGTCAAGTCTG	*Desulfobacterium* sp.	(Daly *et al.*, 2000)
DBBII 121F [b]	66	CGCGTAGATAACCTGTCTTCATG	*Desulfobulbus* sp.	(Daly *et al.*, 2000)
DBBII 1237R [b]	66	GTAGTACGTGTGTAGCCCTGGTC	*Desulfobulbus* sp.	(Daly *et al.*, 2000)
DCC140F	65	CTRCCCYYGGATYSGGGATAAC	*Desulfococcus* sp., *Desulfonema* sp., and *Desulfosarcina* sp.	Chapter 3
DCC1273R	65	CTYRCTCTCGCGAGYTCGCTACCCT	*Desulfococcus* sp., *Desulfonema* sp., and *Desulfosarcina* sp.	Chapter 3

[a] Primer's short name used in the reference or in this study; numbers refer to the annealing position on the 16S rDNA according to Brosius *et al.* (Brosius *et al.*, 1978)
[b] Modified in position or sequence from the reference
[c] Based on probe DFM1107 from the reference

For this purpose, DFM 140F/ 842R -amplified PCR products of clone inserts were initially diluted 1: 10 in sterile H_2O (deionized). 5 µl of the diluted product was then used in a restriction digest containing 0.5 µl of enzyme and 2 µl of enzyme buffer in a 20-µl total volume, according to the manufacturer' s instructions. Per condition, 10 clones were selected for sequence analysis of clone inserts. DNA bands from 16S rDNA and *dsrB*-derived DGGE patterns were excised from the polyacrylamide gels and dissolved in 50 µl of H_2O (deionized). 1 µl of the solution was used in a PCR amplification reaction using the corresponding specific primers. The obtained fragment was cloned using TOPO-TA cloning vector kit (Invitrogen). Clones containing recombinant vectors were examined for the presence of the appropriate 16S rDNA or *dsrB* insert using the vector primers M13F/ M13R, followed by PCR with the GC-63F/ 518R primer set, or by the GC-DSRp2060F/ DSR4R primer set, respectively. Cloned fragments were compared in DGGE with the community fingerprint of the parent aquifer to identify which signals from the community fingerprint were cloned. Clone inserts with different DGGE-patterns were sequenced (Westburg Genomics, The Netherlands).

4.2.10. Phylogenetic analysis

Identical DFM 140F/ 842R 16S rDNA sequences were recognized by analysis of phylogenetic trees and manual comparisons, in which sequences with more than 99% similarity were defined as identical, and these sequences were used for further phylogenetic analysis as operational taxonomic unit (OTU). All sequences were submitted to GenBank for preliminary analysis using the program BLASTN (http://www.ncbi.nlm.nih.gov/BLAST) to identify putative close phylogenetic relatives. In order to translate *dsrB* sequences into protein sequences, they were submitted to the 'transeq' algorithm of the EMBOSS program (version 1.9.1., BEN, Belgium). The obtained protein sequences were imported in an alignment of DsrB protein sequences of SRB strains of the δ-*proteobacteria*, low G+C Gram positive bacteria and sulfate-reducing *Archaea*. Phylogenetic analyses of 16S rRNA-genes

and DsrB proteins were performed with the Bionumerics software (version 2.50, Applied Maths, Belgium). Distance-based evolutionary trees were constructed using the neighbor-joining algorithm of Saitou and Nei (1987). Distances were conducted using the Kimura 2 parameter. The topography of the branching order within the dendrogram was evaluated by applying the Maximum Parsimony character based algorithm in parallel combined with bootstrap analysis with a round of 1000 samplings. The DsrB protein sequences of *Thermodesulfovibrio yellowstonii* (AAC24112) and *Thermodesulfovibrio islandicus* (AAK83214) were used as the outgroup to root the *dsrB*-derived protein tree, whereas the 16S rRNA gene sequence of *Archaeoglobus fulgidus* (Y00275) was included to root the 16S rRNA gene tree.

4.2.11. Nucleotide accession numbers

The nucleotide sequences obtained in this study were deposited in the GenBank database under the accession no. AY731475 (clone T10_9(6)1B), AY731476 (clone T10_3(6)5D), AY731477 (clone T10_3(6)7D), AY731413 to AY31425 (*dsrB* DGGE band sequences) and AY731458 to AY731474 (16S rDNA DGGE band sequences). 16S rRNA- and *dsrB* gene sequence information of strain JG32A is available under the accession nos. AY780358 and AY787791.

4.3. Results

4.3.1. Most Probable Number (MPN) cell counts

The density of the indigenous SRB community, present in aquifer material derived from the site, was determined through a most probable number (MPN) counting method using Postgate B medium (Postgate, 1984). SRB cell density was around 10^2 MPN counts g^{-1} aquifer. After 24 weeks incubation under various experimental conditions, MPN counts were applied in triplicate to aquifer of all batch setups.

The results indicate that the amendment of HRC, molasses and methanol remarkably stimulated the SRB population density, just as the addition of yeast extract in the form of Postgate C medium (- C, - SO_4^{2-}), with cell densities of 10^7 – 10^8 MPN counts g^{-1} aquifer. Amendment with acetate had the least stimulating effect on SRB-growth, with MPN counts increasing no more than 10-fold.

4.3.2. Sulfate reduction and heavy metal precipitation

Except for the control setups, SO_4^{2-}concentrations dropped rapidly in all batch experiments (Table 4.3): after 6 weeks, 50% removal was observed in the batch tests amended with lactate + Postgate C, molasses, and molasses + vitB12, whereas similar removal rates were found after 8 weeks for the conditions with HRC®, acetate + Postgate C, methanol, and methanol + vitB12. This demonstrates a strong promotion of sulfate-reduction activity when extra carbon sources are applied. In parallel, there was a steady increase in alkalinity, from pH 5.6 to pH 6.2-6.8. However, the setups with acetate or acetate + vitB12 showed no more than 11% and 33% SO_4^{2-}removal, respectively, even after 24 weeks. A decrease in redox-potential (E_h) from -90 mV to values below -100 mV to –200 mV was only observed for the conditions with Postgate C + acetate, Postgate C + lactate, molasses, molasses + vitB12, and HRC® + vitB12. The drop in E_h was most remarkable when yeast extract was added in the form of Postgate C medium: within 6 weeks, the E_h was decreased from – 90 mV to -280 mV.

The aqueous phase of the batch experiments was analyzed in function of time for its metal concentrations. Only the results for Zn removal are shown here (Table 4.4) since they were representative for all other metals that were monitored. An overview of Cd, Co, Ni and Fe-removal is given in Annex I. Especially, the evolution in Zn and Cd concentrations were very similar. There was a substantial Zn removal rate for all setups except for the controls (aquifer + groundwater, and aquifer + groundwater + $HgCl_2$) and the acetate/ acetate + vitB12 amended

conditions: up to ≥ 99.5% of initial Zn concentration was removed, as it dropped on the average from 180 mg l^{-1} to 0.45 mg l^{-1}.

Table 4.3 Evolution of the SO_4^{2-} concentration (mg l^{-1}) in function of time (weeks) for the different batch setups

experimental setup	T0	T6	T8	T10	T13	T16	T24
1. A+G	1450	1380	1270	1290	1350	1380	1300
2. A+G $HgCl_2$	1390	1350	1340	1330	1420	1360	1290
3. A+G acetate	1440	1360	1060	1080	1110	1050	980
4. A+G lactate	1340	780	770	740	710	700	670
5. A+G methanol	1480	860	750	500	400	330	300
6. A+G ethanol	1590	960	980	970	970	950	870
7. A+G molasses	1580	540	600	490	430	410	330
8. A+G HRC®	1310	830	670	520	290	150	10
9. A+G acetate Postg C	1380	1030	470	330	240	5	5
10. A+G lactate Postg C	1420	480	170	120	70	5	10
11. A+G acetate VitB12	1570	1290	1140	1050	1080	1020	1040
12. A+G lactate VitB12	1630	1270	1220	1130	1100	950	790
13. A+G methanol VitB12	1530	820	660	570	580	410	330
14. A+G ethanol VitB12	1510	870	890	890	830	770	750
15. A+G molasses VitB12	1460	470	490	310	260	230	150
16. A+G HRC® VitB12	1280	920	670	450	210	80	40
17. A+G (1/2) lactate	1510	890	810	810	790	920	770
18. A+G (1/2) lactate VitB12	1510	800	820	800	740	720	650

[a] A: aquifer; G: groundwater; PgC: Postgate C medium (Postgate, 1984) without C-source or SO_4^{2-}; VitB12: vitamin B12; (1/2): aquifer/ groundwater ratio= 1.3 instead of 2.3

The most remarkable results for metal removal from solution were those for Cd. Already after 6 weeks, the concentration of Cd was dramatically lowered for all the experimental setups. Moreover, after 10 weeks Cd concentrations dropped from an average of 23 mg l^{-1} to concentrations below the detection limit of 5 µg

l^{-1}, except for the two controls. However, after 24 weeks a slight increase in soluble Zn and Cd could be detected. With regard to Co removal, there was a strong removal of 99% in the setups with HRC®, molasses, methanol and lactate or acetate + Postgate C, with Co concentrations declining from 32 mg l^{-1} to 0.2 mg l^{-1} within 13 weeks.

Table 4.4 Evolution of the Zn concentration (mg l^{-1}) in function of time (weeks) for the different batch setups

experimental setup	T0	T6	T8	T10	T13	T16	T24
1. A+G	207.0	172.0	145.9	153.8	148.3	144.2	151.0
2. A+G $HgCl_2$	188.0	173.0	153.1	159.6	148.6	149.9	158.1
3. A+G acetate	187.0	145.0	15.74	22.89	25.32	12.77	9.19
4. A+G lactate	199.0	0.399	0.61	0.23	0.36	0.01	0.54
5. A+G methanol	192.0	0.909	0.46	0.24	0.36	0.01	0.32
6. A+G ethanol	194.0	4.78	5.67	6.26	7.01	10.24	0.76
7. A+G molasses	192.0	0.227	0.35	0.10	0.33	1.92	0.47
8. A+G HRC®	181.0	0.67	0.38	0.23	1.13	0.88	0.44
9. A+G acetate PgC	166.0	15.5	0.22	0.15	0.24	0.05	0.47
10. A+G lactate PgC	184.0	0.024	0.06	0.05	0.16	0.89	0.61
11. A+G acetate VitB12	160.0	115.0	2.73	0.26	0.68	1.14	1.88
12. A+G lactate VitB12	169.0	66.3	40.85	7.55	0.31	0.04	0.81
13. A+G methanol VitB12	179.0	0.192	0.35	0.07	0.28	0.01	0.62
14. A+G ethanol VitB12	186.0	4.175	4.01	4.45	5.38	4.92	1.08
15. A+G molasses VitB12	188.0	0.039	0.18	0.14	0.25	0.01	0.40
16. A+G HRC® VitB12	179.0	0.35	0.15	0.21	0.01	0.01	0.72
17. A+G (1/2) lactate	186.0	0.149	0.16	0.31	1.67	0.01	0.96
18. A+G (1/2) lactate VitB12	171.0	0.212	0.77	0.24	0.30	0.01	0.60

[a] A: aquifer; G: groundwater; PgC: Postgate C medium (Postgate, 1984) without C-source or SO_4^{2-}; VitB12: vitamin B12; (1/2): aquifer/ groundwater ratio= 1.3 instead of 2.3

In the lactate amended batch tests about 85% of the soluble Co precipitated, whereas removal ratios in the setups with acetate and ethanol were merely 35%

to 50%. Concerning the evolution in soluble Ni, a 52% and 84% reduction was observed for the conditions with molasses and molasses + vitB12 respectively, and this already after 6 weeks. Final removal ratios of 80 to 85% were after 24 weeks observed for Ni. For the conditions that were supplied with methanol or methanol + vitB12, the initial Ni concentration was reduced with 90% after 16 weeks. The most remarkable Ni removal was observed when HRC® was added: 91% of the initial Ni was precipitated, which implies that Ni concentration had decreased from 81 mg l^{-1} to 7 mg l^{-1}. Comparable results were obtained when Postgate C medium was added. The remaining setups showed a steady decrease in Ni concentrations after 24 weeks, no more than 10 to 30% of total Ni being removed.

After 8 weeks, black precipitates indicative for iron sulfide formation were noted. Accordingly, soluble Fe-levels dropped during the course of the experiment with at least 80% in the molasses- and methanol-amended batch tests. When HRC® or Postgate C medium were added, initial Fe-concentrations were reduced for 95% (from 100 mg l^{-1} to 5 mg l^{-1}). For the remaining conditions, only 30% to 50% of Fe was precipitated. A lower aquifer/ groundwater ratio for the condition with lactate resulted in a lower sulfate reduction- and metal removal rate. This was in contrast to the condition with lactate + vitB12, where a lower aquifer/ groundwater ratio resulted in a higher decrease in sulfate- and metal concentrations.

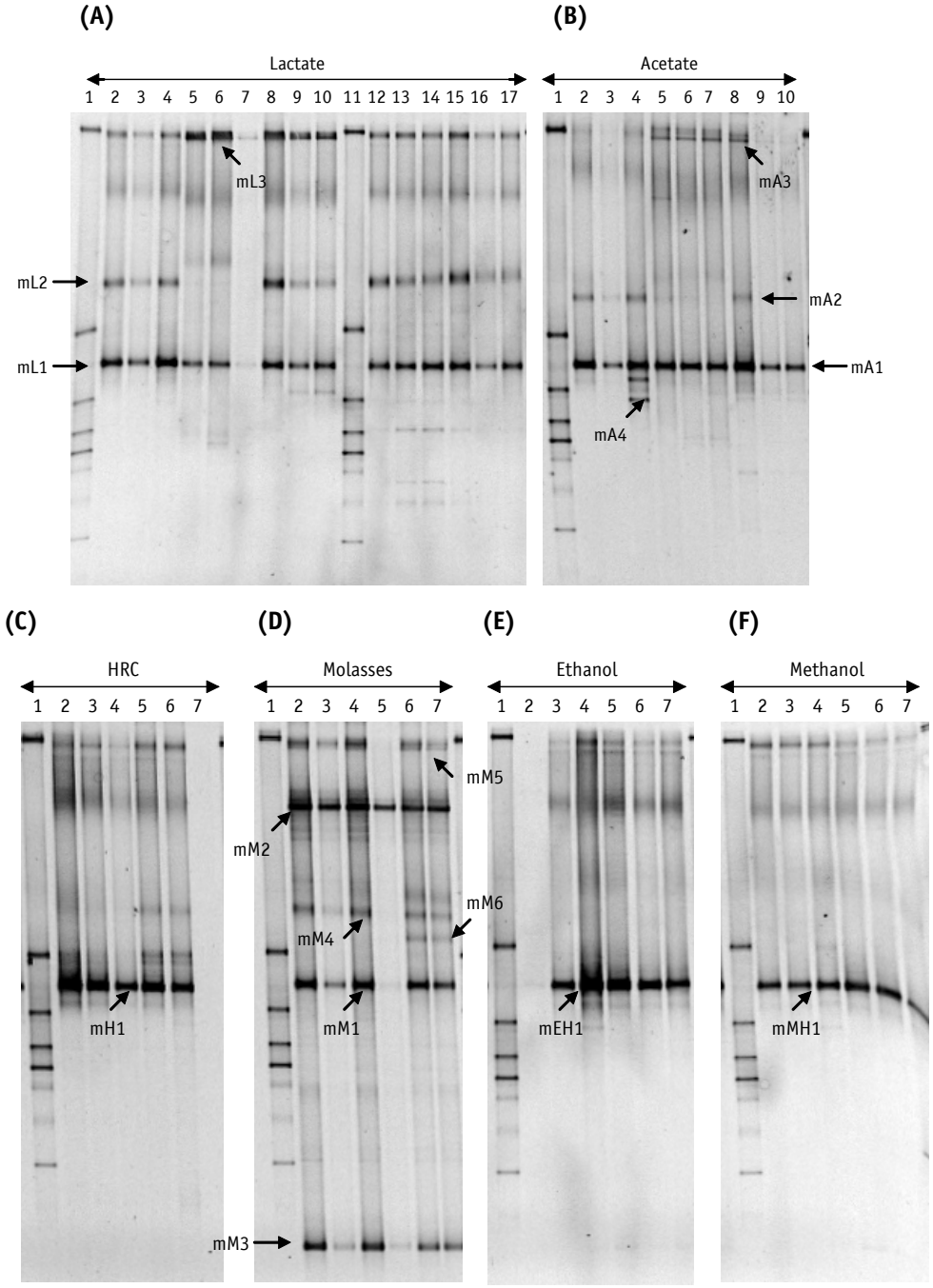
4.3.3. Characterization of the SRB-community by SRB subgroup and genus specific PCR detection of 16S rRNA genes

SRB subgroup and genus specific PCR primer for the 16S rRNA gene were used to rapidly characterize the SRB communities in the batch tests in response to carbon source, VitB12 and yeast extract amendments. From 6 weeks on, in accordance with the results of sulfate- and heavy metal-removal, sulfate reducers were detected in all experimental conditions, with the exception of the controls (data

Figure 4.1 Community fingerprints by eubacterial 16S rDNA-based DGGE analysis obtained for the different batch setups. DNA from three different time points (8, 13 and 16 weeks after starting the experiments) was used. Cloned 'bands' are indicated within the aquifer fingerprint based on the comparison of migration profiles of pure clones and the soil profile. Legend: A, aquifer; G, groundwater; T, time (weeks). **(A) DGGE profiles of the setups with lactate.** Lanes: 1, DGGE marker; 2, A + G + lactate T8; 3, A + G + lactate T13 ; 4, A + G + lactate T16; 5, A + G + lactate + Postgate C T8; 6, A + G + lactate + Postgate C T13; 7, A + G + lactate + Postgate C T16 ; 8, A + G + lactate + vitB12 T8; 9, A + G + lactate + vitB12 T13; 10, A + G + lactate + vitB12; 11, DGGE marker; 12, A + G (1/2) + lactate T8; 13, A + G (1/2) + lactate T13; 14, A + G (1/2) + lactate T16; 15, A + G (1/2) + lactate + vitB12 T8; 16, A + G (1/2) + lactate + vitB12 T13; 17, A + G (1/2) + lactate + vitB12 T16. **(B) DGGE profiles of the setups with acetate.** Lanes: 1, DGGE marker; 2, A + G + acetate T8; 3, A + G + acetate T13 ; 4, A + G + acetate T16; 5, A + G + acetate + Postgate C T8; 6, A + G + acetate + Postgate C T13; 7, A + G + acetate + Postgate C T16 ; 8, A + G + acetate + vitB12 T8; 9, A + G + lactate + vitB12 T13; 10, A + G + acetate + vitB12. **(C) DGGE profiles of the setups with HRC.** Lanes : 1, DGGE marker; 2, A + G + HRC® T8; 3, A + G + HRC® T13; 4, A + G + HRC® T16; 5, A + G + HRC® + vitB12 T8; 6, A + G + HRC® + vitB12 T13; 7, A + G + HRC® + vitB12 T16. **(D) DGGE profiles of the setups with molasses.** Lanes: 1, DGGE marker; 2, A + G + molasses T8; 3, A + G + molasses T13; 4, A + G + molasses T16; 5, A + G + molasses + vitB12 T8; 6, A + G + molasses + vitB12 T13; 7, A + G + molasses + vitB12 T16. **(E) DGGE profiles of the setups with ethanol.** Lanes: 1, DGGE marker; 2, A + G + ethanol T8; 3, A + G + ethanol T13; 4, A + G + ethanol T16; 5, A + G + ethanol + vitB12 T8; 6, A + G + ethanol + vitB12 T13; 7, A + G + ethanol + vitB12 T16. **(F) DGGE profiles of the setups with methanol.** Lanes: 1, DGGE marker; 2, A + G + methanol T8; 3, A + G + methanol T13; 4, A + G + methanol T16; 5, A + G + methanol + vitB12 T8; 6, A + G + methanol + vitB12 T13; 7, A + G + methanol + vitB12 T16.

not shown). Amplicons were only obtained with primer sets for the subgroup *Desulfotomaculum* sp.-*Desulfosporosinus* sp. (primer pair DFM 140F/ 842R), and the genus *Desulfosporosinus* (primer pair DSP 140F/ 1107R). For the acetate-amended batch tests, clone libraries were constructed with the DFM 140F/ 842R PCR fragments in order to investigate whether the SRB-population exclusively existed of members of the genus *Desulfosporosinus*, or if there also had been an enrichment of members of the genus *Desulfotomaculum*. All clones obtained from the condition acetate + Postgate C and acetate + vitB12, grouped within 1 OTU, represented by clone T10_9(6)1B (accession no. AY731475). Its sequence affiliated most closely to *Desulfosporosinus meridiei* (98% sequence similarity), isolated from gasoline-contaminated aquifer material (Robertson *et al.*, 2000). For the acetate amended setup, 2 OTU's were identified, each comprising 50% of

Figure 4.1


(A)
Lactate
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
mL3
mL2
mL1
(B)
Acetate
1 2 3 4 5 6 7 8 9 10
mA3
mA2
mA1
mA4
(C)
HRC
1 2 3 4 5 6 7
mH1
(D)
Molasses
1 2 3 4 5 6 7
mM5
mM2
mM6
mM4
mM1
mM3
(E)
Ethanol
1 2 3 4 5 6 7
mEH1
(F)
Methanol
1 2 3 4 5 6 7
mMH1

the DFM 140F/ 842R clones. The OTU represented by clone T16_3(6)7D (accession no. AY731477) showed 96% sequence similarity to the 16S rRNA-gene of *Desulfotomaculum* sp. DEM-Kme99-2, which was detected by cloning rice bulk soil DNA (Stubner and Meuser, 2000). The OTU represented by clone T10_3(6)5D (accession no. AY731476) closely matched *D. meridiei* (97% sequence similarity).

4.3.4. Analysis of eubacterial community structure by 16S rRNA-based DGGE

In order to obtain an insight in the whole community composition and dynamics during the course of the ISMP process, 16S rRNA-based DGGE fingerprints derived from the different batch setups where compared to each other, as well as analyzed at different time points (8, 13 and 16 weeks). Profiles generated from the abiotic control and the non-carbon supplemented setup are not shown since bands were hardly visible on the DGGE-gel. Different bands within one fingerprint were cloned and sequenced (Figure 4.1). Table 4.5 shows the nearest protein match based on BLASTN analysis of the cloned sequences, while Figure 4.2 shows the position of clone sequences in a 16S rDNA phylogenetic tree.

A comparison of DGGE fingerprints showed relative stability of the bacterial community profiles between the different treatments in function of time. One strong, common DGGE band was revealed for all experiment conditions (Figure 4.1). To determine its gene sequence and to identify the corresponding bacterial strain, the band was excised, cloned and sequenced from the DGGE fingerprints of the lactate (band mL1), acetate (band mA1), molasses (band mM1), HRC (band mH1), methanol (band mMH1) and ethanol (band mEH1) amended setups. The obtained sequences exhibited more than 99% sequence identity with each other and affiliated for 98% to the 16S rRNA-gene of *D. meridiei,* which is consistent with the sequencing results of the DFM 140F/ 842R clones T10_9(6)1B and T10_3(6)5D. DNA band mL2 was revealed in the banding pattern of the lactate-amended setups; this band co-migrated with bands mA2 (present in the acetate-amended setups) and mM4 (detected in the molasses amended setup).Their

Table 4.5 Cloned 16S rRNA gene sequences retrieved from batch experiments

Amended C-source	DGGE band designation	Accession no.	Nearest match in BLASTN analysis	Accession no.	Sequence identities
Acetate	mA1	AY731459	*Desulfosporosinus meridiei*	AF076247	98%
	mA2	AY731460	*Desulfosporosinus limneticum*	AJ582757	99%
	mA3	AY731461	*Anaerospora hongkongensis*	AY372052	92%
	mA4	AY731462	*Desulfotomaculum* sp. DEM-KMe99_2	AJ276565	95%
Lactate	mL1	AY731463	*Desulfosporosinus meridiei*	AF076247	98%
	mL2	AY731464	*Desulfosporosinus limneticum*	AJ582757	99%
	mL3	AY731465	*Anaerospora hongkongensis*	AY372052	92%
Molasses	mM1	AY731466	*Desulfosporosinus meridiei*	AF076247	98%
	mM2	AY731467	*Desulfosporosinus* sp. LauIII	AJ302078	98%
	mM3	AY731468	*Cellulomonadaceae* strain FJS31	AB078828	98%
	mM4	AY731469	*Desulfosporosinus limneticum*	AJ582757	98%
	mM5	AY731470	*Anaerospora hongkongensis*	AY372052	92%
	mM6	AY731471	*Campylobacter* sp. NO3A	AY135396	91%
HRC®	mH1	AY731472	*Desulfosporosinus meridiei*	AF076247	98%
Ethanol	mEH1	AY731473	*Desulfosporosinus meridiei*	AF076247	98%
Methanol	mMH1	AY731474	*Desulfosporosinus meridiei*	AF076247	98%

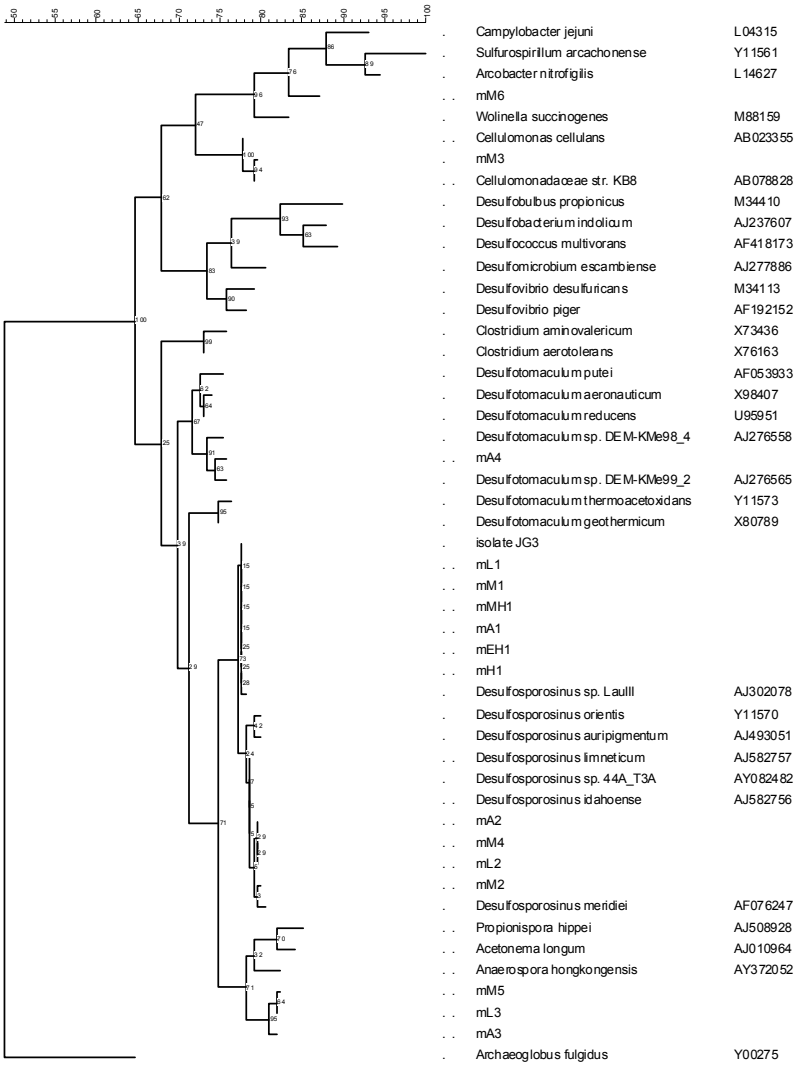


Figure 4.2 Phylogenetic analysis of 16S rDNA-gene fragments detected in the DGGE fingerprints of the batch experiments. The evolutionary tree was generated by the neighbor joining method, evolutionary distances were generated using the Kimura 2 parameter. The Maximum Parsimony algorithm was used to evaluate branching orders. Bootstrap resampling (1000 replicates) of the tree was performed to provide confidence estimates for the inferred topologies. Percentages of bootstrap support are indicated at the branch points. An out-group of the 16S rRNA-gene of *Archaeoglobus fulgidus* was included to root the tree. The bar at the top indicates the estimated evolutionary distance.

corresponding sequences were 99% similar to each other and were closely related to *Desulfosporosinus* sp. 44a-T3a (98%-99% identity), which was found in a ZnS-

producing biofilm of a subsurface acid mine drainage system (Labrenz and Banfield, 2000) and to an isolate of a lake sediment, *Desulfosporosinus limneticum* (Ramamoorthy *et al.*, 2003).

For the molasses-supplemented setups, two additional intense bands were observed. One DNA band, designated as mM2, melted under weaker denaturing conditions. Its 16S rDNA sequence showed the highest similarity (98% identity) with the 16S rDNA of *Desulfosporosinus* sp. LauIII, which was isolated from mining-impacted freshwater lake sediments (Kuesel *et al.*, 2001). The sequence of DNA band mM3 corresponded to that of the 16S rRNA gene of *Cellulomonadaceae* strain KB8 (98% identity), which was associated with degradation of rice plant residues (Akasaka *et al.*, 2003), and to *Cellulomonas cellulans* (93%) (Takeuschi, 1999). Strains of the genus *Cellulomonas* have the capacity to degrade starch and cellulose (Chaudhary *et al.*, 1997), which are both present in molasses. For the acetate-supplemented setup an additional bright DNA-band, mA4, was observed at time point T16 (16 weeks). Its sequence exhibited 95% identity to the 16S rRNA gene of *Desulfotomaculum* sp. DEM-Kme99-2, this in accordance with the sequencing results of the *dsrB* clone T16_3 (6)7D. Fainter bands on DGGE profiles were also sequenced with the intention of identifying putative non-sulfate reducing strains, which might have an important effect on the growth and activity of the SRB-community. Sequencing of bands mL3, mA3, mM5 revealed the presence of strains of the family *Acidaminococcaceae*, with the closest cultured relative *Acetonema longum* (91%-92% identity), and *Anaerospora hongkongensis* (92% similarity) which is a member of the family *Peptococcaceae*. The lack of more available sequences closely related to mL3, mA3 and mM5 renders their phylogenetic placement tenuous. Nevertheless, their sequences cluster closely to *Anaerospora hongkongensis* and divergently with other genera of the *Peptococcaceae* family, such as *Desulfosporosinus* and *Desulfotomaculum*, thereby excluding relationship with sulfate-reducers (Figure 4.2). In addition, DNA sequence analysis of band mM6, which was obtained in the setup with molasses + vitB12, demonstrated the presence of ε-*proteobacteria*. The clone

Figure 4.3 Community fingerprints by *dsrB*-based DGGE analysis obtained for the different batch setups. DNA from three different time points (8, 13 and 16 weeks after starting the experiments) was used. Cloned 'bands' are indicated within the aquifer fingerprint based on the comparison of migration profiles of pure clones and the soil profile. Legend: A, aquifer; G, groundwater; T, time (weeks). **(A) DGGE profiles of the setups with lactate.** Lanes: 1, DGGE marker; 2, A + G + lactate T8; 3, A + G + lactate T13 ; 4, A + G + lactate T16; 5, A + G + lactate + Postgate C T8; 6, A + G + lactate + Postgate C T13; 7, A + G + lactate + Postgate C T16 ; 8, A + G + lactate + vitB12 T8; 9, A + G + lactate + vitB12 T13; 10, A + G + lactate + vitB12; 11, DGGE marker; 12, A + G (1/2) + lactate T8; 13, A + G (1/2) + lactate T13; 14, A + G (1/2) + lactate T16; 15, A + G (1/2) + lactate + vitB12 T8; 16, A + G (1/2) + lactate + vitB12 T13; 17, A + G (1/2) + lactate + vitB12 T16. **(B) DGGE profiles of the setups with acetate.** Lanes: 1, DGGE marker; 2, A + G + acetate T8; 3, A + G + acetate T13 ; 4, A + G + acetate T16; 5, A + G + acetate + Postgate C T8; 6, A + G + acetate + Postgate C T13; 7, A + G + acetate + Postgate C T16 ; 8, A + G + acetate + vitB12 T8; 9, A + G + lactate + vitB12 T13; 10, A + G + acetate + vitB12. **(C) DGGE profiles of the setups with HRC.** Lanes : 1, DGGE marker; 2, A + G + HRC® T8; 3, A + G + HRC® T13; 4, A + G + HRC® T16; 5, A + G + HRC® + vitB12 T8; 6, A + G + HRC® + vitB12 T13; 7, A + G + HRC® + vitB12 T16. **(D) DGGE profiles of the setups with molasses.** Lanes: 1, DGGE marker; 2, A + G + molasses T8; 3, A + G + molasses T13; 4, A + G + molasses T16; 5, A + G + molasses + vitB12 T8; 6, A + G + molasses + vitB12 T13; 7, A + G + molasses + vitB12 T16. **(E) DGGE profiles of the setups with ethanol.** Lanes: 1, DGGE marker; 2, A + G + ethanol T8; 3, A + G + ethanol T13; 4, A + G + ethanol T16; 5, A + G + ethanol + vitB12 T8; 6, A + G + ethanol + vitB12 T13; 7, A + G + ethanol + vitB12 T16. **(F) DGGE profiles of the setups with methanol.** Lanes: 1, DGGE marker; 2, A + G + methanol T8; 3, A + G + methanol T13; 4, A + G + methanol T16; 5, A + G + methanol + vitB12 T8; 6, A + G + methanol + vitB12 T13; 7, A + G + methanol + vitB12 T16.

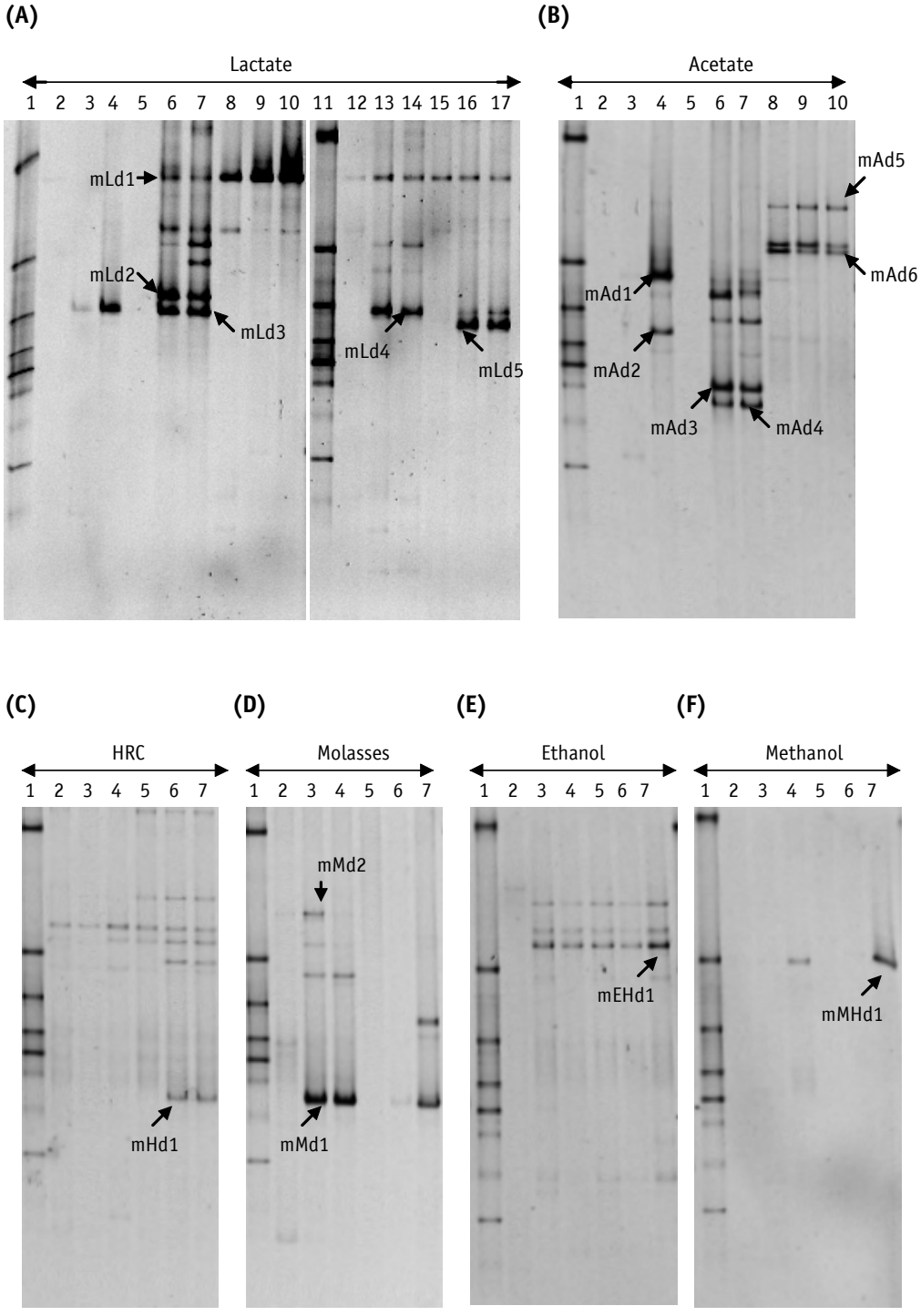
sequence matched for 91% to the 16S rRNA-genes of *Campylobacter* sp. NO3A (Hubert *et al.*, 2003) and *Wollinella succinogenes* (Wesley *et al.*, 1995). Since sequence similarities are low, we cannot ascertain what function or activity is carried out by this bacterium within the overall microbial community.

In general, the results clearly demonstrate that molasses enriched a distinct population. The addition of Postgate C and vitamin B12 had a limited influence on the community composition, but it cannot be ruled out that these amendments did not have an effect on the bacterial community's overall activity.

4.3.5. DGGE analysis of dsrB-gene fragments

The response of the indigenous SRB-community to the different batch conditions and its dynamics during the course of the ISMP process was further examined by

Figure 4.3


(A)
Lactate
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
mLd1
mLd2
mLd3
mLd4
mLd5
(B)
Acetate
1 2 3 4 5 6 7 8 9 10
mAd1
mAd2
mAd3
mAd4
mAd5
mAd6
(C)
HRC
1 2 3 4 5 6 7
mHd1
(D)
Molasses
1 2 3 4 5 6 7
mMd2
mMd1
(E)
Ethanol
1 2 3 4 5 6 7
mEHd1
(F)
Methanol
1 2 3 4 5 6 7
mMHd1

dsrB-based DGGE, a specific biomarker for SRB (Chapter 3). Different bands within one fingerprint were cloned and sequenced. Due to the degeneracy of the DSRp2060F primer, the possibility exists that a multiple-band DGGE fingerprints for single SRB strains might appear, thereby causing overestimation of the SRB-population diversity. However, previous results showed that such extra bands had minor intensities (Chapter 3). Therefore, in this study, we emphasized on the brightest bands (Figure 4.3).

Surprisingly, the *dsrB*-based DGGE profiles showed greater diversity between experimental conditions than the 16S rDNA-based DGGE patterns. For example, the *dsrB*-based DGGE banding patterns of setups with lactate + Postgate C and acetate + Postgate C are more complex than for the lactate or acetate amended setups, suggesting that a more complex community of sulfate-reducing strains is created when Postgate C medium is supplied. Hence, this increased diversity might explain why these conditions are so efficient in heavy metal-precipitation. Also, the addition of vitB12 results in a totally different *dsrB*-DGGE fingerprint for the lactate- and acetate amended setups, indicating that other SRB-strains gain the upper hand under these conditions. However, all but one of the DsrB protein sequences obtained from cloning and sequencing of excised DNA bands, showed sequence similarity (80% to 84% identity) with the DsrB protein sequences of *Desulfotomaculum thermoacetoxidans*, *Desulfotomaculum thermosapovorans*, and other thermophilic members of the genus *Desulfotomaculum* that is monophyletic with δ-*proteobacterial* Dsr sequences (Klein *et al.*, 2001) (Table 4.6). The DsrB protein sequences of cloned bands form a divergent cluster with the DsrB sequences of the thermophilic *Desulfotomaculum* strains, and due to subtle mutual sequence distinctions, they split into 2 separate lineages (Figure 4.4). DNA-bands with a similar melting behavior (e.g. mLd2, mLd3, mLd4, mLd5; mHd1 and mMd1; mEHd1 and mAd6) cluster within the same lineage. The deduced DsrB protein sequence of band mAd1 affiliated for 83% to the DsrB protein sequence of *Desulfotomaculum putei*. No *dsrB* gene sequences were found to affiliate with those of the genus *Desulfosporosinus*, although their presence was indisputably

Table 4.6 Cloned *dsrB* gene sequences retrieved from batch experiments

Origin	DGGE band designation	Accession no.	Nearest match in BLASTX analysis		
			Protein (Accession no.)	Host	Protein identities
Acetate	mAd1	AY731425	AAK58405	*Desulfotomaculum putei*	83%
	mAd2	AY731414	AAK83690	*Desulfotomaculum thermosapovorans*	81%
	mAd3	AY731415	AAK83690	*Desulfotomaculum thermosapovorans*	80%
	mAd4	AY731416	AAK83690	*Desulfotomaculum thermosapovorans*	81%
	mAd5	AY731417	AAK83692	*Desulfotomaculum thermoacetoxidans*	80%
	mAd6	AY731418	AAK83692	*Desulfotomaculum thermoacetoxidans*	81%
Lactate	mLd1	AY731420	AAK83692	*Desulfotomaculum thermoacetoxidans*	84%
	mLd2	AY731419	AAK83690	*Desulfotomaculum thermosapovorans*	83%
	mLd3	AY731413	AAK83690	*Desulfotomaculum thermosapovorans*	82%
	mLd4	AY731421	AAK83690	*Desulfotomaculum thermosapovorans*	81%
	mLd5	AY731412	AAK83690	*Desulfotomaculum thermosapovorans*	82%
Molasses	mMd1	AY731423	AAK83690	*Desulfotomaculum thermosapovorans*	83%
	mMd2	AY731422	AAK83692	*Desulfotomaculum thermoacetoxidans*	80%
HRC®	mHd1	AY731424	AAK83690	*Desulfotomaculum thermosapovorans*	83%
Ethanol	mEHd1	AY731414	AAK83692	*Desulfotomaculum thermoacetoxidans*	81%
Methanol	mMHd1	AY731458	AAK82967	*Desulfotomaculum acetoxidans*	83%

Figure 4.4

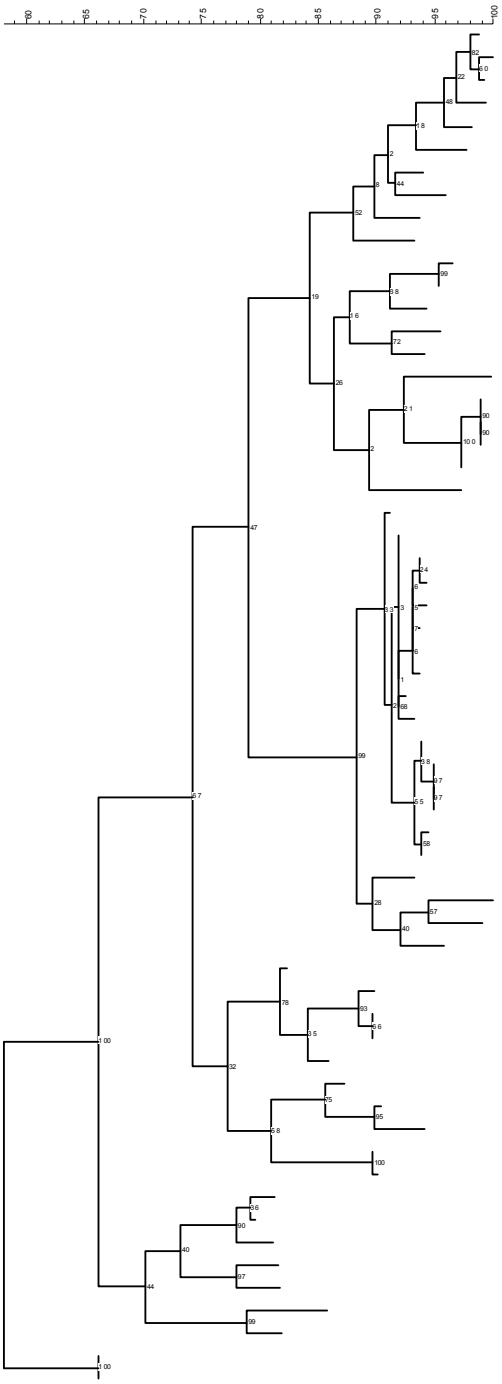

100
95
75
70
75
80
85
90
95
100
. Desulfobacter postgatei AAL57470
. Desulfobacter curvaturus AAL57472
. Desulfobacter latus AAC24098
. Desulfobacterium vacuolatum AAL57480
. Desulfobacterium autotrophicum AAL57438
. Desulfofaba gelida AAK83202
. Desulfococcus multivorans AAC24102
. Desulfonema limicola AAC24100
. Desulfobotulus sapovorans AAC24110
. Desulfosarcina variabilis AAK71936
. Desulfovibrio vulgaris BAB55580
. Desulfovibrio termitidis AAL57444
. Desulfovibrio africanus AAG21987
. Desulfovibrio longus BAB55574
. Desulfovibrio aespoeensis AAL99083
. Desulfovibrio desulfuricans subsp. desulfuricans AAK83200
. Desulfomicrobium macestii BAB55560
. Desulfomicrobium norvegicum BAB55558
. Desulfomicrobium baculatum BAB55554
. Desulfomicrobium orale AAM03450
. Desulfonatronum lacustre AAL57452
. isolate JG32A
. mAd4
. mLd2
. mAd3
. mLd4
. mLd5
. mMd1
. mHd1
. mAd2
. mLd3
. mAd5
. mLd1
. mMd2
. mMHd1
. mEHd1
. mAd6
. Desulfotomaculum acetoxidans AAK82967
. Desulfotomaculum thermoacetoxidans AAK83692
. Desulfotomaculum thermosapovorans AAK83690
. Desulfotomaculum geothermicum AAK58399
. Desulfofustis glycolicus AAL57456
. Desulfobulbus rhabdoformis CAB95039
. Desulfobulbus elongatus AAL57477
. Desulfobulbus propionicus AAG28586
. Desulforhopalus singaporensis AAL57466
. Thermodesulforhabdus norvegica AAK83210
. Desulforhabdus amnigena AAL07559
. Desulfovirga adipica AAK83198
. Thermodesulfobacterium commune AAL78312
. Thermodesulfobacterium thermophilum AAK83212
. Desulfotomaculum putei AAK58405
. Desulfotomaculum aeronauticum AAK58407
. Desulfotomaculum ruminis AAC24104
. Desulfosporosinus orientis AAK82965
. Desulfitobacterium dehalogenans AAL07563
. Archaeoglobus profundus AAC78310
. Archaeoglobus fulgidus AAB17214
. Thermodesulfovibrio islandicus AAK83214
. Thermodesulfovibrio yellowstonii AAC24112

Figure 4.4 Phylogenetic analysis of the derived protein sequences of the *dsrB* gene fragments detected in the DGGE fingerprints of the batch experiments. The evolutionary tree was generated by the neighbor joining method; evolutionary distances were generated using the Kimura 2 parameter. The Maximum Parsimony algorithm was used to evaluate branching orders. Bootstrap resampling (1000 replicates) of the tree was performed to provide confidence estimates for the inferred topologies. Percentages of bootstrap support are indicated at the branch points. An out-group of the DsrB proteins of *Thermodesulfovibrio yellowstonii* and *Thermodesulfovibrio islandicus* were was included to root the tree. The bar at the top indicates the estimated evolutionary distance.

demonstrated by 16S rRNA analysis. These results suggested that the 16S rDNA PCR-DGGE approach might have been biased, for example by preferential amplification of 16S rRNA-genes. However, when a DNA-mixture of *Desulfotomaculum aeronauticum* (DSM 10349) and *Desulfosporosinus auripigmentus* (DSM 13351) was made by mixing equal amounts of equal DNA concentrations (100 ng μl^{-1}), and serial dilutions were PCR amplified with the GC-DSRp2060F/ DSR4R primer set as well as the GC-63F/ 518R primer set, the intensity of PCR amplicons decreased with decreasing DNA-concentration for both primer sets (data not shown).

4.3.6. Isolation of SRB strains

One sulfate reducing bacterium, JG32A, was isolated from the aquifer of the lactate-amended batch-experiments and was identified by its 16S rRNA-gene as a *Desulfosporosinus* strain, thereby confirming the sequencing results of 16S rDNA DGGE bands such as mL1, mM1, mMH1, mA1, mEH1 and mH1 (Figure 4.2). However, its *dsrB* gene sequence was related to *Desulfotomaculum thermoacetoxidans*, *Desulfotomaculum thermosapovorans*, and other thermophilic members of the genus *Desulfotomaculum* which received their xenologous *dsrB* gene from the δ-*proteobacteria* (Klein *et al.*, 2001). The deduced DsrB protein sequence clustered most closely to the lineage formed by the *dsrB*-DGGE bands mLd2, mLd3, mLd4, mLd5, mHd1, mMd1, mEHd1 and mAd6 (Figure 4.4).

4.4. Discussion

The batch experiments carried out in the context of this paper, which aimed at stimulating the activities of the indigenous SRB communities, demonstrated that these communities were present and that their activities could be used to obtain efficient *in situ* precipitation of the contaminating heavy metals. This opens the possibility to test this concept in the future as an on site demonstration as part of the groundwater strategy for this HM-contaminated site.

The efficiency of different types of C-sources/ electron donors (lactate, acetate, methanol, ethanol, molasses, Hydrogen Release Compound®) to stimulate sulfate-reduction and metal-removal was explored, together with the effect of extra nutrients (vitB12, yeast extract). The ISMP process was found to be promoted in the order HRC > molasses > methanol > lactate > ethanol > acetate. This was not always accompanied by a decrease in E_h below -100 mV to -200 mV, although it is assumed that sulfate reduction can only take place under these conditions (Zehnder, 1998). The drop in E_h was most remarkable when Postgate C medium, omitted from C-sources and sulfate, was added, showing that this medium provides all necessary elements for optimal SRB activity, and thus the best sulfate-reduction and metal-immobilization activities. The yeast extract present in Postgate C is consumed at once by fermenting bacteria, together with traces of potential electron donors other than sulfate, such as oxygen or nitrate, that might negatively influence SRB activity. The addition of vitamin B12 provided no significant stimulation of the ISMP process. Changing the aquifer/ groundwater ratio did not inhibit or kill the SRB. Hence, it is assumed that the ISMP process won't be influenced by small fluctuations in the groundwater's heavy metal concentrations. Cd was the first metal to be removed from the aqueous phase during the batch experiments, closely followed by Zn and Co, then Ni, and finally Fe. This precipitation order, which was also observed by Jong and Parry (2003) is reflected in the trend in solubility products (K_{sp}) of the respective metal sulfides; the log K_{sp} values of CdS (-27.9), ZnS (-23.3), CoS (-21.6) and NiS (-19.4) are

much lower than the log K_{sp} of FeS (-18.1). After 24 weeks, a very small amount of Zn and Cd which represented less than 1% of the total metal content was released back into solution without a simultaneous increase of sulfate concentration or redox potential, suggesting that part of the metal removal was caused by reversible adsorption processes, perhaps by adsorption to biomass. Due to the exhaustion of carbon- and sulfate-sources, part of the SRB community dies, which is accompanied by release of heavy metals that were sorbed to biomolecules that are now being recycled.

Although batch setups are suitable for preliminary feasibility studies for ISMP, they do not reflect the *in situ* situation where sulfate- and HMM-polluted groundwater is supplied continuously. Consequently, some questions remain, particularly about the completeness of metal-precipitation processes, and about the long-term efficacy and especially the sustainability of the ISMP process as a strategy to remedy HMM contaminated groundwater. Hence, the ISMP process has to be studied in lab-scale column experiments, conducted with aquifer and groundwater derived from the contaminated site. This will additionally allow to define optimal process conditions before going into an *on site* bioremediation application.

In order to link the results of the analytical methods to the composition and dynamics of the indigenous SRB-community, molecular methods were applied in function of time to DNA-extracts from the batch experiments. For all experimental conditions, except the controls, the removal of sulfate and heavy metals was accompanied by the PCR-detection of sulfate reducers of the subgroup *Desulfotomaculum* sp.-*Desulfosporosinus* sp. (primer pair DFM 140F/ 842R), and the genus *Desulfosporosinus* (primer pair DSP 140F/ 1107R). In a next step, DGGE analysis was applied to determine differences between bacterial populations in response to different batch conditions, and to assess community dynamics. By using the 16S rRNA PCR primer combination for eubacteria, DGGE fingerprints revealed differences in the whole (i.e. SRB and non-SRB) microbial community, whereas DGGE analysis of *dsrB* gene fragments obtained with the SRB-specific

primers visualized differences in the SRB populations. The bacterial community was characterized by cloning and sequencing of excised DNA bands. In general, the 16S-rDNA-based DGGE approach revealed little diverse and stable microbial communities and confirmed the predominance of SRB of the genus *Desulfosporosinus*, with the occasional appearance of a member of the genus *Desulfotomaculum*. Several other molecular surveys have reported the presence of *Desulfotomaculum* sp. under a variety of harsh environmental conditions, such as uranium mine tailings (Chang *et al.*, 2000) and heavy metal-contaminated estuarine sediments (Tebo and Obraztsova, 1998). Their capability to produce spores and to utilize a variety of electron donors seems to facilitate their adaptation harsh polluted environments (Liu *et al.*, 2003). The amendment of molasses resulted, compared to the application of the defined C-sources, in a more complex community fingerprint and in the presence of non-SRB, such as ε-*proteobacteria* and *Cellulomonas* species. Aiming at an on site applied ISMP process as the remediation approach, the amendment of molasses might cause difficulties in controlling the composition and activity of the microbial population, given that such a crude substrate will enrich a diverse, unpredictable microbial population, which might have an adverse effect on the SRB community.
The *dsrB*-based DGGE community profiles, when compared to the 16S rDNA-based analysis, revealed much more diversity between different treatments, suggesting that they provide a better reflection of the SRB-community composition. Moreover, the PCR-detection of *dsrB* genes seems to be closely linked to a certain threshold of sulfate-removal, although this can only be established by quantitative PCR. None of the sequenced DNA bands were related to *dsrB* gene sequences of the genus *Desulfosporosinus* although this species was predominantly detected with the 16S rDNA-based analyzes. Instead, these sequences formed a divergent lineage with the *dsrB* gene sequence cluster found in *Desulfotomaculum*, which received their xenologous *dsrB* gene from the δ-*proteobacteria* (Klein *et al.*, 2001). A sulfate-reducing strain JG32A was isolated from the batch experiments, and it was shown that its 16S rRNA gene affiliated

with the genus *Desulfosporosinus*, while its *dsrB* gene sequence formed a divergent lineage with *Desulfotomaculum dsrB* gene sequences, which received their xenologous *dsr* genes from δ-*proteobacteria*. Although horizontal transfer of *dsrB* genes has been well described by Klein *et al.* (2001), *dsr*-gene sequences of some genera were not included in their study (e.g. *dsr*-genes of *Desulfomicrobium* strains, newly published *dsr*-genes). Moreover, the GenBank database contains only one *dsr* sequence of the *Desulfosporosinus* genus, namely the *dsr* gene of *Desulfosporosinus orientis*. Moreover, several studies describe *dsr* genes that form a divergent branching clade with the *Desulfotomaculum dsrB* xenologs (Baker *et al.*, 2003; Chang *et al.*, 2001; Dhillon *et al.*, 2003; Nakagawa *et al.*, 2002). Therefore we hypothesize that the batch experiments enrich members of the *Desulfosporosinus* genus that possess a non-orthologous *dsr*-gene, thus explaining the differences found in strain identification when using the 16SrRNA or *dsrB* based methods, respectively.

The next step towards an on site pilot test for ISMP will be the setup of a series of column experiments, with process conditions that are selected based on the above mentioned results. By applying the described molecular tools together with physical-chemical analysis, it can be investigated whether the same SRB community is enriched and which type of C-source is most effective in promoting and sustaining its growth and sulfate-reduction activity.

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CHAPTER 5

Column Experiments to Assess the Effects of Carbon Sources on the Efficiency of *in situ* Precipitation of Zn, Cd, Co and Ni in Contaminated Groundwater

Abstract

The potential of *in situ* metal precipitation (ISMP) as a treatment technology for mildly acidic metal (Zn, Ni, Co, Cd, Fe) and sulfate contaminated groundwater was explored in a series of column experiments, using lactate, molasses or HRC® as supplemented electron donor and C-source for the indigenous community of sulfate-reducing bacteria (SRB). In addition, the effect of N/P nutrient amendment was investigated.

Although all carbon sources drastically reduced the sulfate and heavy metal content in the columns effluent within 8 weeks, unexpected temporal decreases in the efficiency of the ISMP process accompanied by the release of precipitated metals were observed for most conditions tested. The most dramatic observation of the failing ISMP process was observed within 12 weeks for the molasses amended column. Lowering the chemical oxygen demand (COD) to [SO_4^{2-}] ratio from 1.9 to 0.4 did not alter the outcome of sulfate reduction and metal precipitation efficiency in this setup. Remarkably, after half a year of inactivity, the bacterial sulfate reduction was recovered in the molasses setup when the original COD/ SO_4^{2-}ratio of 1.9 was applied again. Intentional disruption of the lactate- and HRC®-supplies resulted in an immediate stagnation of the ISMP processes and a rapid release of precipitated metals in the columns' effluents.

However, the ISMP process could be restored after substrate amendment. Denaturing gradient gel electrophoresis (DGGE) and phylogenetic analysis of 16S rRNA- and *dsr* (dissimilatory sulfite reductase) genes were successfully used to link the results of the ISMP process to the community composition of sulfate reducing bacteria.

Our results suggest that in future field applications of the ISMP process a continuous substrate supply and eventually removal of metal-sulfide precipitates is required for the long-term viability of this bioremediation approach.

5.1. Introduction

Environmental pollution by toxic metals is a threatening problem for the ecology and the human health in affected areas. In the case of aqueous heavy metal (HM) contamination, there has been a growing interest for remediation technologies based on the biological immobilization of the metals through microbial reduction and precipitation (Gadd, 2004). Sulfate-reducing bacteria (SRB) are known for their capacity to reduce and precipitate heavy metals as metal sulfides, which form stable precipitates due to their low solubility product (Blowes *et al.*, 2000). The activity of SRB has been applied for the removal of heavy metals from contaminated wastewater, acid mine drainage or contaminated groundwater plumes in on-site reactive barriers or zones (Benner *et al.*, 1999; Janssen and Temminghoff, 2004; Waybrant *et al.*, 2002), as well as *ex situ* in bioreactors (Dvorak *et al.*, 1992; Elliott *et al.*, 1998) or constructed/ natural wetlands (McIntire *et al.*, 1990; Webb *et al.*, 1998). In the case of contaminated groundwater, the creation of an *in situ* reactive zone, where biological sulfate reduction is promoted by injecting the appropriate substrates, offers a cost-effective alternative to off-site pump-and-treat technologies (Suthersan and Yardley, 1997).

Before going to the on site pilot scale application of an *in situ* metal precipitation (ISMP) process, its feasibility has to be investigated in batch

experiments followed by column experiments where sulfate reducing conditions are created in a more realistic aquifer model system (Janssen and Temminghoff, 2004). The conclusion of these preliminary studies will eventually lead to a go-no go decision for the dimensioning of an on site application. Successful bioremediation relies on the management of soil microbial populations responsible for the desired bioremediation activity. Thus, in order to optimize the ISMP process, an insight is needed in the composition and activity of the indigenous SRB-community, as well as information on the way its composition and activity are affected by process conditions, such as the added type of C-source/ electron donor, or by the presence of other prokaryotes (e.g. fermenting bacteria, methane producing *Archaea*, acetogens). This can be obtained by combining physico-chemical analytical techniques with molecular tools for monitoring the abundance, diversity and dynamics of the indigenous sulfate-reducing community within the whole microbial community. Ultimately, molecular detection and quantification of specific (functional) genes, and if possible, measurement and quantification of (functional) gene expression should provide a clear indication of whether the ISMP process design has selected the optimal microbial populations (Rogers and McClure, 2003).

In this study column experiments were conducted as the follow up of batch experiments (Chapter 4) to assess the efficiency of different C-sources and electron donors (lactate, molasses and HRC®) to promote and maintain sulfate reduction and metal sulfide precipitation in aquifer material and groundwater sampled at the site of a nonferrous company. This site contains a groundwater plume with a severe heavy metal (Zn, Cd, Ni, Co)-contamination, low pH, high sulfate-concentration and a naturally high Fe-concentration. The biological sulfate reduction process in the column experiments was evaluated by combining classical analytical methods (measurement of HM-concentration, SO_4^{2-}-concentration, pH, dissolved organic carbon (DOC)) with molecular methods (DGGE fingerprinting and phylogenetic sequence analysis) based on either the 16S rRNA-gene or the *dsr* (dissimilatory sulfite reductase) -gene, which is a

specific biomarker for SRB (Wagner *et al.*, 1998). Emphasis was placed on the sustainability of heavy metal immobilization under a range of process conditions, including the COD/ SO_4^{2-}-ratio and disruption of substrate supply.

5.2. Materials and Methods

5.2.1. Aquifer and groundwater samples

The sampling location is at the site of a nonferrous company in the Kempen (Belgium). Its aquifer and groundwater is contaminated with Zn, Cd, Ni and Co, and has a naturally high Fe concentration. Groundwater has a low pH and contains high sulfate-concentrations. Aquifer and groundwater samples from this site were previously used in batch experiments to evaluate the presence of indigenous communities of SRB (Chapter 4). For the column experiments samples were obtained within 1 meter distance from the former sampling points. Their composition is shown in Table 5.1. Groundwater samples were collected from a network of small-diameter temporary wells at 10 m below soil surface. Water was pumped using a peristaltic pump and stored in glass bottles. Aquifer material was sampled from a 10 m depth within a few meters of the water sampling wells using a macro-core soil sampler. Glass bottles and cores were stored at 4°C prior to their use.

5.2.2. Setup of column experiments

Sulfate reduction experiments were carried out in Plexiglas columns having an internal diameter of 4.0 cm and a height of 50 cm, which were designed and constructed at Vito. Columns were filled with saturated aquifer material under N_2 atmosphere in an anaerobic glove box (Coy Laboratory Products, Inc.).

Table 5.1 Composition of aquifer and groundwater used in column experiments

Parameters	Analysis aquifer
Dry matter	81.2%
Clay fraction (<2 µm)	21.3%
Sand fraction 50-2000 µm	73.4%
Loam fraction 2-50 µm	63%
Zn (mg kg^{-1})	1630
Co (mg kg^{-1})	102
Cd (mg kg^{-1})	158
Ni (mg kg^{-1})	433
Fe (mg kg^{-1})	39300
DOC %C	0.13
DIC %C	<0.01
DC %C	0.13
Total N (mg kg^{-1} dm)	86
Total S (mg kg^{-1})	914
Buffer capacity	
pH 4.3 mmol/l	0.04
pH 8.3 mmol/l	0.45

Parameters	Analysis groundwater	Norm (mg l^{-1})
pH	4.1	
E_h (mV)	-26	
Zn (mg l^{-1})	444	0.5
Cd (mg l^{-1})	53.5	0.005
Ni (mg l^{-1})	144	0.04
Fe (mg l^{-1})	34.5	
Co (mg l^{-1})	31.2	0.1
SO_4^{2-}(mg/l)	1460	
PO_4^{3-}P (mg/l)	<0.15	
Total P (mg/l)	0.15	
NO_3-N (mg/l)	<1	
Total N (mg/l)	3.0	
DOC mg/l C	3.3	
DIC mg/l C	2.5	
DC mg/l C	5.8	

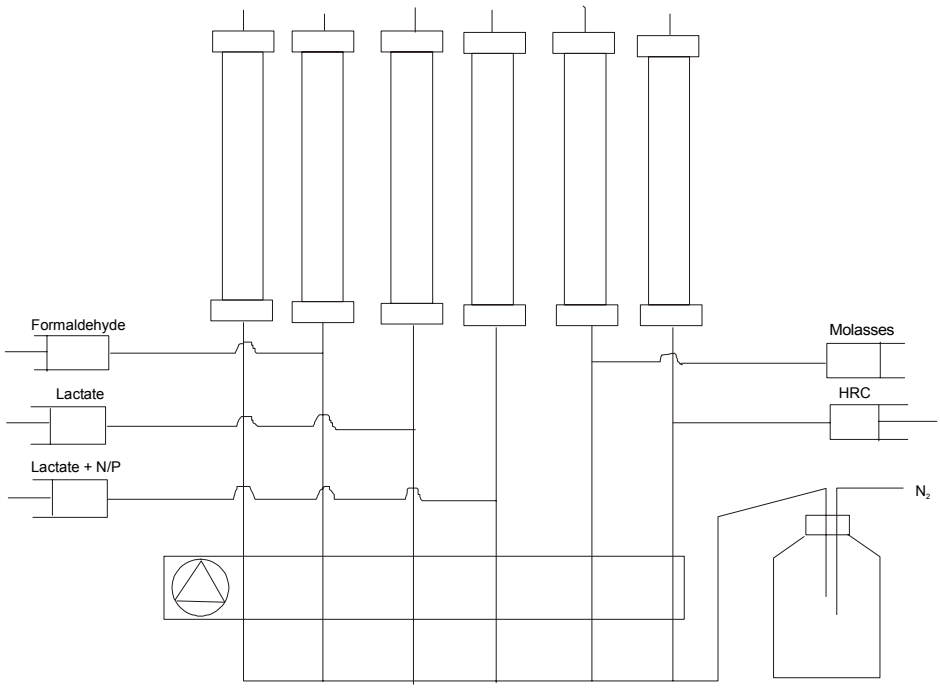


Figure 5.1 A schematic diagram of the upflow column setups used in this study.

Entrapped air was removed by pumping groundwater through the columns from the bottom to the top until the aquifer material was flooded by a 3-cm water layer. The fully saturated columns were allowed to equilibrate for one night. Then, columns were infiltrated upwardly with groundwater via a peristaltic multi-channel pump (Watson Marlow 205S) and stainless steel wire. Amendment solutions were added to the groundwater with a syringe pump (Harvard Apparatus). A schematic presentation of the column experiments is given in Figure 5.1. The inward flow velocity was set at 25 ml/ day; together with a pore volume of 50%, this resulted in a hydraulic retention time of 12.5 days. The column overflow was collected in waste jugs, which were weighed frequently to determine whether the flow rates remained stable over time. Groundwater was flushed in advance with N_2 and kept under N_2-pressure by means of a collapsible Teflon bag to prevent oxidation of Fe^{2+} and to maintain its redox potential.

Experiments were performed at room temperature, which was controlled at approximately 23°C.

In a previous study (Chapter 4), we explored the applicability of acetate, lactate, molasses, HRC®, methanol and ethanol as C-source and electron donor for promoting bacterial sulfate reduction in batch experiments with aquifer and groundwater from the contaminated site. The results pointed out that acetate was the least suitable substrate to accomplish SRB activity, thus eliminating it from further ISMP studies, whereas HRC® gave the best metal precipitation results at the considered HM-polluted site. Due to safety issues, many industries are not keen on ethanol or methanol for on site remediation technologies. Molasses is economically and regulatory most favorable (Janssen and Temminghoff, 2004). For these reasons, HRC® and molasses seemed the best options as substrate for column experiments. Although the use of lactate would result in high operational costs (Hammack *et al.*, 1994), we applied it to draw a comparison between lactate as a pure substrate and molasses and HRC® as complex substrates, where lactate is produced as the result of their (bio)degradation. The column supplemented with lactate + N/P was considered as an extra control. For determination of the N/ P ratio, the ratio C: N: P in the cell wall of anaerobic bacteria (500: 10: 1 mol ratio) was taken into account. Since some SRB have the capacity to use NO_3^- as an alternative electron acceptor (Ito *et al.*, 2002; Okabe *et al.*, 2003), NH_4Cl was selected as N-source. Extra phosphorus was added as KH_2PO_4. The following column setups were used as controls: one without additional carbon amendment to account for consumption of indigenous carbon sources, and one in which the microbial population was poisoned with formaldehyde to account for abiotic processes.

Table 5.2 describes the subsequent steps in the column amendments. Dilutions of the substrate solutions with the groundwater flowing past the delivery location resulted in a groundwater carbon-concentration of 0.02% (w/v). After 26 weeks, the continued failure of the ISMP process in the molasses amended column led to the decision to try to stimulate this process by lowering the ratio of chemical

Table 5.2 Time table showing the variations in influent solution for the column setups[a]

	Additions to groundwater					
Time (weeks)	Column 1	Column 2	Column 3	Column 4	Column 5	Column 6
0	none	0.175% (w/v) formaldehyde	6.5 mM lactate	6.5 mM lactate + 1.5 mM NH_4Cl + 0.17 mM KH_2PO_4	0,15% (w/v) molasses	0,13% (w/v) HRC®
26	end		none	43.75 mM lactate + 10.03 mM NH_4Cl + 1.15 mM KH_2PO_4	0.03% (w/v) molasses	none
41			6.5 mM lactate			0,13% (w/v) HRC®
51					0,15% (w/v) molasses	

[a] Blank fields indicate no change in setup for the considered column

oxygen demand (COD) to [SO_4^{2-}] (mg O_2/ mg SO_4^{2-}) from 1.9 to 0.4. At the same time, the COD/ SO_4^{2-} ratio was increased from 0.38 to 3.0 in the lactate plus N/P-amended column in order to study the influence of methane producers (MP).
These ratios were defined based on the observation of Choi and Rim (1991) that MP were very competitive at COD/ SO_4^{2-} ratio of 1.7 to 2.7 and that MP predominated at high COD/ SO_4^{2-} ratios, while SRB predominated when the value of this ratio decreased. The observations of Prasad *et al.* (1988) were also taken into account, namely that MP prevailed over SRB for at COD/ SO_4^{2-}ratio around 1. It was noticed that there was a substantial difference between the initial COD/ SO_4^{2-}ratios of the molasses-amended column and the lactate + N/P-amended column, although the carbon-concentration at the columns inlet was calculated as 0.02% (w/v). This is a consequence of the heterogeneous and unsteady composition of molasses which is in turn affected by the presence of bacterial contamination. In fact the molasses solution had to be changed every 2 to 3 days due to growth of contaminating bacteria. As the COD/ SO_4^{2-}ratio of 0.4 did not improve the ISMP process after 25 weeks (i.e. 51 weeks from starting the experiments) in the molasses-amended column, it was at that time decided to supply the column with the original amount of molasses at COD/ SO_4^{2-}ratio of 1.9. After 26 weeks, the substrate supply was disrupted for both the lactate and HRC amended columns to determine the long-term viability of the ISMP technology, and 15 weeks later (~ 41 weeks after the start of the column experiments), the original conditions were restored to investigate whether the SRB communities would be able to recover their metal precipitation capacities. The non-amended column was stopped after 26 weeks, as no ISMP was observed.

5.2.3. Analytical methods

Groundwater samples of column experiments were collected at the in- and outlet of the columns and without preceding filtration analyzed throughout the experiment for sulfate concentration, soluble Zn, Ni, Cd, Co and Fe concentrations

and pH. Sulfate concentrations were determined turbidimetrically by barium sulfate precipitation, using the Spectroquant Sulfate Cell Test (Merck) and measuring turbidity in a UV-visible spectrophotometer at wavelength 525 nm. Concentrations of Zn, Co, Cd and Fe were measured by ICP-AEMS (Induction Coupled Plasma Atomic Emission Mass Spectrometry) using an ATOMCOMP model 750 (Jarrell Ash, Europe), while the Ni concentration was determined by AAS (Atomic Absorption Spectrometry) using a Perkin Elmer 51000 ZL. The pH was measured with a Liq Glass pH electrode (Hamilton). Redox potential of samples was measured with an Oxytrode Platinum Redox electrode (Hamilton). Semi-quantitative analysis of methane- and hydrogen production was performed periodically during the first phase of the experiments (i.e. from T= 0 to T= 26 weeks) by respectively GC-FID (Gas Chromatography-Flame Ionisation Detection) and GC-RGD (Gas Chromatography-Reductive Gas Detection) in an isothermal GC RGA5 Process Gas Analyzer (Trace Analytical, California, USA). For this purpose, effluent was collected via Viton tubing and cupper needles in glass flasks that had been made gastight and were flushed in advance with N_2-gas. Dissolved organic carbon (DOC) was measured by acid hydrolysis, combustion and infrared detection.

Sulfate reduction, DOC degradation and Zn, Ni, Co and Cd precipitation rates were calculated as percentage of the differences measured between the feed and effluent solutions. Metal precipitation rates were subsequently used to calculate mass balances and percentages of metal release by using the equations described by Kaksonen *et al.* (2003). As for Fe^{2+}, it was noticed that the effluent concentrations in all columns were significantly higher than the influent concentration. Some orange-brown spots, which are the typical product of local enrichments of Fe^{3+}-compounds such as $Fe(OH)_3$ could be detected on the bottom of groundwater bottles and were attributed to Fe^{2+} oxidation in the influent. Nevertheless, in the columns aquifer these Fe^{3+}-compounds can be reduced by microbial dissimilatory Fe-reduction or abiotically by inorganic compounds such as sulfides. On the other hand, precipitation of Fe^{2+} as $FeCO_3$ also might have had

an effect on the eluates Fe-concentration. Together, these processes hamper the calculation of representative Fe-removal rates. Therefore, we based our calculations on the initial Fe concentration measured in the groundwater, i.e. 34.5 mg l^{-1}, this on the assumption that this value was most representative for the total Fe-concentration available for bacterial sulfate reduction.

5.2.4. Extraction and purification of community DNA

Columns were provided with hexagonal screws every 10 cm for aquifer sampling. Aquifer samples were taken periodically at 10 cm and 30 cm above the column's inlet. DNA was extracted from the columns aquifer as described previously (see Chapter 3). For PCR purposes, the DNA concentration was measured spectrophotometrically (NanoDrop® ND-1000 spectrophotometer, NanoDrop Technologies Inc.) and adjusted to a concentration of 100 ng μl^{-1}.

5.2.5. Polymerase Chain Reaction

PCR on the extracted DNA was performed in a 100 µl volume. Eubacterial 16S rRNA-genes of ~450 bp were PCR amplified with primers GC40-63F (5'-GC clamp-CAGGCCTAACACATGCAAGTC-3') and 518R (5'-ATTACCGCGGCTGCTGG-3') (El-Fantroussi *et al.*, 1999). PCR primers ARC-344F (5'-ACGGGGYGCAGCAGGCGCGA-3') and ARC-915R (5'-GTGCTCCCCCGCCAATTCCT-3') targeting archaeal 16S rRNA-genes were used in a PCR-protocol formulated by Casamayor *et al.* (2002) for the amplification of a ~570 bp 16S rDNA fragment. *DsrB*-genes of SRB were PCR amplified with primers GC-DSRp2060F (5'-GC clamp-CAACATCGTYCAYACCCAGGG-3') and DSR4R (5'-GTGTAGCAGTTACCGCA-3') (Wagner *et al.*, 1998) as described in Chapter 3. For the specific detection of methane producers, a primer set was used that amplified the α-subunit of the *mcr* gene, which encodes the methyl coenzyme-M reductase (MCR), a key enzyme in the production of methane and hence ubiquitous in methanogens (Thauer, 1998). Primers McrA-F (5'-

GGTGGTGTMGGATTCACAARTAYGCWACAGC-3') and McrA-R (5'-TTCATTGCRTAGT TWGGRTAGTT-3') were used following the instruction of the authors (Luton *et al.*, 2002). PCR amplification was performed in a Biometra T3 Thermocycler. Positive controls containing purified DNA from suitable reference organisms were included in all of the PCR amplification experiments along with negative controls (no DNA added). The PCR products were examined on 1.5% ethidium bromide-stained agarose gels.

5.2.6. Denaturing gradient gel electrophoresis

Community patterns based on 16S rRNA and *dsrB* genes were generated using DGGE analysis (Muyzer and Smalla, 1998). The applied method has been described in Chapter 3. A DGGE marker was included on the gels for normalization of banding patterns. The digitized images of 16S rDNA-DGGE gels were analyzed by the Bionumerics program (Bionumerics Version 1.01, Applied Maths, Belgium) to generate densitometric profiles. Bands were considered when the peak height relative to total peak height exceeded 1%, in accordance with Iwamoto *et al.* (2000). The Pearson correlation coefficient was used to calculate similarities and to produce a distance matrix, since this is recommended for use with data originating from DGGE profiles (Boon *et al.*, 2002). The UPGMA clustering algorithm (Michener and Sokal, 1957) was used to calculate the dendrograms of each DGGE gel using the Bionumerics software.

5.2.7. Cloning and sequencing of 16S rRNA- and dsr-gene fragments

DNA bands of 16S rDNA-based and *dsrB*-based DGGE fragments were excised from the 8% polyacrylamide gels and dissolved in 50 µl of H_2O (deionized). 1 µl of the solution was used in a PCR amplification reaction using the corresponding specific primers. The obtained fragment was cloned into the pCR®2.1-TOPO® plasmid vector and *Escherichia coli* TOP10 cells using TOPO-TA cloning vector kit

(Invitrogen). Clones containing inserts were selected for sequencing (Westburg Genomics, The Netherlands) as described previously (Chapter 3). TOPO-TA clone libraries were constructed with PCR-amplicons of the ARC-344F/ ARC-915R primer set; clones containing the appropriate ~570 bp fragment were randomly picked for sequence analysis.

5.2.8. Phylogenetic analysis

Sequences were submitted to GenBank for preliminary analysis using the program BLASTN (http://www.ncbi.nlm.nih.gov/BLAST) to identify putative close phylogenetic relatives. In order to translate *dsrB* sequences into protein sequences, they were submitted to the 'transeq' algorithm of the EMBOSS program (version 1.9.1., BEN, Belgium). The obtained protein sequences were imported in an alignment of DsrB protein sequences of SRB strains of the δ-*proteobacteria*, low G+C Gram positive bacteria and sulfate-reducing *Archaea*. Deduced sequences were edited manually to remove regions of ambiguous positional homology. Phylogenetic analyses of 16S rRNA-genes and DsrB proteins were performed with the Bionumerics software (version 2.50, Applied Maths, Belgium). Distance-based evolutionary trees were constructed using the neighbor-joining algorithm of Saitou and Nei (1987). Distances were conducted using the Kimura 2 parameter. The topography of the branching order within the dendrogram was evaluated by applying the Maximum Parsimony character based algorithm in parallel combined with bootstrap analysis with a round of 1000 samplings. The DsrB proteins of *Thermodesulfovibrio yellowstonii* (AAC24112) and *Thermodesulfovibrio islandicus* (AAK83214) were used as the outgroup to root the *dsrB* tree, whereas the 16S rRNA gene sequence of *Archaeoglobus fulgidus* (Y00275) was included to root the 16S rRNA gene tree. The 16S rRNA gene of *Sulfolobus acidocaldarius* (D14876) was the root of the crenarchaeal tree according to Sandaa *et al.* (Sandaa *et al.*, 1999).

5.2.9. Nucleotide accession numbers

The nucleotide sequences obtained in this study were deposited in the GenBank database under the accession no. AY731478 to AY731487 (crenarchaeal sequences), AY731499 to AY31503 (*dsrB*-based DGGE fragment sequences) and AY731488 to AY731498 (16S rDNA-based DGGE fragment sequences).

5.3. Results

5.3.1. Sulfate-reduction and metal-removal

As expected from previous batch experiments (Chapter 4) all three carbon sources initially supported sulfate reduction and metal precipitation. Cd was retained for 99% in all columns throughout the experiments, even for a while in the column where microbiota were poisoned with formaldehyde (data not shown), indicating that Cd was easily adsorbed by the aquifer material.

a) Performance of the lactate-amended column

Within 8 weeks, the sulfate-concentration in the eluate decreased with > 50% compared to the concentration in the feed (Figure 5.2). This was accompanied by substantial removal of Zn (75%) and Co (55%), whereas attenuation of the Ni-concentration started (Figure 5.3, 5.4, 5.5). Meanwhile, the effluent pH increased from ~4.5 to a value of 5.8 – 6.5 and the effluent's DOC decreased from ~370 mg l^{-1} to 45– 55 mg l^{-1} (data not shown), corresponding with a removal rate of ~85% and suggesting efficient lactate degradation. The results of Fe-removal rates (Figure 5.6) show that 81% of Fe^{2+} was retained in the column. These observations were corroborated by measurement of the E_h values, which decreased from around 50 mV to approximately -110 mV. In the following weeks, sulfate- and metal removal efficiencies showed an increasing trend to a maximum removal

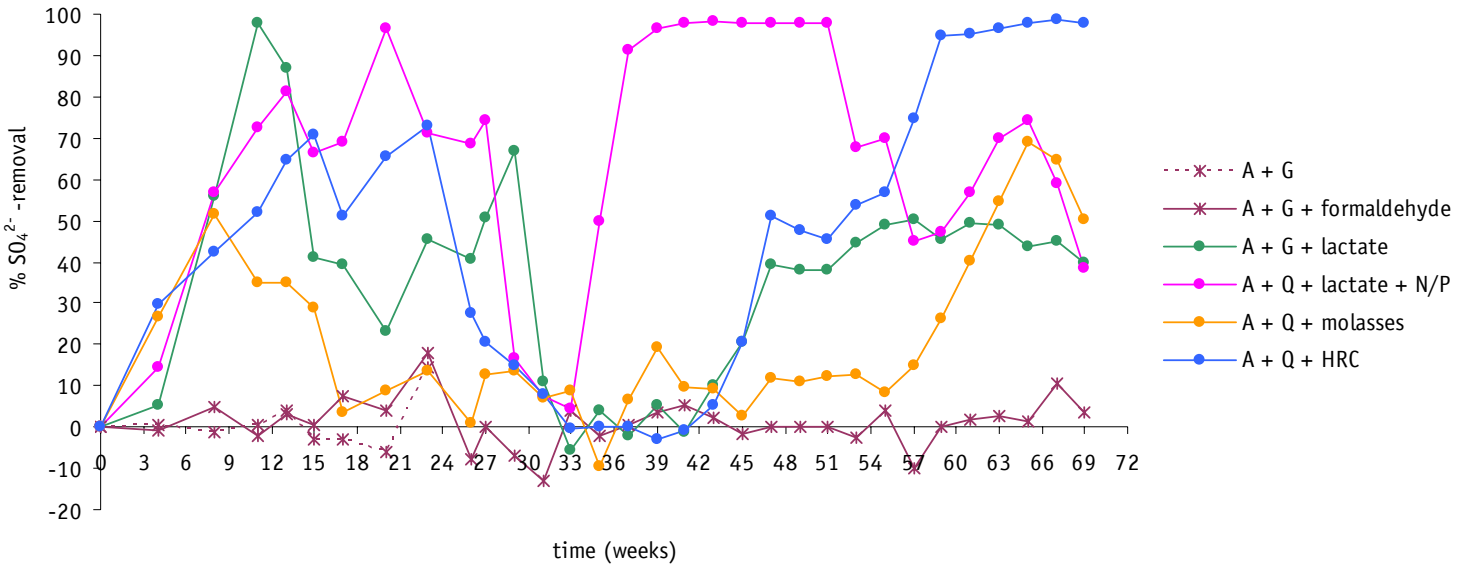


Figure 5.2 Sulfate removal efficiencies in the column experiments. Red lines indicate different phases of the experiments: (1) change of COD to [SO_4^{2-}] ratio from 0.38 to 3.0 in the lactate + N/P-amended column, and from 1.9 to 0.4 in the molasses-amended column; (2) interruption of substrate supply in the lactate- and HRC®-amended columns; (3) re-establishment of substrate-supply in the lactate- and HRC®-amended columns; (4) change of COD to [SO_4^{2-}] ratio from 0.4 to 1.9 in the molasses-amended column; (5) second interruption of substrate supply in the HRC®-amended columns.

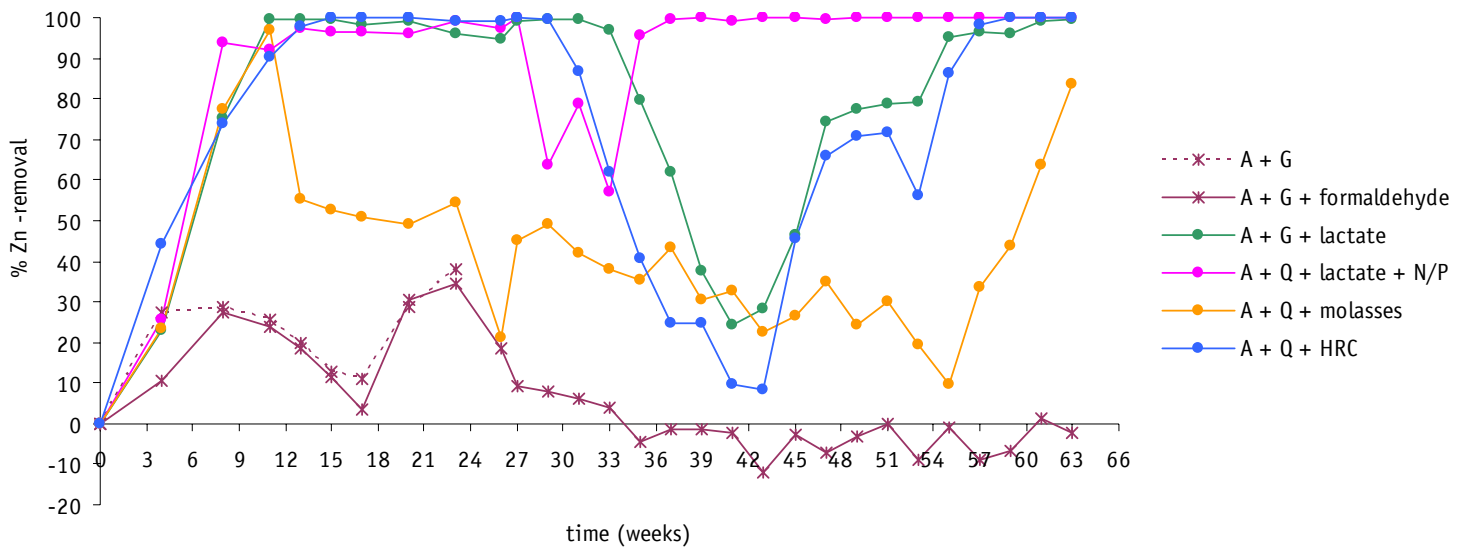


Figure 5.3 Zn-removal efficiencies in the column experiments. Red lines indicate different phases of the experiments: (1) change of COD to [SO_4^{2-}] ratio from 0.38 to 3.0 in the lactate + N/P-amended column, and from 1.9 to 0.4 in the molasses-amended column; (2) interruption of substrate supply in the lactate- and HRC®-amended columns; (3) re-establishment of substrate-supply in the lactate- and HRC®-amended columns; (4) change of COD to [SO_4^{2-}] ratio from 0.4 to 1.9 in the molasses-amended column.

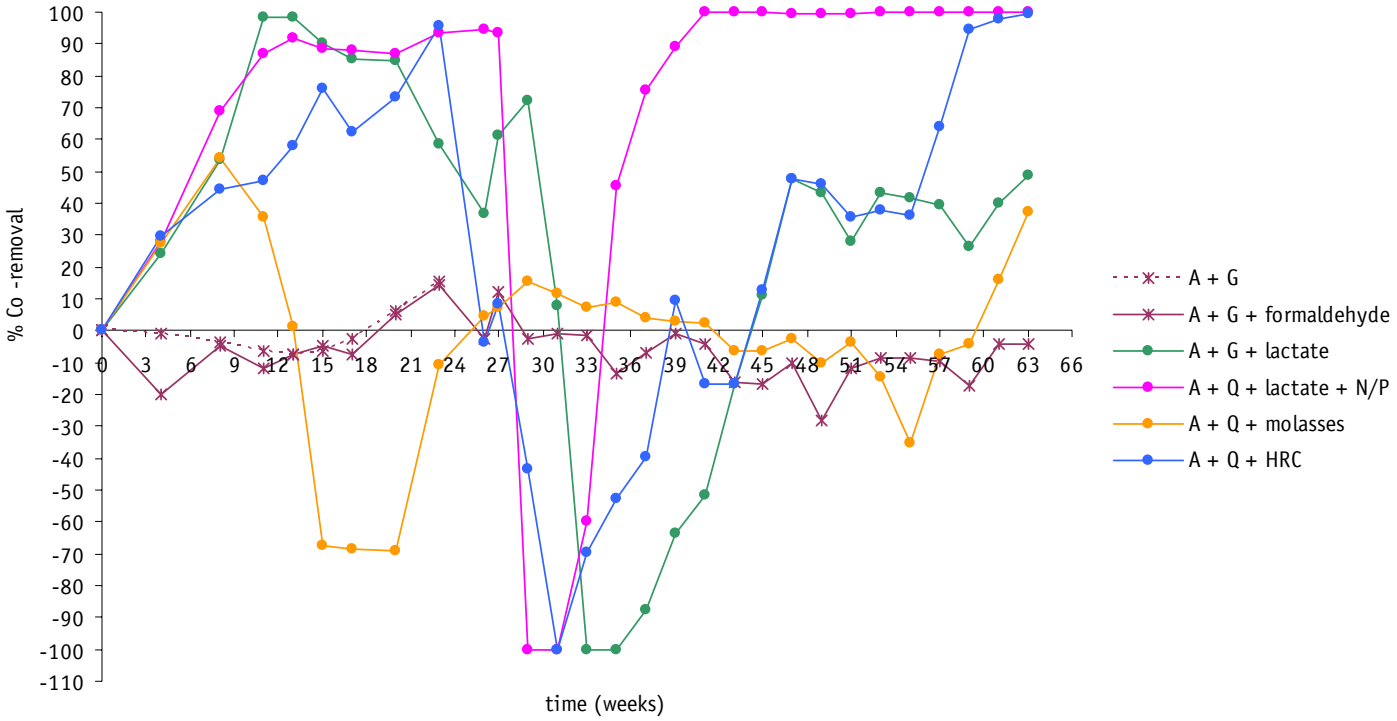


Figure 5.4 Co-removal efficiencies in the column experiments. Red lines indicate different phases of the experiments: (1) change of COD to $[SO_4^{2-}]$ ratio from 0.38 to 3.0 in the lactate + N/P-amended column, and from 1.9 to 0.4 in the molasses-amended column; (2) interruption of substrate supply in the lactate- and HRC®-amended columns; (3) re-establishment of substrate-supply in the lactate- and HRC®-amended columns; (4) change of COD to $[SO_4^{2-}]$ ratio from 0.4 to 1.9 in the molasses-amended column.

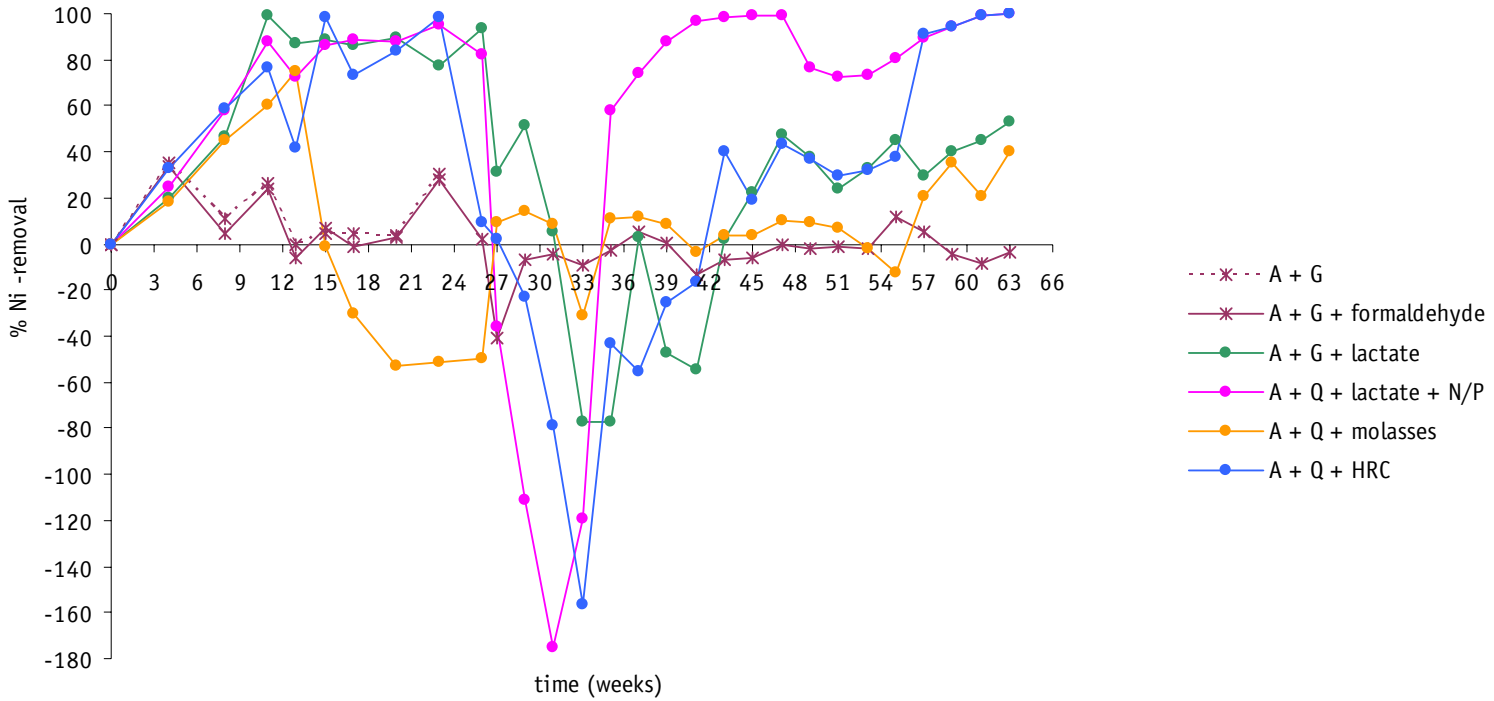


Figure 5.5 Ni-removal efficiencies in the column experiments. Red lines indicate different phases of the experiments: (1) change of COD to $[SO_4^{2-}]$ ratio from 0.38 to 3.0 in the lactate + N/P-amended column, and from 1.9 to 0.4 in the molasses-amended column; (2) interruption of substrate supply in the lactate- and HRC®-amended columns; (3) re-establishment of substrate-supply in the lactate- and HRC®-amended columns; (4) change of COD to $[SO_4^{2-}]$ ratio from 0.4 to 1.9 in the molasses-amended column.

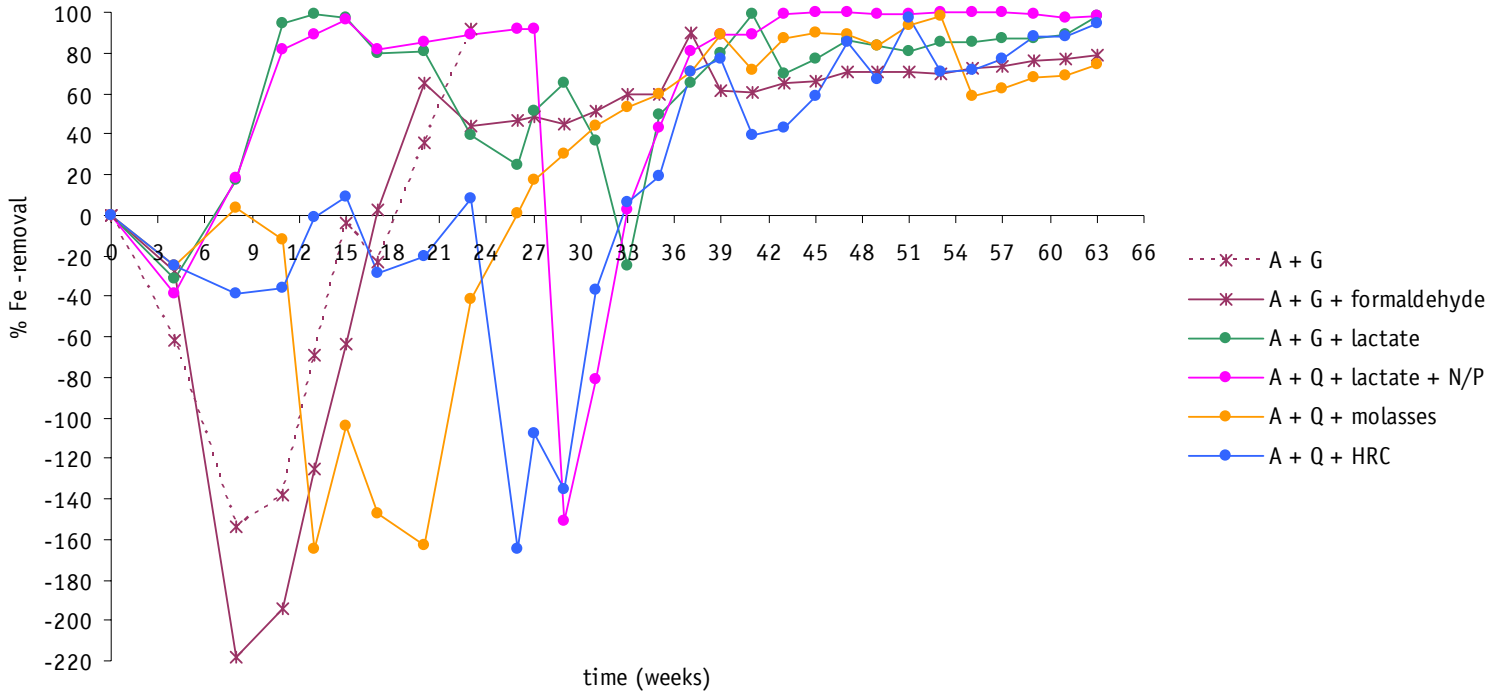


Figure 5.6 Hypothetical Fe-removal efficiencies in the column experiments. Red lines indicate different phases of the experiments: (1) change of COD to [SO_4^{2-}] ratio from 0.38 to 3.0 in the lactate + N/P-amended column, and from 1.9 to 0.4 in the molasses-amended column; (2) interruption of substrate supply in the lactate- and HRC®-amended columns; (3) re-establishment of substrate-supply in the lactate- and HRC®-amended columns; (4) change of COD to [SO_4^{2-}] ratio from 0.4 to 1.9 in the molasses-amended column.

efficiency of 99% Zn, 89% Ni, and 98% Co. At T= 15 weeks, the SO_4^{2-} consumption started fluctuating. Thereupon, minor variations were observed in Zn and Ni-removal rates, although the Co concentrations in the column's effluent began to increase slightly with time.

In order to study the stability of the metal immobilization, the lactate-supply was intentionally disrupted at T= 26 weeks. The activity of the indigenous SRB-community immediately dropped and within 5 weeks, sulfate reduction had come to a stop. This was accompanied with a gradual decrease of pH, increase of E_h, and leaching of Fe, Ni and Co, but no release of Cd and Zn was observed. Calculating mass balances pointed out that in the case of Co, a total amount of ~2096 mg was retained in the column during the first phase of the experiment, but upon suspending substrate supply, a total of ~402 mg or 19% of precipitated Co was washed out over a 10 week period between T= 32 weeks and T= 42 weeks. As for Ni, ~ 6875 mg was retained from T= 0 tot T= 26 weeks, but ~ 262 mg of precipitated Ni was released into solution in the first 12 weeks after termination of substrate supply, which corresponds to a leaching of 3.8%. To reinstate an efficient ISMP process, after having stopped the lactate supply for 15 weeks (~41 weeks after starting up the experiment), lactate amendment was reapplied to its corresponding column. However, although sulfate reduction was rapidly restored, sulfate removal rates did not exceed 52%, even after 28 weeks (~69 weeks after starting up the experiment). As to date, Zn-removal efficiencies of >99%, together with Co- and Ni-removal rates of about 50% were obtained and Fe-removal was re-established within 3 weeks. The pH stabilized at around a constant value of pH= 5.7 and up to 68% of DOC was being removed.

b) Performance of the lactate + N/P-amended column

The bacterial sulfate-reduction was rapidly established with a sulfate removal rate of >50% within 8 weeks (Figure 5.2), which was accompanied by substantial removal of Zn (93%) and Co (69%), whereas attenuation of the Ni concentration

started (Figure 5.3, 5.4, 5.5). Also 98% of Fe was retained from the solution (Figure 5.6). In the following weeks, these rates had an increasing trend. As with the lactate-amended column, the effluent E_h decreased to approximately -110 mV, the effluent pH increased from 4.5 to 6.5, and >80% of DOC was degraded.

In order to investigate after 26 weeks the effect of the fluctuations in COD to [SO_4^{2-}] ratio on the ISMP process, this ratio was changed from 0.38 to 3.0 (i.e. DOC= 2000 mg l^{-1}) (Table 5.2) with the intention of stimulating the growth of methanogens. The bacterial sulfate reduction dropped rapidly and after 5 weeks (i.e. after a total of ~33 weeks), no more sulfate removal was observed while the degradation of DOC came to a stop. Meanwhile, Fe, Ni- and Co concentration in the column's effluent became remarkably higher than their concentrations in the influent as an indication of metal leaching processes. Although only minor variations in pH and E_h could be observed, this metal leaching is congruent with the E_h-pH behavior of Co-O-H-S, Ni-O-H-S and Fe-O-H-S systems under the given conditions. According to mass balance calculations, a total amount of ~1320 mg Co had accumulated in the column during the first phase of the experiment, but in the first 6 weeks after changing COD/ SO_4^{2-} ratio, a total of ~93 mg, or 7% of retained Co was washed out. About 6937 mg of Ni was retained, but ~843 mg, or 12%, leached into solution in the first 6 weeks after switching the COD/ SO_4^{2-} ratio. No mass balances were calculated for Fe since there were too many uncertainties about the inlet Fe^{2+}-concentrations. The temporary failure of the ISMP process turned out to be the result of shock loading of the substrate since the sulfate reduction activity quickly recuperated: within 4 weeks (i.e. after a total of ~37 weeks), sulfate was removed for 99% and subsequent to this, removal rates of all metals increased to > 98%. The maximum ISMP efficiency persisted throughout the following 27 week period, while the pH value stabilized around 6.2 to 6.8, with only 50% of incoming DOC being removed. Then, the sulfate reduction rate began to fluctuate, showing a downward tendency. Despite this fluctuation, no significant decline in metal removal efficiencies has so far been observed.

c) Performance of the molasses-amended column

Also for this setup, a bacterial sulfate reduction rate > 50% was observed within 8 weeks (Figure 5.2), which was accompanied by a decrease in E_h from around 50 mV to approximately -110 mV. At the same time, a substantial removal of Zn (75%) and Co (55%) from the effluent was observed, whereas attenuation of the Ni concentration started (Figure 5.3, 5.4, 5.5). No Fe removal was observed (Figure 5.6). The effluent pH increased from pH= 4.0 to 5.7, and the influent's DOC varied from 200 mg l^{-1} to 1250 mg l^{-1}, which was probably a result of the heterogeneity of molasses and growth of contaminating bacteria; nevertheless, a decrease in DOC could be observed throughout the column, varying from 65% to 75% degradation.

However, after 11 weeks, sulfate- and heavy metal concentrations suddenly increased dramatically in the column's effluent. In addition, the effluent's pH dropped with one unit, the E_h increased from -110 mV to a value around -5 mV and DOC-consumption stagnated. Moreover, 4 weeks later (~15 weeks after starting the column experiment), some retained Co and Ni were released into the effluent, which is congruent with the E_h and pH behavior of Co-O-H-S and Ni-O-H-S systems. Mass balances showed that a total amount of ~88 mg Co had been retained in the column during the first 13 weeks of the experiment, but in the next 12 weeks, until 25 weeks, a total of ~179 mg of precipitated Co was washed out. This suggests that not only all of the accumulated Co but also some formerly precipitated/ adsorbed Co was released into the effluent. The mass balance for Ni showed that about 175 mg was retained from T= 0 tot T= 15 weeks, but ~94.5 mg of precipitated Ni was released into solution in the next 12 weeks, which corresponds to a leaching of 54%. Only a minor release of Zn was observed, while no increase in the effluent's Cd-concentration was detected during this period (data not shown).

Between T= 8 and T= 26, the production of methane and hydrogen was measured semi-quantitatively every 2 to 3 weeks, and it was found that these gasses were

present in the effluent of all columns, except the abiotic control (data not shown). Estimated concentrations were low: the CH_4 concentrations fluctuated from 0.01 – 0.03 ppm, whereas the H_2 concentration varied from 0.001 – 0.005 ppm. No striking differences in CH_4 production could be noticed between the different column set-ups or in function of time. On the other hand, H_2 production was increased a 100-fold in the molasses amended column at T= 15 weeks and T=17 weeks.

In order to study the influence of methanogenic prokaryotes on the performance of the molasses-amended column, the COD/ SO_4^{2-}ratio was modified from 1.9 to 0.4 (i.e. DOC= 60 – 100 mg l^{-1}) after 26 weeks (Table 5.2), but no positive effect on sulfate or heavy metal removal could be detected: Ni and Co were no further released into the effluent, the ISMP process remained negligible over a period of 25 weeks (~51 weeks after starting up the experiment). On the other hand, Fe removal rates increased rapidly (Figure 5.6); however, as with the control set-ups, this could be attributed to Fe^{2+} being oxidized to Fe^{3+}, or to a lack of a microbial or chemical reducing environment (E_h >15 mV). In the hope that the indigenous SRB community was not completely eliminated and that its sulfate-reduction activity could be regained, the COD/ SO_4^{2-}-ratio was altered back to its original value of 1.9. Indeed, 6 weeks later, the bacterial sulfate reduction was restored, and this was accompanied by a gradual increase in metal removal efficiencies, pH and DOC-consumption. Moreover black spots, presumably FeS precipitates, became visible in the column aquifer, and although Fe removal suddenly dropped, this was instantly followed by increasing removal ratios. Altogether, these results suggest that Fe^{3+}-reduction had taken over from Fe^{2+}-oxidation. Also, sulfate removal rates of 74% were established within 14 weeks (~65 weeks after starting up the experiment) and the effluent's E_h was decreased from ~15 mV to ~ -100 mV at that time.

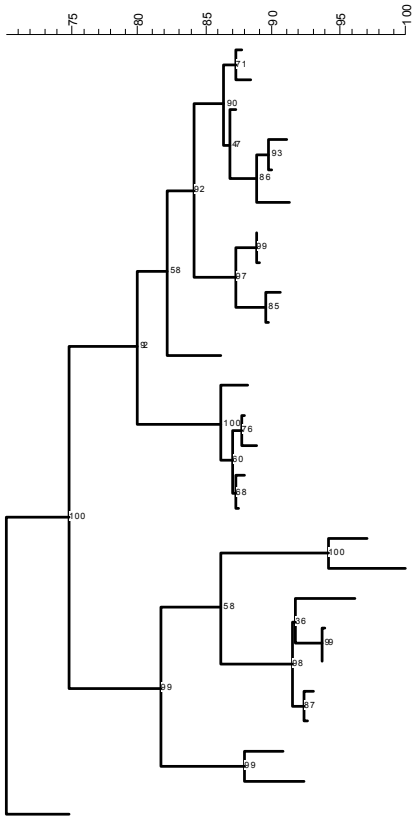
Figure 5.7 Phylogenetic analysis of non-thermophilic crenarchaeal sequences detected in the column experiments. The evolutionary tree was generated by the neighbor joining method, evolutionary distances were generated using the Kimura 2 parameter. The Maximum Parsimony algorithm was used to evaluate branching orders. Bootstrap resampling (1000 replicates) of the tree was performed to provide confidence estimates for the inferred topologies. Percentages of bootstrap support are indicated at the branch points. An out-group of the 16S rRNA-gene of the thermophilic crenarchaeote *Sulfolobus acidocaldarius* was included to root the tree. The bar at the top indicates the estimated evolutionary distance.

d) Performance of the HRC®-amended column

8 weeks after initializing the experiment, the bacterial sulfate reduction rate was > 50% (Figure 5.2) together with removal rates of 75% for Zn and 45% for Co, whereas attenuation of the Ni-concentration was observed (Figure 5.3, 5.4, 5.5). No Fe-removal was observed (Figure 5.6). Compared to the other set-ups, the influent of the HRC® amended column had a decreased pH (3.5). In this set-up, an increase up to pH=5.0 was observed. This was accompanied with a decrease in the effluent's DOC from ~ 520 mg l^{-1} to 300-150 mg l^{-1}, meaning that 42% to 71% of DOC is consumed. As for the lactate amended column, concentrations of SO_4^{2-} started fluctuating after 15 weeks. Thereupon, only minor variations were observed in Zn, Cd and Ni-removal rates but Co concentrations in the columns effluent began to increase slightly over time.

Since HRC® slowly releases lactic acid when hydrated, we examined whether the HRC® amended column behaves similar to the lactate-fed column. Therefore, we caused an intentional process failure by disrupting the HRC® addition at the same time as the substrate supply was stopped for the lactate amended column (T= 26 weeks). Unfortunately, the issue was obscured because sulfate and metal removal already showed a declining trend from T= 24 weeks in the HRC®-amended column. After disruption of the HRC® supply, the activity of the indigenous SRB-populations showed a further decrease and within 5 weeks, bacterial sulfate reduction had come to a stop. This was accompanied by a gradual decrease of the pH, increase of the E_h, and leaching of Fe, Ni and Co into the effluent. A total of ~1100 mg Co had accumulated in the column before interrupting substrate

Figure 5.7



kL_Ar_3	A + Q + Lactate (inlet, T25)
kLNP_Ar_3	A + Q + Lactate + N/P (outlet, T25)
kLNP_Ar_2	A + Q + Lactate + N/P (outlet, T13)
kL_Ar_2	A + Q + Lactate (outlet, T13)
kL_Ar_1	A + Q + Lactate (inlet, T13)
Uncultured crenarchaeote VAL18	AJ131321
Unidentified crenarchaeote clone pLemB389	U59995
Unidentified crenarchaeote clone pLemB108	U59988
Unidentified crenarchaeote clone pGrfB98	U59985
kM_Ar_3	A + Q + Molasses (outlet, T13)
Uncultured crenarchaeote clone pSL123	U63345
kM_Ar_2	A + Q + Molasses (outlet, T13)
kH_Ar_2	A + Q + HRC (outlet, T13)
kM_Ar_1	A + Q + Molasses (intlet, T13)
kLNP_Ar_1	A + Q + Lactate + N/P (intlet, T13)
Unidentified crenarchaeote strain VIARC-2	AJ240005
Uncultured crenarchaeote SB95-61	U78202
Archaeal sp. clone SBAR5	MB80175
Unidentified archaeon SCA1154	U62814
Unidentified archaeon SCA1175	U62819
Unidentified crenarchaeote clone pGrfA23	U59973
Unidentified archaeon SCA1145	U62811
Unidentified crenarchaeote clone pGrfA50	U59979
Archaeal sp. clone FFSB2	X96689
Archaeal clone FFSB3	X96690
Sulfolobus acidocaldarius	

Figure 5.8 Population fingerprints by eubacterial 16S rDNA PCR-DGGE analysis obtained for the different column setups. DNA from different time points (13, 18, 25, 31, 38, 51 and 56 weeks) was used. Cloned 'bands' are indicated within the aquifer fingerprint based on the comparison of migration profiles of pure clones and the soil profile. Legend: T, time (weeks); I, 10 cm from inlet; E, 30 cm from inlet. **(A) DGGE profiles of the lactate-amended column.** Lanes: 1, DGGE marker; 2, T13 E; 3, T13 I; 4, T18 E; 5, T18 I; 6, T25 E; 7, T25 I ; 8, DGGE marker ; 9, T31 E; 10, T31 I; 11, T38 E; 12, T38 I; 13, T51 E; 14, T51 I; 15, T56 E; 16, T56 I. **(B) DGGE profiles of the lactate + N/P -amended column.** Lanes: 1, DGGE marker; 2, T13 E; 3, T13 I; 4, T18 E; 5, T18 I; 6, T25 E; 7, T25 I ; 8, T31 E; 9, DGGE marker; 10, T31 I; 11, T38 E; 12, T38 I; 13, T51 E; 14, T51 I; 15, T56 E; 16, T56 I. **(C) DGGE profiles of the molasses-amended column.** Lanes: 1, DGGE marker; 2, T13 E; 3, T13 I; 4, T18 E; 5, T18 I; 6, T25 E; 7, T25 I ; 8, T31 E; 9, DGGE marker; 10, T31 I; 11, T38 E; 12, T38 I; 13, T51 E; 14, T51 I; 15, T56 E; 16, T56 I. **(D) DGGE profiles of the HRC®-amended column.** Lanes: 1, DGGE marker; 2, T13 E; 3, T13 I; 4, T18 E; 5, T18 I; 6, T25 E; 7, T25 I ; 8, DGGE marker ; 9, T31 E; 10, T31 I; 11, T38 E; 12, T38 I; 13, T51 E; 14, T51 I; 15, T56 E; 16, T56 I.

supply, and 260 mg or 23.6% was washed out between T= 29 weeks and T= 39 weeks. With regard to Ni, ~4623 mg of Ni was retained, but ~2028 mg, or 44%, leached into solution in the first 16 weeks following substrate cut off.

To reinstall after a 15 weeks interruption (~41 weeks after starting up the experiment) an efficient ISMP process, the HRC® solution was fed again to the column. The sulfate removal rate rapidly increased, and after 18 weeks (i.e. after a total of ~59 weeks), a removal efficiency of > 97% was achieved. This was accompanied by restoration of metal removal efficiencies, reaching values of >99%. DOC values in the effluents gradually decreased, whereas pH values increased from 4.0 to 5.5.

5.3.2. Molecular analysis of microbial communities

PCR primer sets ARC-344F/ ARC-915R (Casamayor *et al.*, 2002) and McrA-F/ McrA-R (Luton *et al.*, 2002) were applied to examine for the presence of methane producing prokaryotes. Although in all column setups, except for the abiotic control, a PCR amplicon was obtained with the archaeal primer set ARC-344F/ ARC-915R, there was no detection of *mcrA*-genes with the primers McrA-F/ McrA-R (data not shown). Analysis of randomly cloned ARC-344F/ ARC-915R PCR

Figure 5.8

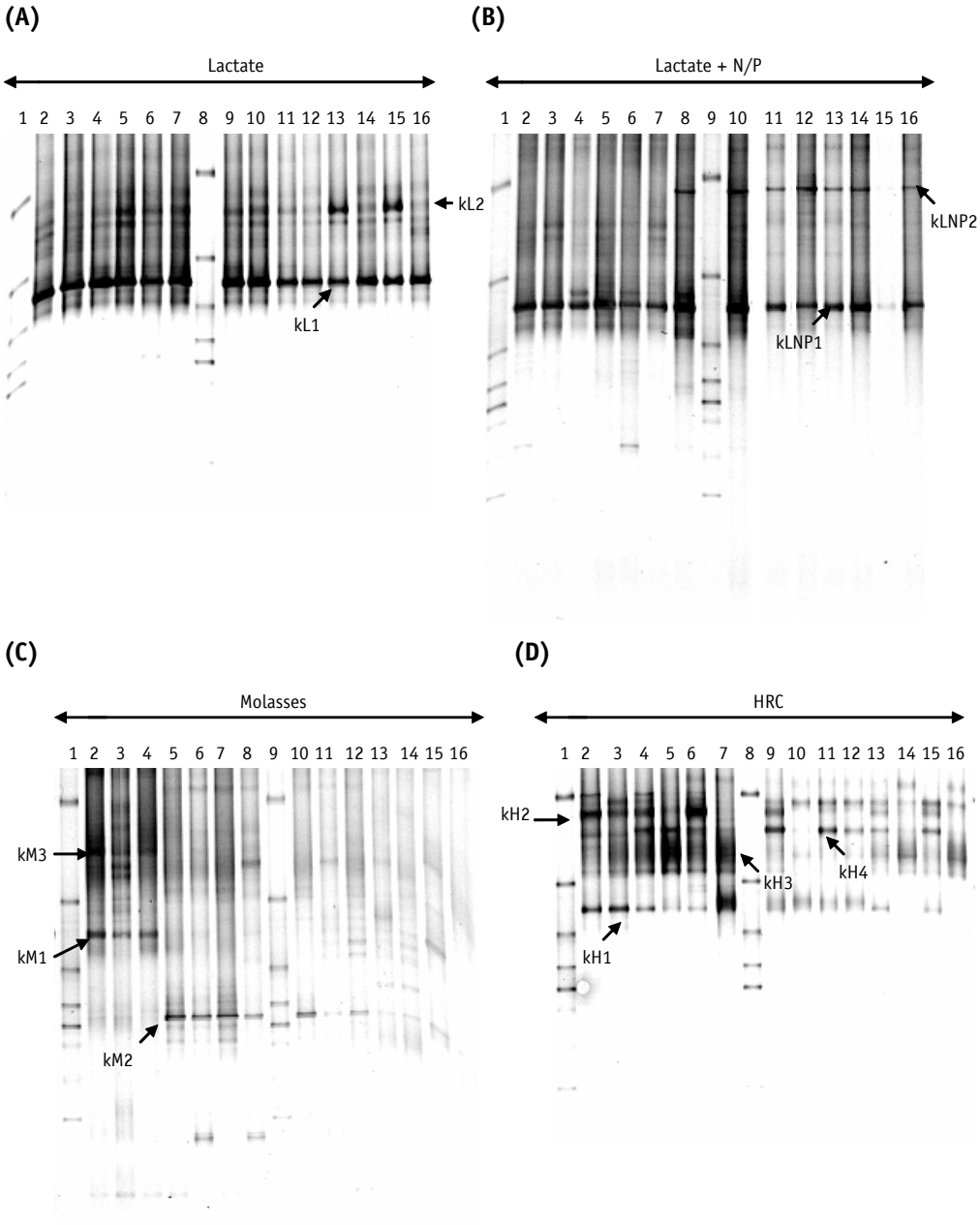
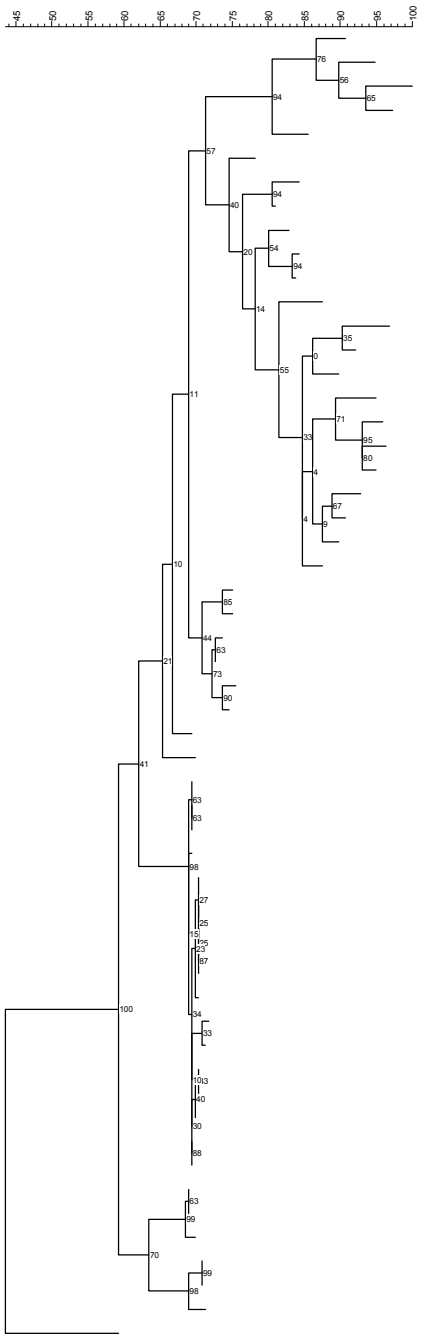

(A)
Lactate
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
kL2
kL1
(B)
Lactate + N/P
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
kLNP2
kLNP1
(C)
Molasses
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
kM3
kM1
kM2
(D)
HRC
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
kH2
kH3
kH4
kH1

Figure 5.9 Phylogenetic analysis of 16S rDNA-gene fragments detected in the DGGE fingerprints of column experiments. The evolutionary tree was generated by the neighbor joining method, evolutionary distances were generated using the Kimura 2 parameter. The Maximum Parsimony algorithm was used to evaluate branching orders. Bootstrap resampling (1000 replicates) of the tree was performed to provide confidence estimates for the inferred topologies. Percentages of bootstrap support are indicated at the branch points. An out-group of the 16S rRNA-gene of *Archaeoglobus fulgidus* was included to root the tree. The bar at the top indicates the estimated evolutionary distance.

amplified 16S rDNA showed close similarities (96 to 98% sequence identity) to a unique and globally distributed lineage in the kingdom of the *Crenarchaeota* that is phylogenetically distinct from currently characterized crenarchaeotal species (Figure 5.7). No sequences were found within the kingdom of the *Euryarchaeota*, which comprises methanogenic *Archaea*. Thus, no methane producers were detected using PCR. In order to obtain an insight in the whole community composition and dynamics during the course of the ISMP process, 16S rDNA-based DGGE fingerprints derived from the different columns where compared with each other, as well as analyzed at different points in time (13, 18, 25, 31, 38, 51 and 56 weeks). The response of the indigenous SRB-community to the different conditions and its dynamics in the course of the ISMP column experiments was examined by *dsrB*-based DGGE, a specific biomarker for SRB (Chapter 3). Profiles generated from the abiotic control and the non-carbon amended column are not shown since bands were hardly visible on the DGGE-gel. Different bands within one fingerprint (Figures 5.8, 5.11) were cloned and sequenced.

In general, 16S rDNA-based DGGE analysis revealed that the SRB population consisted exclusively of members of the genus *Desulfosporosinus* (Figure 5.9). This was in accordance to the results of the previously performed batch experiments (Chapter 4). In the HRC® amended column, this *Desulfosporosinus* population was more diverse compared to the other setups. Interruption of HRC® supply caused the community fingerprints to become more faint, but when the substrate amendment was restored, the original community gradually re-established. In the molasses-amended columns, 16S rDNA-based DGGE fingerprints revealed the presence of an additional DNA band, kM3, whose sequence clustered within the

Figure 5.9



..	kM4	
.	Campylobacter jejuni	L04315
.	Sulfurospirillum arcachonense	Y11561
.	Arcobacter nitrofigilis	L14627
.	Wolinella succinogenes	M88159
.	Desulfonatronum lacustre	Y14594
.	Desulfovibrio desulfuricans	M34113
.	Desulfovibrio piger	AF192152
.	Desulfovibrio gracilis	U53464
.	Desulfomicrobium baculatum	AF030438
.	Desulfomicrobium escambiense	AJ277886
.	Desulforhabdus amnigenus	X83274
.	Desulfonema limicola	U45990
.	Desulfococcus multivorans	AF418173
.	Desulfobacca acetoxidans	AF002671
.	Desulfobulbus rhabdoformis	U12253
.	Desulfocapsa thiozymogenes	X95181
.	Desulfofustis glycolicus	X99707
.	Desulforhopalus singaporensis	AF118453
.	Desulfomusa hansenii	AF321820
..	Desulfofaba gelida	AF099063
.	Desulfobacterium indolicum	AJ237607
.	Desulforegula conservatrix	AF243334
.	Desulfotomaculum sp. DEM-KMe98_4	AJ276558
.	Desulfotomaculum sp. DEM-KMe99_2	AJ276565
.	Desulfotomaculum aeronauticum	X98407
.	Desulfotomaculum reducens	U95951
.	Desulfotomaculum nigrificans	X62176
.	Desulfotomaculum putei	AF053933
.	Desulfotomaculum geothermicum	X80789
.	Desulfotomaculum thermoacetoxidans	Y11573
.	kM1	
.	kH1	
..	kL1	
.	kLNP1	
..	Desulfosporosinus idahoense	AJ582756
.	Desulfosporosinus sp. 44A_T3A	AY082482
.	Desulfosporosinus sp. P3	AF427080
.	kH4	
..	Desulfosporosinus limneticum	AJ582757
..	kL2	
.	Desulfosporosinus orientis	Y11570
.	Desulfosporosinus auripigmentum	AJ493051
.	kH2	
.	Desulfosporosinus meridiei	AF076247
..	kLNP2	
.	kH3	
.	Desulfosporosinus sp. LauIII	AJ302078
.	Ethanologenbacterium harbin strain MD-19	AY434722
.	Clostridium sp. rennanqilfy3	AY363375
..	kM2	
.	Clostridium aerotolerans	X76163
.	Clostridium xylanolyticum	X71855
.	Clostridium aminovalericum	X73436
.	Archaeoglobus fulgidus	Y00275

class of the ε-*proteobacteria*, with the 16S rRNA-genes of *Campylobacter* sp. NO3A (Hubert *et al.*, 2003) and *Sulfurospirillum arcachonense* (Finster *et al.*, 1997) as the closest cultured matches (90% sequence identity). Since sequence similarities are low, we cannot ascertain what function or activity is carried out by this bacterium. However, at T= 18 weeks, when sulfate- and metal removal had dropped significantly, the indigenous community profile shifted towards a pattern that was dominated by band kM2, whose sequence was related to hydrogen producing strains such as *Clostridium* sp. rennanqilfy 3 (Ren *et al.*, 2003b) and *Ethanologenbacterium harbin* strain MD-19 (Ren *et al.*, 2003a) (both with 97% sequence similarity). The presence of the species from which band kM2 was derived might explain the H_2-peak at T= 15 weeks and T= 17 weeks, as was detected by GC-RGD. In the period that the COD/ SO_4^{2-}-ratio was decreased, faint community profiles were obtained, which suggested that little bacterial growth occurred. Changing the COD/ SO_4^{2-}-ratio back into its original value did not immediately affect the intensity of the banding pattern but DNA band kM1, which corresponded with a *Desulfosporosinus* strain, reappeared and its increase in intensity coincided with a gradual increase in sulfate reduction and metal precipitation rates.

UPGMA clustering of the eubacterial 16S rRNA-based gene DGGE profiles of the different column set-ups demonstrated that the bacterial community was very stable with little diversity in the lactate amended columns (Figure 5.10, profiles with faint bands were not included to avoid resolution loss in clustering analysis). Two main clusters were observed: one cluster contained the DGGE profiles of the HRC®-amended column during the second phase of the ISMP experiments, which was probably because some DNA bands had become too faint

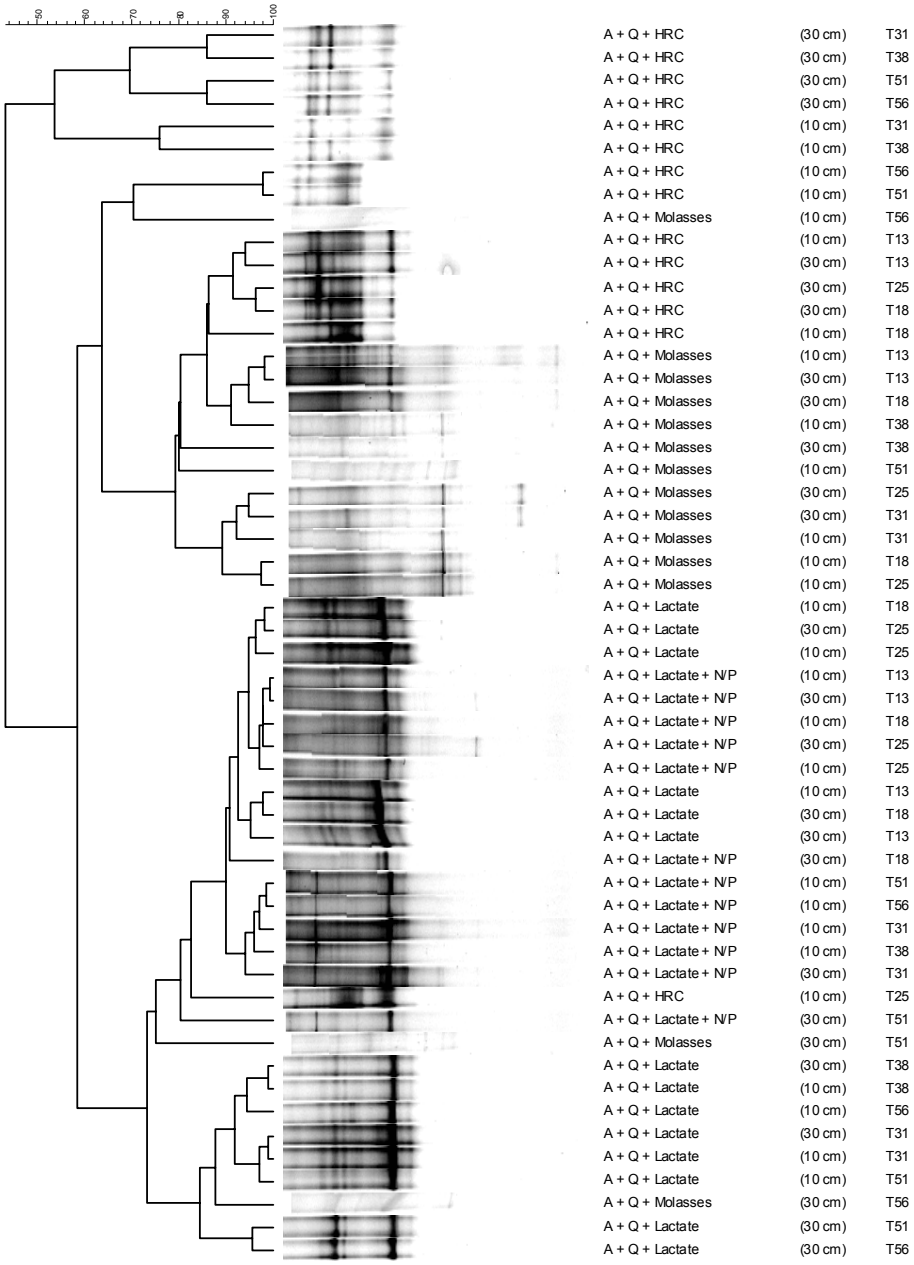


Figure 5.10 UPGMA clustering of community profiles obtained by eubacterial 16S rRNA gene PCR-DGGE analysis obtained for the different column setups at different points of time. Clustering was performed using the Pearson moment-based similarity coefficient. Similarity percentages between populations are indicated above.

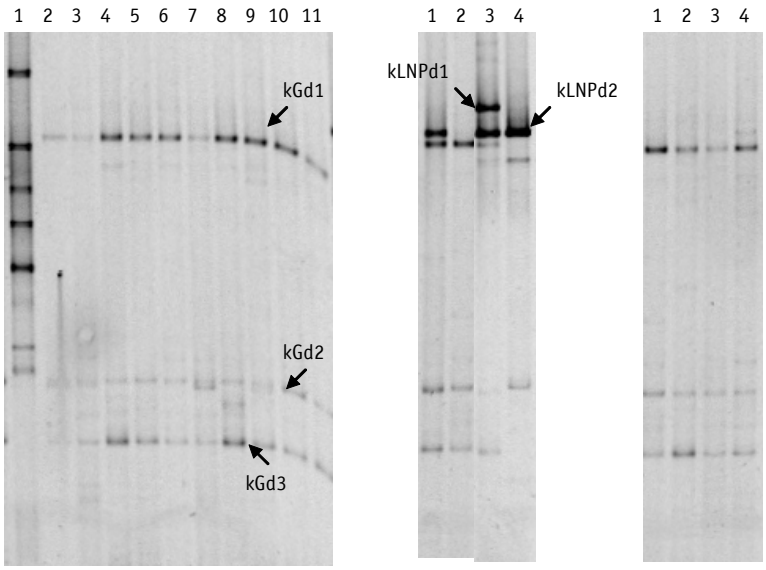
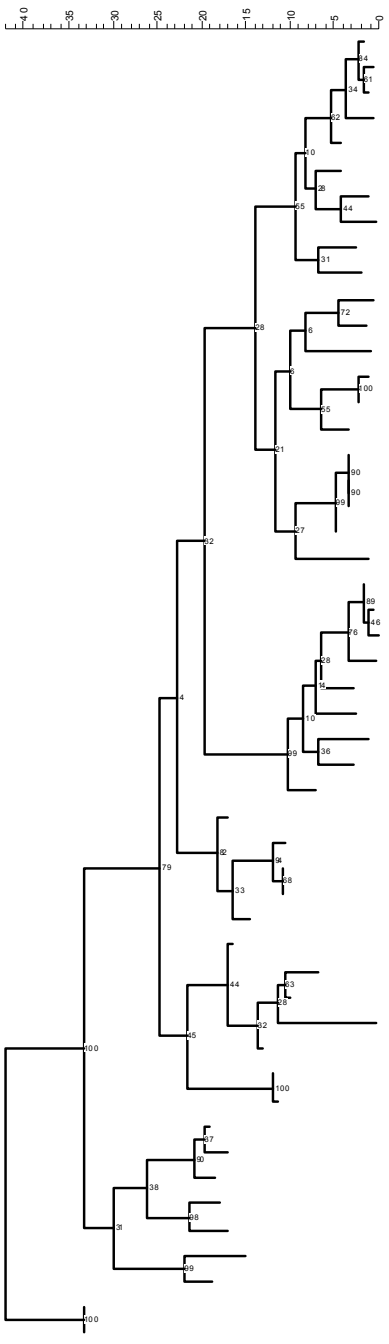


Figure 5.11 *dsrB* PCR-DGGE population fingerprints obtained for the different column setups. DNA from different time points (13, 18, 25, 31, 38, 51 and 56 weeks) was used, but only the most relevant profiles are shown. Cloned 'bands' are indicated within the aquifer fingerprint based on the comparison of migration profiles of pure clones and the soil profile. DNA from three different time points (13, 18, 25, 31, 38, 51 and 56 weeks) was used. Cloned 'bands' are indicated within the aquifer fingerprint based on the comparison of migration profiles of pure clones and the soil profile. Legend: A, aquifer; G, groundwater; T, time (weeks); I, 10 cm from inlet; E, 30 cm from inlet. **(A) DGGE profiles at T= 13 weeks.** Lanes: 1, DGGE marker; 2, A + Q E; 3, A + Q I; 4, A + Q + lactate E; 5, A + Q + lactate I; 6, A + Q + lactate + N/P E; 7, A + Q + lactate + N/P I ; 8, A + Q + molasses E; 9, A + Q + molasses I; 10, A + Q + HRC® E; 11, A + Q + HRC® I. Identical patterns were obtained at T= 18 weeks, except for the molasses-amended setup (no PCR amplicon). **(B) DGGE profiles of the lactate + N/P -amended column at T= 31 weeks and T= 38 weeks.** Lanes: 1, A + Q + lactate + N/P E T31; 2, A + Q + lactate + N/P I T31; 3, A + Q + lactate + N/P E T38; 4, A + Q + lactate + N/P I T38. At T= 51 weeks and T= 56 weeks, DGGE profiles were identical to the profiles of T= 38 weeks. **(C) DGGE profiles of the lactate and HRC® -amended columns at T= 51 weeks.** Lanes: 1, A + Q + lactate E; 2, A + Q + lactate I; 3, A + Q + HRC® E; 4, A + Q + HRC® I. Identical patterns were obtained at T= 56 weeks.

Figure 5.12



.Desulfobacter postgatei	AAL57470
.Desulfobacter curvaturus	AAL57472
.Desulfobacter latus	AAC24098
.Desulfobacterium autotrophicum	AAL57438
.Desulfobacterium vacuolatum	AAL57480
.Desulfobotulus sapovorans	AAC24110
.Desulfococcus multivorans	AAC24102
.Desulfonema limicola	AAC24100
.Desulfofaba gelida	AAK83202
.Desulfosarcina variabilis	AAK71936
.Desulfovibrio longus	BAB55574
.Desulfovibrio aespoeensis	AAL99083
.Desulfovibrio desulfuricans subsp. desulfuricans	AAK83200
.Desulfovibrio vulgaris	BAB55580
.Desulfovibrio termitidis	AAL57444
.Desulfovibrio africanus	AAG21987
.Desulfomicrobium macestii	BAB55560
.Desulfomicrobium norvegicum	BAB55558
.Desulfomicrobium baculatum	BAB55554
.Desulfomicrobium orale	AAM03450
.Desulfonatronum lacustre	AAL57452
.kGd1	
.kLNPd1	
.kLNPd2	
.kGd2	
.Desulfotomaculum acetoxidans	AAK82967
.Desulfotomaculum geothermicum	AAK58399
.Desulfotomaculum thermoacetoxidans	AAK83692
.Desulfotomaculum thermosapovorans	AAK83690
.kGd3	
.Desulfofustis glycolicus	AAL57456
.Desulfobulbus rhabdoformis	CAB95039
.Desulfobulbus elongatus	AAL57477
.Desulfobulbus propionicus	AAG28586
.Desulforhopalus singaporensis	AAL57466
.Thermodesulforhabdus norvegica	AAK83210
.Desulfovirga adipica	AAK83198
.Desulforhabdus amnigena	AAL07559
.Desulfoarculus baarsii	AAK83216
.Desulfacinum infernum	AAL57462
.Thermodesulfobacterium commune	AAL78312
.Thermodesulfobacterium thermophilum	AAK83212
.Desulfotomaculum aeronauticum	AAK58407
.Desulfotomaculum ruminis	AAC24104
.Desulfotomaculum putei	AAK58405
.Desulfosporosinus orientis	AAK82965
.Desulfitobacterium dehalogenans	AAL07563
.Archaeoglobus profundus	AAC78310
.Archaeoglobus fulgidus	AAB17214
.Thermodesulfovibrio islandicus	AAK83214
.Thermodesulfovibrio yellowstonii	AAC24112

Figure 5.12 Phylogenetic analysis of *dsrB*-gene fragments detected in the DGGE fingerprints of column experiments. The evolutionary tree was generated by the neighbor joining method, evolutionary distances were generated using the Kimura 2 parameter. The Maximum Parsimony algorithm was used to evaluate branching orders. Bootstrap resampling (1000 replicates) of the tree was performed to provide confidence estimates for the inferred topologies. Percentages of bootstrap support are indicated at the branch points. An out-group of the DsrB proteins of *Thermodesulfovibrio yellowstonii* and *Thermodesulfovibrio islandicus* were included to root the tree. The bar at the top indicates the estimated evolutionary distance.

to be considered in the clustering calculations; the second cluster consisted of the remaining profiles, and was further split into two groups, one composed offingerprints derived from the lactate and lactate + N/P amended setups, and the other of both the molasses and HRC®-amended setup during the first phase of the ISMP experiment. This result indicated that initially very similar bacterial communities were enriched, independent of the type of amendment. Amplification of the *dsrB*-gene turned out to be strongly related to the activity of the sulfate reduction and the ISMP process. At T= 13 weeks, the *dsrB*-based DGGE profiles of the different columns that showed sulfate reduction activity were highly similar and showed little diversity (Figure 5.11A). At T= 31 weeks, only with material from the lactate + N/P amended column the *dsrB* gene could be amplified (Figure 5.11B), which is in accordance with the observation that only this column showed significant sulfate reduction at that time. Moreover, a shift had occurred in the *dsrB*-based DGGE profile, this as a result of the increased COD/ SO_4^{2-} ratio, which stimulated the ISMP process. At T= 38 weeks, after restoring the carbon-source amendment, a *dsrB*-based DGGE pattern similar to the earlier profiles (Figure 5.11C) was obtained for the setups with lactate and HRC®.
In contrast to the sequencing results of the 16S rRNA genes, none of the sequenced *dsrB*-based DGGE bands clustered with *dsrB* gene sequences of the genus *Desulfosporosinus* (Figure 5.12). Instead, they formed a divergent lineage with *Desulfotomaculum dsrB* gene sequences, which received their xenologous *dsr* gene from δ-*proteobacteria* (Klein *et al.*, 2001). This observation corresponds to *dsrB* gene sequencing results of our batch experiments (Chapter 4).

5.4. Discussion

In a previous study (Chapter 4), we explored the applicability of acetate, lactate, molasses, HRC®, methanol and ethanol as C-source and electron donor to promote bacterial sulfate reduction in batch experiments, this with regards to applying an ISMP process as a remediation tool for heavy metal-contaminated groundwater at the site of a nonferrous industry. Based on the results of these batch tests, column experiments were conducted with lactate, molasses and HRC® as the next step in the preliminary study for a go-no go decision for dimensioning an on site application of the ISMP process that applies the activity of the indigenous population of SRB. Column experiments are pivotal because they allow investigating the ISMP process in a more realistic aquifer (Janssen and Temminghoff, 2004). Special attention was given to the sustainability of the precipitation process in circumstances of changing COD to [SO_4^{2-}] ratios or when the substrate supply was disrupted.

Initially, it seemed that the setup with molasses was the most optimal condition for ISMP since HM removal efficiencies and sulfate-consumption rates were higher and increased faster compared to the other setups. However, the sulfate-removal rate suddenly dropped after 12 weeks. Thereupon, metal concentrations dramatically increased in the column's effluent. Although over-saturation of the aquifer (Diels *et al.*, 2003) might have caused some of this metal increase, calculation of Ni and Co mass balances indicated that most of the observed metal increase was due to the release of retained metal ions into the effluent. Metal-O-H-S systems and processes such as the adsorption of metals to different clay minerals and calcareous soils (Garcia-Sanchez *et al.*, 1999), the complexation to organic matter (Davis, 1984) or silica (Delolme *et al.*, 2004), adsorption to biologically produced metal sulfides (Jong and Parry, 2004), adsorption to (Davis, 1984) and/ or co-precipitation with iron sulfide (Moore *et al.*, 1989) are strongly dependent of E_h and pH. Indeed, it was found that metal leaching was preceded by the loss of a reducing environment and a decrease in pH. The fact that

retained Fe, Ni and Co are substantially released into solution and no leaching of Zn and Cd was observed, is reflected in the trend in solubility products (K_{sp}) of the respective metal sulfides; the log K_{sp} values of CdS (-27.9) and ZnS (-23.3) are much lower than the log K_{sp} of CoS (-21.6), NiS (-19.4) and FeS (-18.1). Moreover, the solubilization of metal sulfide complexes with a high log K_{sp} value (FeS, NiS, CoS) might result in a shift in chemical equilibrium: the released sulfides will readily react with leaching Zn and Cd ions to form ZnS and CdS again. This could explain why little or no leaching was observed for Zn and Cd.

Growth of sulfate-reducers could not be boosted by decreasing the COD/ SO_4^{2-} ratio. Nevertheless, molecular analysis using the 16S rRNA gene as a target showed that the SRB community survived for more than half a year and by reinstating the original COD/ SO_4^{2-} ratio, its sulfate reduction activity was re-established.

Also in the lactate-HRC®- and lactate + N/P-amended columns unexplainable decreases in sulfate- and metal removal-rates were sometimes observed over shorter periods. Intentional disruption of substrate supply in the lactate- and HRC®-amended setups resulted in a strong drop in the sulfate- and metal-removal rates, followed by release of retained metals. Significant differences could be noticed between the percentages of metal ions that were released in the effluents, which might be explained by differences in E_h and pH as well as by heterogeneity of the column aquifers. As in the molasses-amended column, molecular analysis showed that the SRB community survived and that their activity could be restored by substrate application.

In the case of an intentional disruption of substrate supply, the ISMP process failed most likely because growth and activity of the indigenous SRB community came to a stop due to the lack of a carbon and electron donor. However, the cause of the suddenly observed temporal shortcomings of the ISMP process in the presence of different substrates was not immediately clear. It was first thought to be the result of competition between methanogenic prokaryotes (MP) and sulfate reducers, since the formation of small CH_4 amounts (0.01 – 0.03 ppm) was

detected. There is a classical paradigm of SRB predominating MP in the presence of non-limiting sulfate concentrations, because SRB have kinetic and thermodynamic advantages in the competition for common substrates (hydrogen, acetate) (Ward and Winfrey, 1986; Widdel, 1988). However, according to recent studies, methanogens can outcompete SRB even in the presence of non-limiting sulfate concentrations; this observation was explained by variable sulfide toxicities (Parkin *et al.*, 1990), mass transfer limitations (Nielsen, 1987), or differences in microbial colonization and adhesion properties (Iza *et al.*, 1986). Nevertheless, there was methane production in all setups even when metal- and sulfate-removal was highly efficient, suggesting that SRB and MP might co-exist in a synergistic way, e. g. the sulfate reducers oxidize lactate into acetate, which is in turn consumed by methanogens. Recently, it was demonstrated that some SRB (mainly the so-called "incomplete" oxidizers, which oxidize their substrates incompletely to e.g. acetate) can grow syntrophically with MP (Oude-Elferink *et al.*, 1998; Raskin *et al.*, 1996).

In order to elucidate whether MP were present in our columns and if competition or syntrophy was occurring between the SRB and MP populations, the community of *Archaea* was characterized by PCR using the archaeal specific primer pair ARC-344F/ARC-915R (Casamayor *et al.*, 2002), followed by cloning and sequencing of PCR amplicons. This approach only revealed the presence of non-thermophilic *Crenarchaeota*. Little is still known about the physiological characteristics and ecological significance of this novel group of organisms and they are only distantly related to methane producing *Euryarchaeota* (Buckley *et al.*, 1998). Subsequently, we tried without success to use a PCR primer set for the specific detection of *mcrA*-genes (Luton *et al.*, 2002), which are believed to be a specific biomarker for methanogens (Thauer, 1998). These results indicate that methanogens do not constitute a major fraction of the microbial communities that we enrich in our column experiments. This was underlined by the observation that the growth of sulfate-reducers in the molasses-amended column was not favored by decreasing the COD/ SO_4^{2-} ratio. Moreover, in the lactate + N/P

setup, in spite of our efforts to stimulate methane producers by increasing COD/ SO_4^{2-} ratio, the opposite effect was obtained and the ISMP process was significantly enhanced. Analysis of eubacterial 16S rDNA sequences did not reveal the presence of non-sulfate reducers that might have outplayed the SRB community. Therefore, we postulate that the SRB community becomes inhibited by the formed metal sulfides. Liu and Fang (1997) suggested that the inhibition of sulphidogenic activities of biogranules used for the treatment of sulfate-loaded wastewater resulted from the formation of excessive sulphureous precipitates on the bacterial cell surface. This phenomenon was also observed in an anaerobic bioreactor used for treating acid mine drainage (Utgikar *et al.*, 2002). By means of scanning electron micrographs, Utgikar and coworkers (Utgikar *et al.*, 2002) pointed out that metal sulfides could act as barriers preventing the access of reactants (sulfate, organic matter) to the necessary enzymes. Nevertheless, the SRB community retained its ability to perform sulfate reduction, which indicated that the metal sulfides were not lethally toxic to them. It was also observed that re-establishment of sulfate reduction was easily obtained when part of the metal precipitates were dissolved. We assume that similar processes took place during the course of our column experiments, as was indicated by periods of efficient metal and sulfate removal followed by periods of decreased microbial activity, during which excess metals were removed. One should also keep in mind that the molasses amended column, which initially showed the most efficient ISMP process, was the first to fail. This observation has major consequences for the application of the ISMP process, as a continuous risk for unexpected metal release exists.

In conclusion, our results indicate that the ISMP process is highly dependent on SRB-stimulation by substrate amendments and suggest that this remedial approach might not be viable for long-term application unless substrate amendments are continued or environmental conditions are strictly controlled. This will include the removal of affected aquifer material from the metal precipitation zone at the end of the remediation process, or removal of metal

precipitates when the microbial activity decreases. Additional tests are necessary to investigate what will happen when clean groundwater passes through the reactive zone while no more C-sources are amended and all indigenous carbon is consumed. Also, the effect of dramatic increases in sulfate-or HM concentrations on the SRB-community and the concomitant ISMP process needs to be studied in more detail.

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CHAPTER 6

General Discussion and Perspectives

Recently, *in situ* metal precipitation (ISMP) has received much attention for the treatment of heavy metal contaminated-groundwater, especially when dealing with the control of heavy metal contaminated groundwater plumes at sites where more invasive physical-chemical approaches are too expensive or simply can not be implemented. The success of the *in situ* immobilization of metals relies on the presence and management of the indigenous community of sulfate-reducing bacteria (SRB). The ability of the SRB to maintain constant metal precipitation activity also needs to stand the test of time. This implies that competition or syntrophy with other non-sulfate reducing strains such as methanogens, Fe-reducers, acetogens or fermenting bacteria should be taken into account. Moreover, the SRB community should withstand fluctuating environmental conditions often encountered with industrial groundwater plumes, including significant changes in sulfate- and heavy metal-concentration, redox potential and pH that can strongly impact, often in a negative way, the activity and sustainability of the ISMP process.

In this study, we conducted batch and column experiments to explore the feasibility and applicability of ISMP as a remediation strategy for a contaminated groundwater plume, located at the site of a nonferrous industry. This plume is severely enriched with heavy metals (Zn, Cd, Ni, Co), with local conditions of low pH, high sulfate concentration and a naturally high Fe content (Tables 4.1, 5.1). The outcomes of these preliminary studies should eventually lead to a go- no go decision for the design of an on site biological barrier to promote ISMP as the remediation strategy for this metal contaminated groundwater plume. Although

analytical data ([SO_4^{2-}], [HM], pH, DOC,...) are indispensable in the optimization of the design of a large-scale implementation setup, and in the follow-up of biological processes once the ISMP technology is being applied, the use of molecular techniques will provide more detailed information regarding the impacts of process and environmental conditions on the composition and activity of the indigenous SRB population, as well as on that of the non- SRB microbial community members.

6.1. Development of microbial tools to study the SRB community structure and activity

To date, cloning and sequencing of *dsr* gene libraries is the only generally available molecular tool to obtain an insight in the functional diversity and dynamics of SRB communities. Although this is an effective approach, there is a need for alternative methods to rapidly assess the diversity of SRB communities over time and space for the follow-up of bioremediation processes such as ISMP.
In Chapter 3 we developed a *dsrB*-based DGGE method which effectively monitors the abundance, diversity and dynamics of SRB in heavy metal contaminated environmental samples. Its results were compared to those found with PCR amplification of SRB-subgroup and -genus specific primers and DGGE analysis of 16S rDNA. It was shown that the method delivers reliable results and besides had the important advantage that minor populations of SRB in a highly diverse microbial community were not overlooked. Moreover, the PCR- detection of *dsrB* genes seemed to be closely linked to a certain threshold of sulfate removal, although this needs to be established (confirmed) by quantitative PCR. Recently, a competitive PCR method was developed based on the amplification of the *dsrAB* gene for assessing the abundance of sulfate- reducing microorganisms in estuarine sediments (Leloup *et al.*, 2004). Although competitive PCR has the advantage that the contaminants co-extracted with nucleic acids have similar effects on the amplification of the target and competitor sequences, it requires a

labor-intensive and time-consuming post-amplification analysis (Raeymaekers, 2000). Real-time PCR is a highly specific, sensitive and rapid alternative which was recently used for the enumeration of SRB groups in rice field soil; yet, the quantification was based on the 16S rRNA gene (Stubner, 2004).

In order to only address the active members of the microbial community, it is desirable to apply the DGGE fingerprinting technique, as well as any other method to analyze community composition (with emphasis on the active members) to community RNA, rather to community DNA. Unlike RNA, extracellular DNA derived from dead organisms can persist several months or years due to absorption of DNA on clay minerals (Demaneche *et al.*, 2001). Moreover, the majority of the members of bacterial communities in environmental samples are non-growing or dormant (including survival as spores), but not dead (Novitsky, 1987). This might cause misinterpretation of DGGE profiles and consequently a misunderstanding of the actually ongoing ISMP process. For example, although *dsrB*-based DGGE profiles of the samples MF02, MF03, MF04 and MF05 revealed differences and similarities in SRB- community composition at the different sampling sites (Figure 3.2C), it did not allow the unambiguous identification of those SRB strains that were the key players during the ISMP process, nor did it enable us to link the on site ISMP process to "real" SRB-activity. Messenger RNA (mRNA) is the product of gene expression and has a high turnover rate; therefore, mRNA provides a direct link to the transcriptional activity in cells. Recently, Koizumi *et al.* (2003) compared DGGE profiles from amplified 16S rDNA and reversely transcribed 16S rRNA fragments in mesophilic lake sediment; their results suggested that RNA- based analysis can efficiently reveal active microbial communities and provide direct information for bioremediation process control in environments inhabited by complex microbial communities. Tang *et al.* (2004) were able to relate results of reverse transcription (RT)-PCR of the *dsr* gene to the production of H_2S in an anaerobic digester of municipal waste. Thus, the detection of *dsrB* mRNA will improve our understanding of the sulfate- reduction and concomitant metal precipitation activity of the SRB strains during

bioremediation processes. The use of the *dsrB*- gene in a real-time (RT)-PCR approach on environmental mRNA will give information about the functional activity of SRB, which is especially interesting during the field application of the ISMP process as a decision tool for process adjustments. The *dsrB* gene sequences obtained from the excised and cloned DGGE gel-separated DNA bands should form the basis for a species-specific, quantitative PCR analysis, thereby allowing to follow-up the activity of the most active strains within the SRB communities that we identified in our aquifer samples. This will, however, require the development of reliable community mRNA extraction protocols suitable for the extraction of mRNA from contaminated samples.

6.2. Preliminary experiments for the selection of the optimal carbon source to stimulate SRB activity

The sulfate reduction and concomitant heavy metal precipitation activities of SRB strongly depend on the choice of the carbon/ nutrition source which is, in turn, depending upon the nature and nutritional content of the contaminated site itself. As a result, no universal strategy for the treatment of metal-contaminated plumes can be given, on the contrary each case will require the necessary feasibility studies to determine the optimal conditions. Hence, the ISMP process should first be studied and optimized at a lab-scale, with aquifer and groundwater samples from the field. The conclusions of these preliminary studies should eventually lead to a go-no go decision for the dimensioning and installation of an on site application.

Batch experiments, described in Chapter 4, had as primary aim to identify the most optimal carbon source to stimulate the ISMP process. The results of these experiments showed that an indigenous SRB community was present at the study site (Figures 4.2, 4.4), that the activity of this indigenous SRB population could be stimulated by amendment with several carbon sources (Table 4.3), and that

the SRB community activity could be used to obtain an efficient *in situ* precipitation of the contaminating heavy metals (Table 4.4, Annex I).

In Chapter 5, the batch tests were followed by column experiments where sulfate reduction could be evaluated in a more realistic aquifer system (Janssen and Temminghoff, 2004). Based on the results of batch tests, molasses, HRC® and lactate were selected as C-source/ electron donor as they were found to provide the best stimulation of sulfate reduction and concomitant heavy metal removal by the indigenous SRB community.

As expected from the batch experiments, all three carbon sources initially supported sulfate reduction and metal removal (Figures 5.2 to 5.6). The precipitation order of metals is reflected by the trend of the solubility products (K_{sp}) of their respective metal sulfides. However, although during the batch tests the sulfate reduction- and metal removal-efficiencies followed the order HRC® > molasses > lactate, the molasses-amended column initially seemed to be the most optimal condition for ISMP since HM removal efficiencies and sulfate-consumption rates were higher and increased faster compared to the other setups. On the other hand, the HRC®-amended column was the last to start with bacterial sulfate reduction. Moreover, shortcomings of ISMP with regard to the completeness of metal-precipitation processes, its long-term efficacy and its sustainability, were only just noticed in the column tests. These findings underline that up-scaling of preliminary ISMP tests is necessary for a better understanding of the ISMP process before going into an on site bioremediation application.

6.3. Molecular analysis of the indigenous SRB populations

The results of molecular analysis during batch and column tests demonstrated that for all experimental conditions tested the indigenous SRB community was predominated by strains of the genus *Desulfosporosinus* (Figures 4.2, 5.9). Probably, the high concentrations of heavy metals and low pH at the study site

(Tables 4.1, 5.1) resulted in a strong selection for metal tolerant bacteria. Several other molecular surveys have reported the presence of *Desulfotomaculum* sp. under a variety of harsh environmental conditions, such as uranium mine tailings (Chang *et al.*, 2000) and heavy metal contaminated estuarine sediments (Tebo and Obraztsova, 1998). Their capability to produce spores and to utilize a variety of electron donors seems to facilitate their adaptation to the constraints of environmental pollution (Liu *et al.*, 2003). Members of the genus *Desulfosporosinus* are closely related to the genus *Desulfotomaculum* and are Gram positive sulfate reducing bacteria, most of which oxidize their substrates (lactate, ethanol, and methanol) to acetate. In addition, they are capable of autotrophic growth with H_2 and sulfate. Nevertheless, these SRB genera also include strains that oxidize acetate to CO_2, e.g. *Desulfotomaculum acetoxidans*. (Stackebrandt *et al.*, 1997). These characteristics correspond with the finding that, eventually, all types of C-sources/ electron donors used in the batch tests stimulated sulfate reduction (see Chapter 4). Moreover, the *Desulfosporosinus- Desulfotomaculum* subgroup is, together with the *Desulfovibrio* subgroup, the fastest growing group of SRB with doubling times - under favorable growth conditions - around 3-4h (Postgate, 1984), which makes them very interesting components of a metal decontamination process. On the other hand, a mixed culture of SRB has several advantages over pure culture for environmental biotechnology (White and Gadd, 1996). It is intrinsically less liable to contamination from other microorganisms and may also be able to oxidize certain carbon sources more completely than pure cultures (Davidson *et al.*, 1994; Madsen and Aamand, 1992), or utilize sources such as sugars which are not metabolized by pure cultures of SRB (Maree *et al.*, 1987). A mixed culture is also more flexible when process conditions are altered because component organisms possess differing optima for growth and activity (White and Gadd, 1996).

Although the ISMP process depends on the growth and activity of SRB, it may be affected by non- SRB strains such as methanogens, iron- reducers, acetogens, or fermenting bacteria. Therefore, the presence of these strains was investigated by

eubacterial 16S rDNA DGGE (Figures 4.1, 5.8) and by PCR- detection of archaeal 16S rDNA genes as well as *mcrA* genes of methanogenic *Archaea* (Figure 5.7). In order to characterize the non- SRB community, DGGE bands and PCR products were cloned and sequenced (Figures 4.2, 5.9). This allowed identification of e.g. ε-*proteobacteria*, hydrogen producing *Clostridiaceae* and *Crenarchaeota* which, to our knowledge, have no detrimental effects on the SRB community. However, the cloning and sequencing of DGGE bands and PCR amplicons was a laborious work. A microarray containing probes for the functional and/ or 16S rRNA genes of bacterial groups that are frequently associated with SRB and bioremediation processes would greatly advance such an overall picture. A functional microarray with 23.000 probes specific for a broad range of environmentally relevant functions including sulfate, iron and metal reduction, the nitrogen cycle, the carbon cycle, degradation of organic xenobiotics and heavy metal resistance, was recently developed at the Microbial Genomics and Ecology Group of the Oak Ridge National Laboratory, USA, and is currently being tested on different environmental samples (J. Zhou, personal communication).

6.4. Long-term sustainability of the ISMP process

Recently, ISMP was successfully applied in column tests and in a field-scale pilot experiment at a zinc contaminated site using molasses as substrate (Janssen and Temminghoff, 2004). For our study site however, the column experiments indicated that although all three carbon sources initially stimulated the ISMP process, none of them was able to sustain this process, independently whether their supply was continued (Figures 5.2 to 5.6). Moreover, removed metals started to leach into solution again after bacterial sulfate reduction had come to a stop. Results of molecular and analytical analysis pointed out that there was no problem of competition with other, non-sulfate reducing bacteria. Therefore it was postulated that the SRB- community became inhibited by the formed metal sulfides. Liu and Fang (1997) suggested that the inhibition of sulfidogenic

activities of biogranules used for the treatment of sulfate-loaded wastewater resulted from the formation of excessive sulfurous precipitates on the bacterial cell surface. This phenomenon was also observed in an anaerobic bioreactor used for treating acid mine drainage (Utgikar *et al.*, 2002).

To support this hypothesis, further investigations are necessary. First, it might be interesting to investigate how strongly the metals are retained in the aquifer and how easily they are released into soil solution, since the chemical risk of heavy metals is a function of their chemical availability, bioavailability, and toxicity (Kaasalainen and Yli- Halla, 2003). Metal bioavailability and potential toxicity are affected by biological processes such as solubilization or immobilization (Gadd, 2004) whereas the chemical availability is characterized by physico- chemical driven (e.g. changing environmental conditions such as acidity, redox potential, etc.) adsorption/ desorption processes (Guevara- Riba *et al.*, 2004). The latter can be assessed by metal speciation using sequential extraction techniques. Sequential extraction schemes allow to identify various forms or fractions of heavy metals in the examined materials (e.g. the water soluble metal cations, the exchangeable fraction in organic and inorganic components, the fraction bound to Fe and Mn oxides, insolubles precipitated with other soil components, etc.) (Janos *et al.*, 2004). More information about metal sulfide formation, leachate chemistry and the mineralogy of precipitates could also lead to a better understanding of the reversibility of metal retention. This information can be obtained through techniques such as optical petrography, X- ray diffractometry, or scanning electron microscopy equipped with EDX (Zhixun, 1999).

Secondly, the possibility of substrate inhibition, product inhibition and inhibition by H_2S on the SRB community should be ruled out. Substrate inhibition occurs in the presence of excess of substrate (Matthews and van Holde, 1996). Normally, an increase in substrate concentration increases the velocity of the enzymatic degradation reactions during the microbial metabolism. However, large amounts of substrate can have the opposite effect and actually slow down the reaction because substrate molecules follow one another so rapidly when approaching the

enzyme that the correct catalytic placement is not attained. Hence, excess of substrate should be avoided. In addition, product inhibition can arise from acetate, as the breakdowns of anaerobic treatment of water bodies as a result of decreasing pH are often characterized by an increasing concentration of acetic acid (Kus and Wiesmann, 1995). This would be a consequence of incomplete degradation of lactate by the SRB community: acetate accumulates and lactate degradation blocks due to shifts in thermodynamic and kinetic equilibria (Mosche and Jordening, 1999). However, it was noticed that in our columns, pH decreases were rather the effect of a lack of sulfate reducing activity, instead of the underlying cause. In addition, along the period that the columns were performing properly, a substantial amount (> 85%) of DOC was removed, suggesting that the added substrates were almost completely degraded and that substrate or product inhibition are not a major issue. Yet, analysis of volatile fatty acids by GC-MS or GC-FID (Boschker *et al.*, 2001; Greben *et al.*, 2004) is necessary to determine acetate concentrations in the columns effluents. Nevertheless, although product inhibition can occur in static set-ups such as batch tests or reactor designs, it is rather unlikely to occur in a continuous flow-through system such as our column tests or during *in situ* groundwater treatment implementations, as there will undoubtedly exist microorganisms somewhere in the affected aquifer that are able to degrade the acetate being produced.

Inhibition of SRB by H_2S is well known and results from the sulfide ion (Hao, 2000). The distribution of sulfide in aqueous environments into its various forms (S^{2-}, HS^-, dissolved H_2S) is dependent upon the pH. Consequently the toxicity of sulfide is also dependent upon the pH because the undissociated form (free hydrogen sulfide) is responsible for the toxicity and inhibition of the bacteria (Utgikar *et al.*, 2002). In our study, the presence of free heavy metal ions can insulate the SRB from the toxic effects of hydrogen sulfide since they are readily precipitated by the produced sulfide. Compared to other bacterial groups such as methanogens, SRB have relatively high tolerance toward sulfide. Isa *et al* (Isa *et al.*, 1986) reported that a free hydrogen sulfide concentration of 1000 mg l^{-1}

inhibited SRB communities by only 50%. For the conducted column tests, a stoichiometric amount of ~ 330 mg l^{-1} H_2S would be synthesized from the amended electron donor (~ 580 mg l^{-1} lactate, i.e. pure product or in the form of molasses or HRC®), taken into consideration the fact that the hydrogen sulfide formed by dissimilatory sulfate reduction far exceeds the amount that is used by the SRB for cell synthesis (Widdel, 1988) and on the assumption that lactate is completely degraded. In theory, this amount is hardly sufficient for the amount of heavy metals that continuously have to be removed (see Chapter 5), and hence it follows that excess of sulfide is largely avoided.

The laboratory microcosms conducted in this work are valuable research tools for answering questions about the SRB community composition and the way it behaves and functions in response to certain treatments. For the site under investigation, it should be cleared out what will happen when clean groundwater passes through the reactive zone while C-sources aren't supplied any more and all indigenous carbon is consumed. Also, the effects of dramatic changes in sulfate- or HM concentrations, groundwater temperature, and pH on the SRB- community and the concomitant ISMP process need to be studied in more detail.
However, ultimately, the relevance of the microcosm results must be validated on site since the conditions in the laboratory cannot reproduce the exact conditions in the field (Kampichler *et al.*, 2001). Especially the aspect of the long-term sustainability needs to be studied on larger spatial and temporal scales. Our batch tests were monitored during 24 weeks. To date, our column study is running for almost 2 years. This is too long; by reducing the time for lab-scale experiments and a faster decision-making about conditions to be tested, an *in situ* test could have been running today and undoubtedly would already have provided more relevant and valuable information on the effectiveness and reversibility of the metal immobilization. Also, it should be considered that aquifer environments are characterized by micro-scale variations of both the solid matrix and the liquid phase (Ronen *et al.*, 1987). The ways in which the SRB

operate in such a variable environment and respond to the different chemical fluxes is an open question. Previous studies have illustrated that sulfate reduction activity occurs in a heterogeneous manner throughout the terrestrial subsurface (Albrechtsen and Winding, 1992; Krumholz *et al.*, 1997; McMahon and Chapelle, 1991; Ulrich *et al.*, 1998). For example, low activity regions are often observed in the presence of clay minerals. Wong and coworkers (Wong *et al.*, 2004) reported that clays inhibit sulfate reduction activity in sediments and in pure culture of *Desulfovibrio vulgaris*, which might be a direct effect of $Al^{3+}_{(aq)}$, coming from the Al_2O_3 content of clays, on the bacteria. Hence, soil heterogeneity can also decrease the reliability of microcosm studies.

The fact that precipitated metals are easily released into solution (Figures 5.2 to 5.6) arises doubts about the field applicability of ISMP as a strategy for sustaining groundwater quality at the studied site. Our results suggest that this remedial approach is not viable for long-term application unless substrate amendments are continued or environmental conditions are strictly controlled. However, the continuous addition of soluble nutrients to ensure bacterial activity in field applications is not realistic because it may entail prohibitive costs (Gibert *et al.*, 2004). A solution could be to remove the affected aquifer material from the metal precipitation zone at the end of the remediation process, or remove the metal precipitates when the microbial activity decreases. For this purpose, a large scale implementation set up could be designed in such a way that the contaminating metals are immobilized in a relatively defined region, so that excavation of the affected aquifer material and hence a complete removal of the heavy metals might be possible (Janssen and Temminghoff, 2004); if desired, subsequent metal recovery can be considered.

6.5. Towards a field-scale application

If it is decided to continue the design of an ISMP implementation at the study site via the installation of an on site pilot test, a C-source/ electron donor has to be selected that allows a cost-effective and efficient stimulation of the bacterial sulfate reduction. In this study, we focused on the prospects of using molasses or HRC®. In the case of molasses, there are 3 reasons why prudence is in order: (i) the results of microcosm experiments suggest that amendment of molasses may cause difficulties in controlling the composition and activity of the microbial population, given that such a crude substrate will enrich a diverse, unpredictable microbial population (Table 4.5, Figures 5.8, 5.9), which may have an adverse effect on the SRB community; (ii) the molasses amended column, which initially showed the most efficient ISMP process, was the first to fail (Figures 5.2 to 5.6), probably because of the immediate and massive metal sulfide production; (iii) there are indications that molasses contains components such as cysteine, that have the capacity to bind metal cations (L. Diels, personal communication); if molasses is not completely degraded within the ISMP barrier so that these bound metals are released and subsequently precipitated as metal sulfides, the risk exists that these metals are transported via the groundwater towards neighboring areas. During our experiments, we found no indications that these problems will occur with HRC®. On the other hand, the amendment of HRC® causes a decrease in pH with 1 unit; although this decrease is rapidly quenched by the alkalinity produced by the SRB, it can be expected that periodical injection of HRC® causes periodical pH shocks in the groundwater, thereby threatening the stability of metal sulfide precipitates. This could be improved by adjusting the pH of the injected solution. However, for the choice of buffering solution, one should be aware that adjustment with NaOH, NH_4OH or Na_2CO_3 containing buffers might result in precipitation of metals as hydroxides or carbonates (Haas and Polprasert, 1993; Steed *et al.*, 2000) which are more soluble than metal sulfides (Conner, 1990).

Once the *in situ* pilot test is being applied, it should be assessed whether the SRB community is active and whether the achieved activity can be sustained until the remediation task is completed. Odum (1984) suggested the use of partially permeable outdoor experimental set-ups or *in situ* mesocosm systems to bridge the gap between laboratory microcosms and field experiments. Realism was emphasized as a crucial feature of mesocosms, on the condition that disturbance of abiotic and biotic components due to handling and experimental manipulation are limited to a minimum (Kampichler *et al.*, 1999). Most *in situ* mesocosm systems were designed for the follow-up of physical and chemical parameters in aquifer studies (Mandelbaum *et al.*, 1997). Recently, an *in situ* mesocosm system was designed for the molecular study of microbial communities, consisting of a polyamide membrane pocket filled with aquifer material from the site (Hendrickx *et al.*, 2004). This so-called "sock" is inserted below the groundwater level into a monitoring well at the location of interest. The "socks" can easily be recovered from the aquifer and the community inside, representing the community of the aquifer, can be examined with the appropriate molecular tools. However, it was recommended to carefully select an appropriate "sock" membrane and examine the community structure in and outside the "socks" before starting the actual experiment since some experimental data indicated that the community inside the "socks" was different from that of the surrounding aquifer (Hendrickx, unpublished data).

6.6. Alternative treatment options

An alternative remediation technology for heavy metal contaminated-groundwater plumes could be a permeable reactive barrier. This technique has progressed rapidly over the past decade from lab-scale studies to full-scale implementation (Blowes *et al.*, 2000). Biologically mediated sulfate reduction has been employed in permeable reactive barriers by Benner *et al.* (2002) to treat

metal cations derived from mining activities. They installed a full-scale permeable reactive barrier in a sand aquifer of the Nickel Rim mine site, Ontario, where it intercepts a groundwater plume that is characterized by elevated concentrations of Fe(II), SO_4^{2-} and Ni and a pH between 5 and 6. The reactive barrier contains organic carbon in the form of municipal compost (Benner *et al.*, 1999). Permeable barriers also have a finite treatment capacity (Blowes *et al.*, 2000). The barrier life may be limited by its chemical characteristics, including the total mass of reactive material within the barrier and the reaction rate. Barrier life may also be limited by physical changes such as decreases in porosity and permeability. At many sites, the total reactive material within a barrier installation is theoretically sufficient to remove the target contaminant for tens or hundreds of years. But the rate of the reactions leading to treatment can also decline with time. Monitoring of groundwater chemistry in the Nickel Rim barrier demonstrated a decline of sulfate reduction and metal sulfide precipitation by 30 and 50% respectively over a 3- year period (Benner *et al.*, 2002). This was attributed to the depletion of the more reactive portion of the organic carbon within the barrier, which was caused, in part, by preferential channeling of groundwater flow through portions. Improved treatment can likely be achieved by the thickness of the barrier or by more uniformly distributing the groundwater flow. As with ISMP, the stability of precipitated metal sulfides will determine its long-term applicability and will dictate if the reactive material and contaminants must be eventually removed from the aquifer (Blowes *et al.*, 2000). Within the Nickel Rim barrier, the contaminants are quite stable. Oxidation of sulfides is prevented by limiting the downwards oxygen flux via a clay cap and a 100-m sulfide rich recharge tailing zone. However, in situations where leaching of the heavy metals occurs at unacceptable concentrations, excavation of the barrier would be required.

6.7. General conclusions

ISMP is a feasible process, but the following prerequisites should be fulfilled before this technology becomes an acceptable bioremediation process:

(1) the SRB- specific molecular monitoring methods should be optimized for the follow- up of the metabolically active populations based on their mRNA

(2) preliminary lab-scale experiments should focus on the completeness of metal- precipitation processes, its long-term efficacy and its sustainability when C- sources are depleted or environmental conditions have changed

(3) an on site pilot-scale experiment should be conducted to ensure that the ISMP process behaves *in situ* as predicted from lab-scale experiments

(4) an appropriate *in situ* mesocosm system should be selected for the on site study of the bacterial sulfate reduction and concomitant heavy metal precipitation

(5) an on site implementation of ISMP should be designed as such that no risk of uncontrolled release of heavy metals into the groundwater exists

(6) however, one should never lose sight of the main objective of an *in situ* bioremediation strategy, i.e. being a low-cost and effective alternative for traditional pump-and-treat techniques with sulfate reducing bioreactors

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Annex I Evolution of the Cd concentration (mg l^{-1}) in function of time (weeks) for the different batch setups

experimental setup	T0	T6	T8	T10	T13	T16	T24
1. A+G	30.40	19.90	15.92	10.45	6.13	2.03	1.17
2. A+G $HgCl_2$	30.30	24.00	19.28	13.17	9.64	7.60	4.45
3. A+G acetate	29.10	0.078	0.005	0.005	0.005	0.005	0.005
4. A+G lactate	28.50	0.014	0.10	0.005	0.005	0.005	0.060
5. A+G methanol	28.10	0.164	0.04	0.07	0.005	0.005	0.005
6. A+G ethanol	28.40	0.006	0.063	0.005	0.005	0.005	0.005
7. A+G molasses	26.70	0.063	0.005	0.005	0.005	0.005	0.005
8. A+G HRC®	22.80	0.13	0.005	0.005	0.005	0.005	0.06
9. A+G acetate PgC	19.80	0.011	0.08	0.005	0.005	0.005	0.005
10. A+G lactate PgC	12.10	0.012	0.005	0.005	0.005	0.61	0.005
11. A+G acetate VitB12	20.20	0.917	0.04	0.01	0.005	0.005	0.005
12. A+G lactate VitB12	20.60	0.146	0.017	0.005	0.005	0.005	0.24
13. A+G methanol VitB12	20.20	0.003	0.005	0.005	0.005	0.005	0.20
14. A+G ethanol VitB12	31.90	0.055	0.08	0.005	0.005	0.005	0.10
15. A+G molasses VitB12	28.40	0.034	0.05	0.005	0.03	0.005	160
16. A+G HRC® VitB12	16.70	0.035	0.05	0.03	0.005	0.005	320
17. A+G (1/2) lactate	22.40	0.035	0.50	0.20	0.59	0.005	0.005
18. A+G (1/2) lactate VitB12	26.30	0.059	0.07	0.004	0.003	0.005	0.005

Annex I Evolution of the Co-concentration (mg l^{-1}) in function of time (weeks) for the different batch setups

experimental setup	T0	T6	T8	T10	T13	T16	T24
1. A+G	33.40	32.20	39.48	42.72	37.63	37.25	33.35
2. A+G $HgCl_2$	32.10	33.40	42.06	44.97	38.80	39.61	35.33
3. A+G acetate	32.10	32.10	32.04	30.13	27.84	26.60	21.07
4. A+G lactate	32.70	11.50	11.51	6.84	6.50	5.98	5.65
5. A+G methanol	32.80	18.50	6.69	1.35	1.40	0.50	0.40
6. A+G ethanol	33.50	22.40	24.86	26.53	23.76	22.20	19.17
7. A+G molasses	32.00	12.10	5.43	0.28	1.22	0.15	0.47
8. A+G HRC®	34.00	17.74	10.49	4.57	0. 50	0.09	0.01
9. A+G acetate PgC	32.90	29.50	0.36	0.50	0.40	0.12	0.01
10. A+G lactate PgC	27.20	0.019	0.04	0.05	0.50	0.83	0. 21
11. A+G acetate VitB12	26.40	25.10	23.62	14.58	15.77	15.44	12.63
12. A+G lactate VitB12	27.30	22.30	27.77	25.30	14.16	9.48	4.59
13. A+G methanol VitB12	26.90	6.75	0.48	0.52	0.02	0.48	0.17
14. A+G ethanol VitB12	26.80	21.75	19.17	17.19	15.30	13.90	13.40
15. A+G molasses VitB12	32.30	14.80	19.80	19.56	16.90	16.36	12.51
16. A+G HRC® VitB12	31.90	0.588	1.18	0.51	0.43	0.12	0.03
17. A+G (1/2) lactate	27.70	13.87	0.51	0.42	0.32	0.09	0.05
18. A+G (1/2) lactate VitB12	26.60	10.50	12.08	11.43	11.81	7.87	6.85

Annex I Evolution of the Ni-concentration (mg l^{-1}) in function of time (weeks) for the different batch setups

experimental setup	T0	T6	T8	T10	T13	T16	T24
1. A+G	81.2	76.9	82.0	80.0	72.0	74.0	72.0
2. A+G $HgCl_2$	79.5	78.8	77.0	82.0	75.0	77.0	75.0
3. A+G acetate	79.4	75.5	62.0	64.0	54.0	54.0	49.0
4. A+G lactate	83.1	29.5	24.0	19.0	20.0	18.0	16.0
5. A+G methanol	81.3	52.6	31.0	12.0	8.0	7.0	8.0
6. A+G ethanol	82.1	54.3	49.0	54.0	50.0	51.0	47.0
7. A+G molasses	80.6	39.0	23.0	17.0	7.0	10.0	10.0
8. A+G HRC®	80.4	44.0	25.0	12.0	8.0	7.2	9.0
9. A+G acetate PgC	89.7	76.8	13.0	8.0	7.0	8.0	9.0
10. A+G lactate PgC	75.2	69.0	14.0	7.0	6.0	8.0	11.0
11. A+G acetate VitB12	65.0	59.5	57.0	40.0	36.0	37.0	37.0
12. A+G lactate VitB12	68.3	52.3	59.0	49.0	37.0	32.0	22.0
13. A+G methanol VitB12	64.3	19.5	12.0	7.0	7.0	8.0	12.0
14. A+G ethanol VitB12	80.3	30.3	40.0	37.0	35.0	36.0	39.0
15. A+G molasses VitB12	80.1	12.8	13.0	6.0	7.0	8.0	15.0
16. A+G HRC® VitB12	65.1	29.0	6.0	7.0	7.0	6.5	14.0
17. A+G (1/2) lactate	70.3	27.1	28.0	26.0	24.0	24.0	31.0
18. A+G (1/2) lactate VitB12	79.0	27.3	28.0	27.0	28.0	24.0	32.0

Table 10: Evolution of the Fe-concentration (mg l^{-1}) in function of time (weeks) for the different batch setups

experimental setup	T0	T6	T8	T10	T13	T16	T24
1. A+G	69.20	115.00	104.50	111.40	111.10	108.90	113.40
2. A+G $HgCl_2$	94.50	112.00	103.50	108.60	104.50	106.30	112.90
3. A+G acetate	99.10	111.00	80.48	74.26	77.49	73.59	66.85
4. A+G lactate	100.00	66.90	47.70	40.12	39.50	36.39	34.53
5. A+G methanol	101.00	76.50	47.45	26.43	25.82	16.55	13.44
6. A+G ethanol	100.00	86.40	68.39	72.22	71.99	68.50	65.60
7. A+G molasses	114.00	67.20	45.23	34.30	30.00	25.54	21.69
8. A+G HRC®	113.00	69.83	54.60	43.78	22.43	10.56	3.58
9. A+G acetate PgC	111.00	105.00	44.11	23.52	14.40	2.67	4.32
10. A+G lactate PgC	93.50	22.70	2.85	3.92	3.98	2.20	2.61
11. A+G acetate VitB12	92.00	100.00	67.60	51.20	57.67	54.49	52.58
12. A+G lactate VitB12	88.80	87.00	78.19	66.69	56.12	47.71	35.23
13. A+G methanol VitB12	87.10	54.10	29.34	19.34	22.16	11.45	7.42
14. A+G ethanol VitB12	102.00	80.90	67.82	65.83	63.21	61.32	55.85
15. A+G molasses VitB12	98.00	48.10	37.86	22.46	20.16	16.64	13.79
16. A+G HRC® VitB12	93.60	65.82	41.85	24.33	5.84	4.18	2.46
17. A+G (1/2) lactate	123.00	62.60	55.67	48.74	48.36	38.90	36.32
18. A+G (1/2) lactate VitB12	130.00	63.50	56.94	47.76	46.33	39.43	36.59