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Heavy metal removal by a highly heavy metal tolerant sulfidogenic consortium in anaerobic semi-continuous stirred tank reactors (CSTR): Changes of microbial community structure and abundance

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Thesis for the Degree of Doctor of Science

Heavy metal removal by a heavy metal tolerant sulfidogenic consortium in anaerobic semi-continuous stirred tank reactors (CSTR): Changes of microbial community structure

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Abstract

Nine sulfidogenic consortia, obtained from metal-contaminated sediment in Vietnam, were investigated for their ability to heavy metal tolerance by screening tests in test tubes. A highest heavy metal tolerant sulfidogenic consortium for Cu^{2+}, Zn^{2+}, Ni^{2+}, and Cr^{6+} was selected and further assessed on heavy metal removal efficiency by batch and continuous experiments. For continuous experiments, five semi-continuous stirred tank reactors (referred as R1 to R5) were run in parallel for 12 weeks at heavy metal loading rate of 1.5, 3, 4.5, 6, and 7.5 mg $L^{-1}d^{-1}$ of Cu^{2+}, Ni^{2+}, Zn^{2+}, and Cr^{6+}, respectively. Heavy metal removal efficiencies in all five reactors were estimated through the sulfate-reducing bacteria growth, sulfate reduction, sulfide production and heavy metal precipitation. Simultaneously, the effect of heavy metals on microbial community structure of this consortium were investigated in R1, R2, R3, and R5 by using a combination of denaturing gradient gel electrophoresis (DGGE) of 16S rRNA and dissimilatory sulfite reductase (*dsrB*) gene fragments, 16S rRNA gene cloning analysis and fluorescent *in situ* hybridization (FISH). The results showed that there were no inhibition of bacteria growth and that high heavy metal removal efficiencies of 96-100% for Cu^{2+}, Zn^{2+}, Ni^{2+}, and Cr^{6+} were achieved in R1 (1.5 mg L^{-1} d^{-1}), R2 (3 mg L^{-1} d^{-1}), and R3 (4.5 mg L^{-1} d^{-1}) throughout the experiment and in R4 (6 mg L^{-1} d^{-1}) during the first 8 weeks. The toxic effect of heavy metals on the sulfidogenic consortium was revealed in R5 (7.5 mg L^{-1} d^{-1}), in which no bacteria could survive and almost no heavy metal precipitation was detected after four weeks of operation. The analysis results of the inoculum and reactor samples, obtained respectively by cloning and DGGE techniques, showed that *Desulfovibrio vulgaris* (99% similarity) was dominant in sulfidogenic consortium of all analyzed samples. The abundances ratio of *Desulfovirbiro vulgaris* detect by FISH to total cell counts increased from 46% at the start to 48% in R1 (1.5 mg L^{-1} d^{-1}), to 80% in R2 (3 mg L^{-1} d^{-1}) and R3 (4.5 mg L^{-1} d^{-1}) after two, six and 11 weeks of operation. In contrast in R5 operated with heavy metal loading rate of 7.5 mg L^{-1} d^{-1} *Desulfovibrio vulgaris* greatly decreased from 46% at the start to 22% after one week and failed to be detected by FISH after four weeks of operation. The FISH results, which are consistent with DGGE, indicated that *Desulfovibrio vulgaris* plays a key role in heavy metal reduction in R1, R2 and R3. In R5 after four weeks of cultivation a distinct biomass loss was observed and no positive hybridized cells were detected by group and species specific probes and probes for the domain *Bacteria*. In addition, the anaerobic bacteria such as *Pertrimonas sulfuriphila*, *Clostridium* sp., *Citrobacter amalonaticus*, and *Klebsiella* sp., identified from DGGE

bands and clone library were hypothesized as heavy metal resistant bacteria. The obtained results implied that the investigated sulfidogenic consortium might have potential application for heavy metal biotreatment.

Keywords: Heavy metal removal; sulfate-reducing bacteria (SRB); sulfidogenic consortium; denaturing gradient gel electrophoresis (DGGE); fluorescence *in situ* hybridization (FISH); 16S rRNA cloning analysis; microbial community structure; continuous stirred tank reactor (CSTR).

Kurzfassung

Neun sulfidogene Konsortien, die aus einem mit Schwermetallen belasteten Sediment stammten, wurden auf ihre Eignung zur Behandlung schwermetallhaltiger Abwässer getestet. In Teströhrchen wurde zunächst ihre Toleranz gegenüber verschiedenen Schwermetallen geprüft. Das Konsortium mit der höchsten Toleranz gegenüber Cu^{2+}, Zn^{2+}, Ni^{2+}, and Cr^{6+} wurde ausgewählt, um es in einem semikontinuierlichen Reaktor einzusetzen.

Fünf solcher semikontinuierlichen Reaktoren wurden bei verschiedensten Schwermetallraumbelastungen von 1.5, 3, 4.5, 6 und 7.5 mg L^{-1} d^{-1} (Cu^{2+}, Ni^{2+}, Zn^{2+}, und Cr^{6+}) betrieben. Die Effizienz der Schwermetallentfernung in den verschiedenen Reaktoren wurde anhand des Wachstums von sulfatreduzierenden Bakterien (SRB), der Sulfatreduktion, der Sulfidproduktion und der Schwermetallausfällung bestimmt. Parallel wurde die Auswirkung der verschiedenen Schwermetallkonzentrationen auf die mikrobielle Gemeinschaft mit einer Kombination aus Denaturierender Gradienten Gel Elektrophorese (DGGE), der 16S rRNA Gen Cloning Analyse und Fluoreszenz *in situ* Hybridisierung (FISH) beobachtet. Die Ergebnisse zeigen ein gutes Wachstum der SRB bei gleichzeitig hoher Schwermetallentfernung von 96-100% für Cu^{2+}, Zn^{2+}, Ni^{2+} und Cr^{6+} bei Raumbelastungen von 1.5 mg bis 4.5 L^{-1} d^{-1}. Bei einer Raumbelastung 6 mg L^{-1} d^{-1} machte sich eine Schädigung der Biomasse nach 8 Wochen bemerkbar. Bei einer Raumbelastung 7.5 mg L^{-1} d^{-1} war bereits nach 4 Wochen keine Aktivität der SRB mehr zu detektieren.

Die molekularbiologischen Methoden zeigten ganz klar, dass in allen untersuchten Proben der semikontinuierlich betriebenen Reaktoren *Desulfovibrio vulgaris* (99%) dominant in dem sulfidogenen Konsortium war. Die Abundanz von *Desulfovibrio vulgaris* (FISH) im Vergleich zur Gesamtzellzahl stieg von 46% beim Start auf 48% für eine Raumbelastung von 1.5 mg L^{-1} d^{-1}, auf 80% bei 3 mg L^{-1} d^{-1} und bei 4.5 mg L^{-1} d^{-1} nach 2, 6 und 11 Wochen.

Im Vergleich dazu verringerte sich die Abundanz von *Desulfovibrio vulgaris* bei einer Schwermetallraumbelastung von 7.5 mg L^{-1} d^{-1} von 46% beim Start auf nur noch 22% nach einer Woche. Nach vier Wochen wurde *Desulfovibrio vulgaris* nicht mehr detektiert.

Für die geringeren Schwermetallraumbelastungen von 1.5 mg bis 4.5 L^{-1} d^{-1} wurden weitere anaerobe Bakterien wie *Pertrimonas sulfuriphila*, *Clostridium sp*., *Citrobacter amalonaticus* und *Klebsiella* sp. mit Hilfe der DGGE identifiziert und als schwermetallresistent eingestuft. Die Versuche zeigen deutlich, dass SRB zur Entfernung von Schwermetallen aus der Flüssigphase eingesetzt werden können, wenn die technische Abtrennung der ungelösten Stoffe einwandfrei gelingt.

Schlüsselworte: Schwermetallentfernung; Sulfatreduzierende Bakterien (SRB); Sulfidogene Konsortien; Denaturierende Gradienten Gel Elektrophorese (DGGE), Fluoreszenz *in situ* Hybridisierung (FISH), semikontinuierlicher Reaktor.

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Kieu, Thi Quynh Hoa
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List of abbreviations

ADP	Adenosine diphosphate
AFLP	Amplified fragment-length polymorphism
AMD	Acid mine drainage
AMP	Adenosine monophosphate
APS	Adenosine 5'-phosphosulfate
ARDRA	Amplified ribosomal DNA restriction analysis
ARISA	Automated rDNA internal spacer analysis
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BLAST	Basic local alignment search tool
CSTR	Continuously stirred tank reactor
DDBJ	DNA Data Bank of Japan
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Brauschweig, Germany
Dsr(B)	Dissimilatory sulfite reductase (*β* subunits)
EDS	Energy dispersive x-ray spectrometer
EtBr	Ethidium bromide
FAME	Fatty acid methyl ester analysis
FBR	Fluidized-bed reactor
FISH	Fluorescent *in situ* hybridization
GC%	Guanine plus Cytosine mol% of DNA
*Hae*III	*Haemophilus aegyptius* (GG↓CC)
HRT	Hydraulic retention time
NCBI	National Center for Biotechnology
OTU	Operational taxonomic unit

PAP	Phosphoadenosine 5’-phosphate
PAPS	Phosphoadenosine 5’-phosphosulfate
PCR	Polymerase chain reaction
RAPD	Random amplified polymorphic DNA analysis
rDNA	Ribosomal deoxyribonucleic acid
RISA	Ribosomal rDNA internal spacer analysis
rRNA	Ribosomal ribonucleic acid
SRB	Sulfate-reducing bacteria
SRP	Sulfate-reducing prokaryotes
SSCP	Single-strand conformation polymorphism
TAE	Tris-acetate-EDTA buffer
TGGE	Temperature gradient gel electrophoresis
TRF	Terminal restriction fragment
T-RFLP	Terminal-restriction fragment length polymorphism

CHAPTER 1

State of knowledge

1. Heavy metal pollution

The term 'heavy metal' refers to any metallic element that has a density above 5g/ cm^3 and is toxic or poisonous even at low concentration. There are 90 naturally occurring elements, in which 21 are non-metals, 16 are light metals and the remaining 53 (with As included) are heavy metals (31, 86). Three kinds of heavy metals are of concern, including toxic metals (such as Hg, Cr, Pb, Zn, Cu, Ni, Cd, Co, As, Sn etc.), precious metals (such as Pd, Pt, Ag, Au, Ru etc.) and radionuclides (such as U, Th, Ra, Am, etc.) (110).

The main sources of heavy metal pollution are mining, milling, metal plating, galvanization, tanneries, radiator manufacturing, smelting, bronze, manufacture of batteries, surface finishing and metallurgical industries, discharging a variety of toxic metals such as Fe, Cd, Cu, Ni, Co, Zn, and Pb into the environment. Therefore, wastewaters containing heavy metals represents a serious threat to the environment and human life (48, 13).

1.1. Heavy metal toxicity

Heavy-metal pollution represents an important environmental problem due to their toxic effects. Unlike organic contaminants, which can be degraded into harmless chemical species, heavy metals cannot be degraded. Thus they may accumulate throughout the food chain leads to serious ecological and health problems (17).

In fact that some metals play a key role in functions of living organisms such as iron, zinc, cobalt, copper, magnesium, and calcium. They are used for redox processes, to stabilize molecules through electrostatic interactions, as components of various enzymes, and for regulation of osmotic pressure. However, at high concentrations heavy metal ions form unspecific complex compounds in the cell, which leads to damage cell membranes, alter enzyme specificity, and disrupt cellular functions, as well as damage to the structure of DNA, nerves, liver and bones (86, 55). This might be explained that when a cell faces a high concentration of any heavy metal that is accumulated by such an unspecific system, the specific heavy-metal ion is transported into the cytoplasm in spite of its high concentration,

because these unspecific transporters are constitutively expressed. Thus, the gate cannot be closed. This “open gate” is the first reason why heavy-metal ions are toxic (87).

1.2. Microbial resistance to toxic heavy metals

Microorganisms have adapted to the presence of heavy metals by developing a variety of resistance mechanisms. Six heavy metal resistance mechanisms were described in detail by Bruins et al. as follows (10).

**** Metal exclusion by permeability barrier:***

Bacteria that naturally form an extracellular polysaccharide coating demonstrate the ability to bioabsorb metal ions and prevent them from interacting with vital cellular components. The exopolysaccharide coating of these bacteria may provide sites for the attachment of metal cations.

**** Active transport of the metal away from the microorganisms***

Microorganisms use active transport mechanisms to export toxic metals from their cytoplasm. This mechanism can be chromosomal or plasmid-encoded.

**** Intracellular sequestration of metals by protein binding***

Intracellular sequestration is the accumulation of metals within the cytoplasm to prevent exposure to essential cellular components.

**** Extracellular sequestration***

Metal resistance based on extracellular sequestration

**** Enzymatic detoxification of a metal to a less toxic form***

Some bacteria contain a set of genes that form a resistance operon that not only detoxifies heavy metals but also transports and self-regulates resistance.

**** Reduction in metal sensitivity of cellular targets***

Some microorganisms adapt to the presence of toxic metals by altering the sensitivity of essential cellular components; this provides a degree of natural protection. Protection is achieved by mutations that decrease sensitivity but do not alter basic function or by increasing production of a particular cellular component to keep ahead of metal inactivation.

2. Heavy metal wastewater treatment methods

Although heavy metal cannot be degraded they can be transformed from mobile and toxic forms into their stable immobile and less toxic forms by using chemical, phyisco-chemical and biological methods.

2.1. Chemical methods

Chemical methods such as adsorption, sorption, oxidation and hydrolysis, carbonate precipitation, sulfide precipitation, and co-precipitation, which usually used for heavy metal removal, are present in Table 1-1.

Table 1-1. Heavy metal removal by chemical methods

Methods	**Principles**
Adsorption	Heavy metals are adsorbed to solid phase by cation exchange or chemisorption (5, 112)
Sorption	Heavy metal ions are transferred from solution phase to solid phase by a group of processes includes adsorption and precipitation (5, 112)
Oxidation and hydrolysis	Precipitation of metals as oxides, oxyhydroxides, and hydroxides using NaOH, NH_4OH, CaO or $Ca(OH)_2$ (115)
Carbonate precipitation	Precipitation of metals as carbonates using $CaCO_3$, $NaHCO_3$ or Na_2CO_3 (101)
Sulfide precipitation	Heavy metal react with soluble Na_2S, NaHS, CaS or H_2S (76)
Co-precipitation	Heavy metals such as copper, nickel, zinc, manganese etc. are co-precipitated in iron oxides or cobalt, iron, nickel and zinc are co-precipitated in manganese oxides (100)

2.2. Physico-chemical methods

Conventional physico-chemical methods for removing heavy metal ions such as chemical precipitation, coagulation-flocculation, foam flotation, membrane filtration, ion exchange, electrochemical treatment, and adsorption on activated carbon have been suggested in Table 1-2.

Table 1-2. Heavy metal removal by physico-chemical methods

Methods	Principles
Chemical precipitation	Using a precipitant agent such as lime to convert dissolved heavy metal to insoluble solid phase (106).
Coagulation-flocculation	Sedimentation of colloidal particles by adding a coagulant (99).
Foam flotation	Use of gas bubbles and surfactants to decrease the apparent density of aggregates which float to the liquid/air interface (59).
Membrane filtration	Removing heavy metal through various types of membrane filtration (reverse osmosis, micro/nano/ultrafiltration) (56).
Ion exchange	Exchange of ions on the resin for those in wastewater (93).
Electrodialysis (Electrochemical treatment)	Using an electric potential to transport ionic species through an ion exchange membrane (19).
Membrane electrolysis (Electrochemical treatment)	A chemical process driven by an electrolytic potential, can also be applied to remove metallic impurities from metal finishing wastewater (47).
Electrochemical precipitation (Electrochemical treatment)	Using to modify the conventional chemical precipitation (39, 102).
Adsorption	Using activated carbon to remove heavy metals (62, 73).

2.3. Biological treatment methods

Among methods have been used for treatment of heavy metal contaminated wastewaters, the classical physico-chemical methods (Table 1-2) have been widely used. However, they are expensive when treating large amount of wastewater containing heavy metals in low concentration. In addition, they produce large quantity of sludge. Therefore, alternative heavy metal treatment methods that use the natural capabilities of microorganisms as biological methods are necessary (Table 1-3).

Table 1-3. Heavy metal removal by biological methods

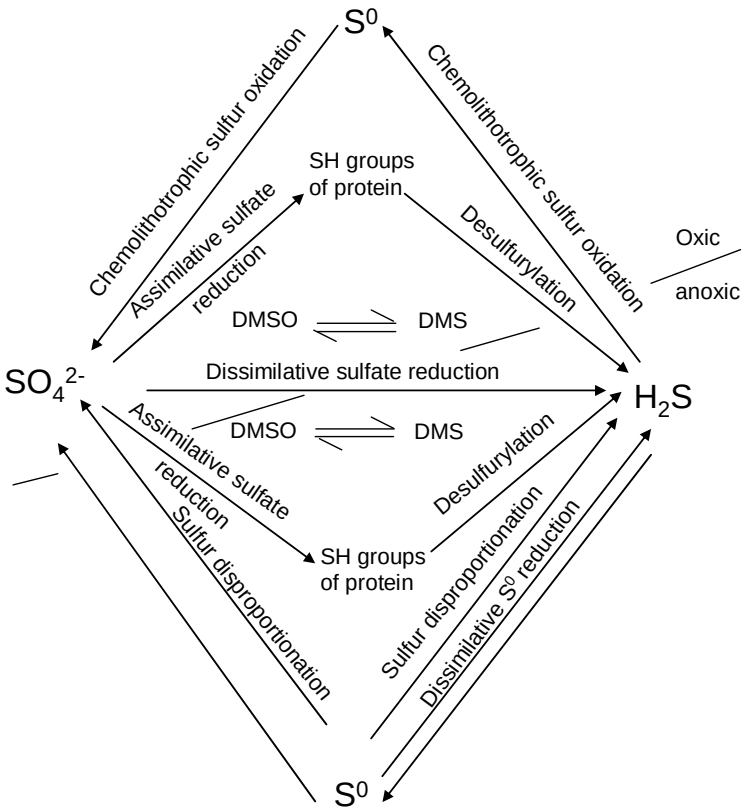
Methods	Principles	Organisms/Materials
Biosorption	A passive uptake process, which can entrap heavy metal ions in the cellular structure and subsequently biosorbed onto the binding sites present in the cellular structure. This method of uptake is independent of the biological metabolic cycle (111, 20).	Bacteria, yeast, fungi, algae, plants, peat, agricultural byproducts, biopolymers
Bioaccumulation (or Intracellular accumulation)	The heavy metal can also pass into the cell across the cell membrane through the cell metabolic cycle. This mode of metal uptake is referred to as “active uptake”. The metal uptake by both active and passive modes can be termed as “bioaccumulation”. Most of studies dealing with microbial metal remediation via growing cells described the biphasic uptake of metals, i.e., initial rapid phase of bosorption followed by slower, metabolism-dependent active uptake of metals (27, 20).	Bacteria, yeast, algae, plants

Complexation	Complexation occurs in two ways: (i) the metals may be involved in non specific binding to cell wall surfaces, the slime layer, or the extracellular matrix or (ii) they may be taken up intracellularly. Both types of metal complexation are used to reduce metal toxicity and mobility (2).	Microorganisms, algae, peat, immobilized siderophores
Methylation	The methylation of metals and metalloids is mediated microorganisms, which enzymatically transfer methyl groups to the metals. The methylated compounds formed differ in their solubility, toxicity, and volatility, and may be eliminated from the system by evaporation (35, 55).	Hg, As, Se, Sn, Te and Pb methylating microorganisms and fungi
Oxidization	Bacterial Fe oxidation is ubiquitous in environments with sufficient Fe^{2+} and conditions to support bacterial growth, such as drainage waters and tailings piles in mined areas, pyretic and hydric soils, drain pines and irrigation ditches, and plant rhizosphes. Iron-oxidizers found in acidic soil environments are acidophilic chemolithotrophs, such as *T. ferrooxidans*, significant for its role in generating acid mind drainage (55, 30).	Fe^{2+} and Mn^{2+}oxidizing microorganims
Dissimilatory reduction	A few bacteria have the capability of reducing toxic heavy metals by a process that couples electron transport to reduction of oxidized metals and the organisms that carry out this process are referred to as dissimilatory metal-reducing bacteria (DMRB) (3, 18, 49)	As^{5+}, Cr^{6+}, Fe^{3+}, Mn^{4+}, Pb^{2+}, U^{6+}, Tc^{7+}, and Co^{3+} reducing microorganisms
Precipitation as metal sulfides by biological H_2S	Hydrogen sulfide produced by the bacteria will react with the cationic metals to give highly insoluble metal sulfides (3).	Sulfate reducing bacteria

3. Sulfate-reducing bacteria (SRB)

SRB constitute a diverse group of prokaryotes (*Archaea* and *Bacteria*) that can be found in many ecosystems including soil, marine waters and sediments, hot spring, oil fields, acid main drainage, rice fields, deep-see hydrothermal vent, sewage, corroding iron, the rumen of sheep and guts of insects, and even in human diseases (91, 88). They are anaerobic microorganisms that oxidize organic compounds by utilizing sulfate as a terminal electron acceptor and generate sulfide and alkalinity (22). They are ubiquitous in anoxic habitats, where they have an important role in the sulfur cycle (Fig. 1-1) and play an important role in many environmental and industrial processes. The activity of SRB in some cases cause a serious problem for industries, such as oil reservoirs (souring) and corrosion because of the production of sulfide. However, these organisms can also be beneficial by removing heavy metals, sulfate or sulfur compounds from wastewater. With the development of new biological molecular techniques, notable advances have been obtained in the taxonomy and phylogeny of this diverse group of prokaryotes (14). Thus, the studies of SRB have been increasing over the last decades. Because sulfate can be utilized as a terminal electron acceptor both by members of the *Bacteria* and *Archaea*, the term sulfate-reducing prokaryotes

are used in some other studies. However, most prokaryotes with sulfate-reducing capacity are *Bacteria*, the term SRB was used in this study to refer to both domain *Bacteria* and *Archaea*.



Key Processes and Prokaryotes in the Sulfur Cycle	
Process	Organisms
Sulfide/sulfur oxidation	($H_2S \rightarrow S^0 \rightarrow SO_4^{2-}$)
Aerobic	Sulfur chemolithotrophs (*Thiobacillus*, *Beggiatoa*, many others)
Anaerobic	Purple and green phototrophic Bacteria, some chemolithotrophs
Sulfate reduction (anaerobic)	($SO_4^{2-} \rightarrow H_2S$) *Desulfovibrio*, *Desulfobacter*
Sulfure reduction (anaerobic)	$S^0 \rightarrow H_2S$ *Desulfuromonas*, Many hyperthermophilic Archaea
Sulfur disproportionation	($S_2O_3^{2-} \rightarrow H_2S + SO_4^{2-}$) *Desulfovibrio*, and others
Organic sulfur compound oxidation or reduction	($CH_3SH \rightarrow CO_2 + H_2S$) (DMSO→DMS)
Desulfurylation	(organic-S→H_2S) Many organisms can do this

Fig. 1-1. Sulfur cycle (from REF. 68). Sulfate-reducign bacteria have a key role in the sulfur cycle.

3.1. Taxonomy and Phylogeny of sulfate-reducing bacteria

SRB are a complex physiological bacterial group, and various properties have been used in traditional classification. The most important of these properties were cell shape, motility, guanine plus cytosine (GC) % content of DNA, type of sulfite reductases (desulfoviridin, P 582, desulforubidin, desulfofuscidin), electron transfer proteins (cytochromes of c_3 and b-types, ferredoxins, flavodoxins and hydrogenase), respiratory menaquinones, fatty acids, optimal growth conditions and complete versus incomplete oxidation of acetate (3, 14, 23). For classification within a particular genus, different electron donors are used. SRB can also be divided into two main groups based on the metabolic functionality. The first group is complete oxidizers (acetate oxidizes) which have the ability to oxidize the organic compound to carbon dioxide (91, 23). The second group is incomplete oxidizes (non-acetate oxidizers) which carry out the incomplete oxidation of the organic compound to acetate and CO_2 (23). The known SRB can be grouped into seven phylogenetic lineages, five within the *Bacteria* and two with the *Archaea* by comparative analysis of 16S rRNA sequences (Fig. 1-2). Most of the sulphate reducers belong to the ~ 23 genera within the *Deltaproteobacteria*, followed by the Gram-positive SRB within the *Clostridia* (*Desulfotomaculum*, *Desulfosporosinus* and *Desulfosporomusa* genera). Three lineages, *Nitrospirae* (*Thermodesulfovibrio* genus), *Thermodesulfobacteria* (T*hermodesulfobacterium* genus) and *Thermodesulfobiaceae* (*Thermodesulfobium* genus), only contain thermophilic sulphate reducers. Within the *Archaea*, SRB belong to the genus *Archaeoglobus* in the *Euryarchaeota*, and to the genera *Thermocaldium* and *Caldirvirga* in the *Crenarchaeota* (79).

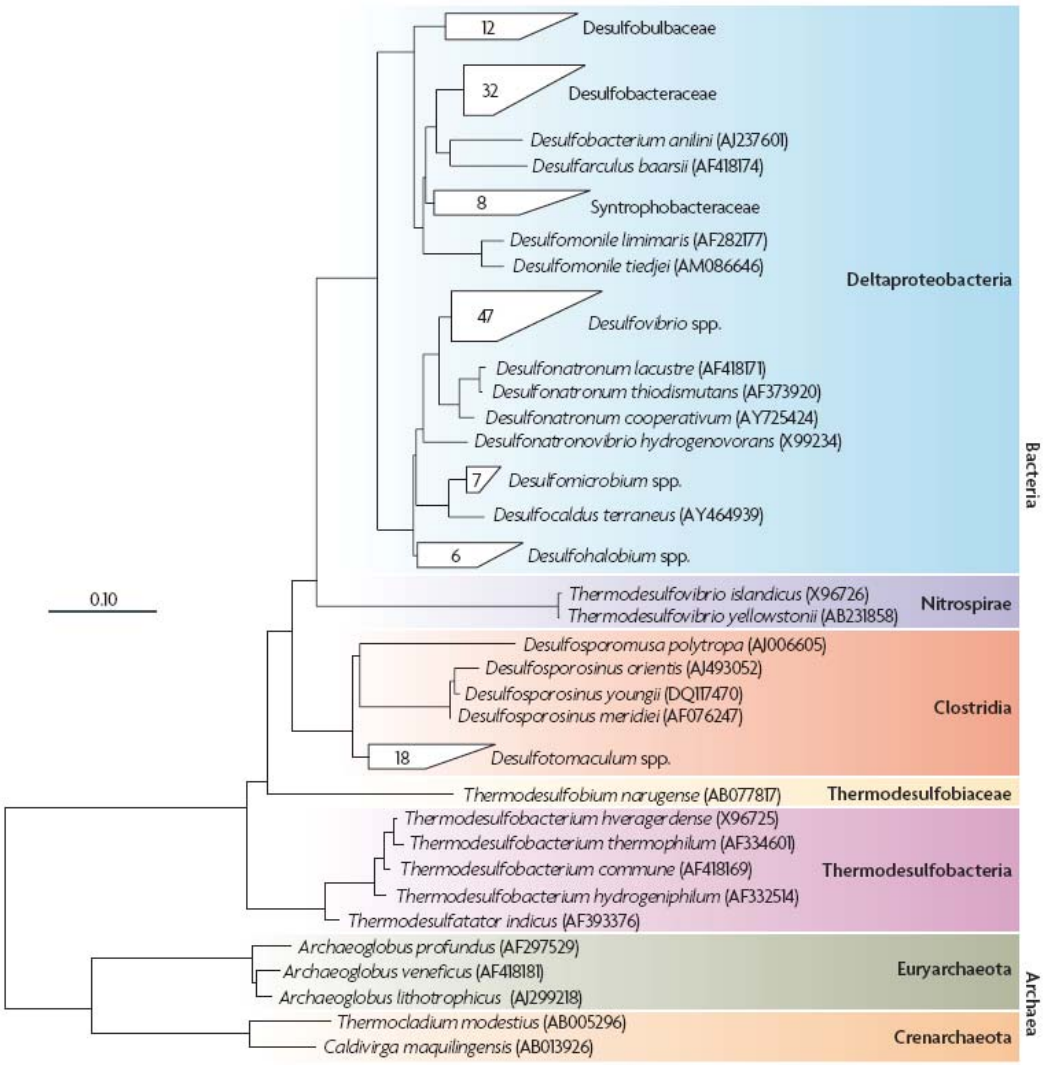


Fig. 1-2. Phylogenetic tree based on nearly complete 16S ribosomal RNA (rRNA) sequence of described sulfate-reducing bacterial species (from REF. 79)

3.2. Biochemistry of sulfate reduction

Sulfate reduction may occur through either assimilatory or dissimilatory pathways (Fig. 1-3). In assimilative sulfate reduction, organisms (e.g. higher plants, algae, fungi, and most bacteria) use sulfate as sulfur source for biosynthesis of amino acids and proteins. In dissimilative sulfate reduction, energy that generated by the ability to utilize sulfate as an electron acceptor is restricted to obligatory anaerobic sulfate reducing. The reduction of SO_4^{2-} to hydrogen sulfide and 8-electron reduction can be described briefly as follows. Sulfate is firstly activated by adenosine triphosphate (ATP), leading to the formation of adenosine phosphosulfate (APS) in both assimilatory and dissimilatory reduction (Fig. 1-3a) with the release of adenosine monophosphate (AMP). In dissimilative sulfate reduction, the sulfate ion

of APS is reduced directly to sulfide (SO_3^{2-}) by the enzyme APS reductase. In assimilative reduction, another phosphorus (P) is added to APS to form phosphoadenosine phosphosulfate (PAPS) (Fig. 1-3b), and then sulfate ion reduced to sulfite with the release of phosphoadenosine 5'-phosphate (PAP). Sulfite is further reduced to hydrogen sulfide by the enzyme sulfite reductase in both cases. In the dissimilative sulfate reduction, sulfide is excreted into the environment, while in the assimilative reduction sulfide formed is immediately converted into organic sulfur compounds, such as amino acids (45). How sulfite is reduced to sulfide is not yet clear. One hypothesis is that the six-electron reduction of sulfite to sulfide catalyzed by sulfite reductase. The pathway of bisulfate reduction to hydrogen sulfide is somewhat controversial and two mechanisms have been proposed. In the first mechanism, also called the trithionate pathway, bisulfate is reduced to sulfide in three steps via the free intermediates, trithionate and thiosulfate. The second mechanism is the direct six-electron reduction of bisulfate to sulfide in one step, catalyzed by the dissimilatory sulfite reductase (DSR), without the formation of free intermediates (3, 68).

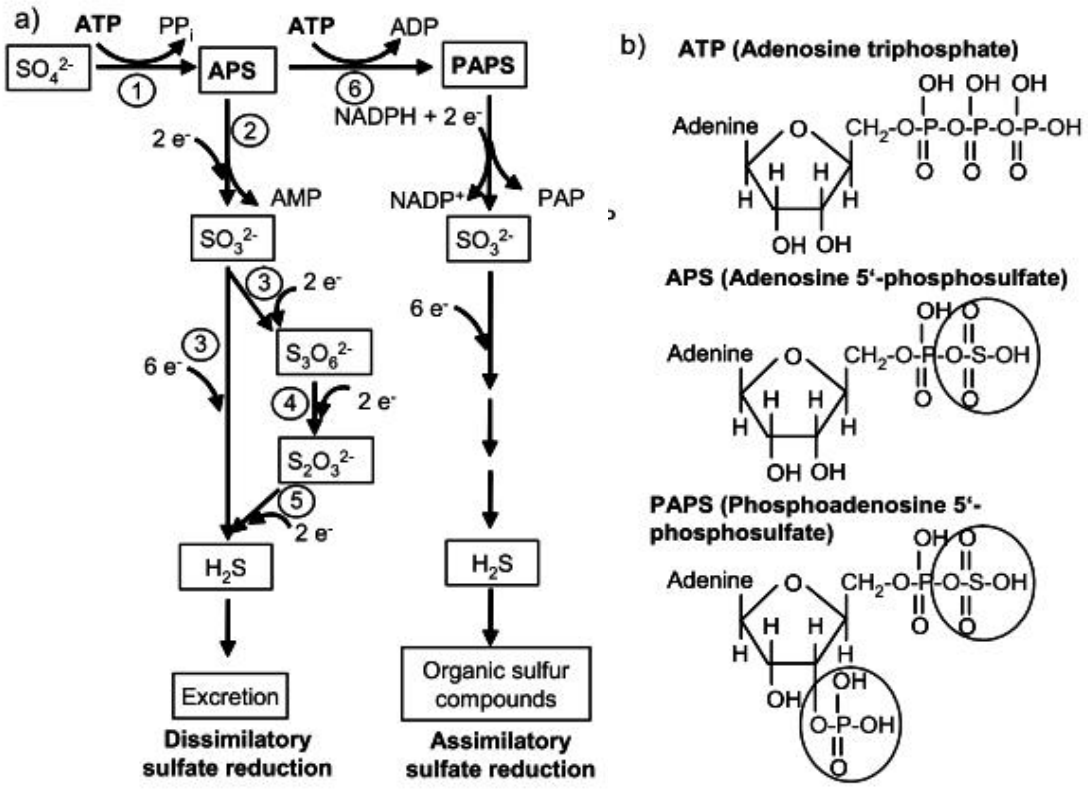


Fig. 1-3. (a) Schemes of assimilative and dissimilative sulfate reduction. (b) Two forms of active sulfate, adenosine 5'-phosphosulfate (APS) and phosphoadenosine 5'-phosphosulfate (PAPS) (from REF. 68)

3.3. Physiology of sulfate reduction

3.3.1. Electron-donor metabolism

Sulfate-reducing bacteria can utilize a variety of electron donors. They have the ability to grow on organic compounds (lactate, pyruvate, malate, succinate, formate, ethanol) (81, 104), simple carbohydrade monomers (glucose, sucrose) (21), short-chain fatty acid (acetate), long-chain fatty acids (36, 75) and aromatic compounds (e.g. benzoate, phenol, toluene, ethylbenzen, and short-chain hydrocarbons (ethane, propane, and butane) (53) (Table 1-5).

Table 1-4. Oxidation of various electron donors coupled to reduction of sulfate and the corresponding Gibbs free energy (from REF. 91)

Electron donors	Reaction	ΔG^o (kJ/reaction)
Hydrogen	$4H_2 + SO_4^{2-} \rightarrow 4H_2O + S^{2-}$	-123.98
Acetate	$CH_3COO^- + SO_4^{2-} \rightarrow H_2O + CO_2 + HCO_3^- + S^{2-}$	-12.41
Formate	$4HCOO^- + SO_4^{2-} \rightarrow 4HCO_3^- + S^{2-}$	-182.67
Pyruvate	$4CH_3COCOO^- + SO_4^{2-} \rightarrow 4CH_3COO^- + 4CO_2 + S^{2-}$	-331.06
Lactate	$2CH_3CHOHCOO^- + SO_4^{2-} \rightarrow 2CH_3COO^- + 2CO_2 + 2H_2O + S^{2-}$	-140.45 or -178.06
Malate	$2(OOCCHCHCOO)^{2-} + SO_4^{2-} \rightarrow 2CH_3COO^- + 2CO_2 + 2HCO_3^- + S^{2-}$	-180.99
Fumarate	$2(OOCCHCHCOO)^{2-} + SO_4^{2-} + 2H_2O \rightarrow 2CH_3COO^- + 2CO_2 + 2HCO_3^- + S^{2-}$	-190.19
Succinate	$4(OOCCH_2CH_2COO)^{2-} + 3SO_4^{2-} + 4CH_3COO^- + 4CO_2 + 4HCO_3^- + 3S^{2-}$	-150.48

3.3.2. Electron-acceptor metabolism

In addition to sulfate (SO_4^{2-}), SRB can use many other electron acceptors for growth depending on the species. They can utilize sulfur compounds (e.g. thiosulfate, sulfite, elemental sulfur, organic sulfur compounds) (92, 60), sulphonates, dimethylsulphoxides (63, 46), and other non sulphure-containing electron acceptors as nitrate and nitrite (3, 78) or CO_2 (52). A few SRB can use toxic heavy metals such as Fe^{3+} (89, 103), U^{6+} (66, 105), Cr^{6+} (70, 105), As^{5+} (85), Tc^{7+} (64), Se^{6+} (105) as the sole electron acceptors. SRB were considered as strict anaerobes. However, growth of SRB with molecular oxygen as electron acceptor has been reported (65).

3.3.3. Effect of pH to the activity of SRB

SRB thrive in the environment with pH range 5-9 (91). Outside this range, specially pH (<5) SRB activity is inhibited especially in heavy metal containing wastewater resulting in the decrease of metal removal capacity due to the inhibition of sulfate reduction and the increase of metal sulfide solubility. However, the presence of SRB in acidic environments has a low pH between 2 and 4 such as acid mine drainage (AMD) have been detected by various researchers. Jong et al. (45) reported that the SRB population was capable of surviving and metabolizing at pH 3.5 in an upflow anaerobic packed bed reactor for at least 20 days. However, the sulfate reduction was substantially reduced only about 1% sulfate removed. Kikot et al. (50) who studied the effect of pH and dissolved heavy metals on the growth of SRB showed that the decrease of pH from 7 to 5 exerted an inhibitory effect on sulfate reduction by the strain and the community. At pH 3.5 the reactor removed 38.3% of influent sulfated and raised the pH of the medium to 5.82 was showed by Elliotte et al. (28). The detection of sulfate reduction at pH 2.5 was observed by Tsukanoto et al. (104).

3.3.4. Effect of Eh to the activity of SRB

SRB were considered as strict anaerobes because they need an anaerobic medium and an anoxic and reduced microenvironment with a redox potential (Eh) lower than -100 mV (91). However, it has been shown recently that SRB are able to tolerate the transient presence of oxygen and can grow in various Eh values (22). Neculita et al. (83) reported that sulfate reduction was observed in passive field bioreactor at positive Eh values. The authors explained that their survival in these adverse conditions may be due to the formation of favorable anoxic microenvironments in the reactive mixtures.

3.3.5. Effect of temperature to the activity of SRB

Postgate (91) showed that SRB can tolerate temperatures from below -5 to 75^{o}C. This is in agreement with other authors. Sahinkaya (96) showed a microbial sulfate reduction at low temperature (8^{o}C) by using waste sludge as a carbon and seed source. The efficient treatment of acid mine drainage in sulfate reducing column experiment was observed at the low temperature (6^{o}C) by Tsukanoto et al (104). Another study of Shinkaya et al. (97) reported that treatment of iron containing wastewater at low (8^{o}C) and high (65^{o}C) temperatures in sulfidogenic fluidized bed reactors (FBR). The growth of SRB is significantly affected by temperature. Nevatalo et al. (84) indicated that the sulfidogenic activity will decrease by 10-40% as the operational temperature of bioreactors with mesophilic SRB decreased to 15-20^{o}C. A significant increase in sulfate reduction rate as temperature increased from 20 to 35^{o}C was observed by Moosa et al. (74).

4. Heavy metal removal by sulfate reduction

The main mechanisms of metal removal precipitation in bioreactors are precipitation in the form of sulfides (Pb^{2+}, Co^{2+}, Cd^{2+}, Cu^{2+}, Ni^{2+}, Fe^{2+}, and Zn^{2+}), hydroxides (Fe^{3+}, Cr^{3+}, and Al^{3+}), and carbonates (Fe^{2+} and Mn^{2+}) (83). In compared to other precipitations, heavy metal precipitation by biological sulfide has advantages of low amounts of residual sludge, lower solubility, highly reactive efficiency, and cost effective. Thus, this method is applied effectively to removal of heavy metal, sulfate or sulfur compounds from wastewater as an attractive alternative over chemical and physico-chemical methods.

**** Principle of metal precipitation by sulfate-reducing bacteria (SRB)***

Metal precipitation by SRB consists of two stages: (i) Sulfate-reducing bacteria (SRB), under anaerobic conditions, use organic compounds such as lactate, acetate, propionate, butyrate, ethanol or combination of hydrogen and CO_2 as carbon and energy sources by utilizing sulfate as an electron acceptor and generate hydrogen sulfide and bicarbonate ion, and (ii) the biologically produced hydrogen sulfide reacts with dissolved metals such as iron, zinc, copper, nickel, etc. to form insoluble metal sulfide precipitates (91, 22, 55).

$$2CH_2O + SO_4^{2-} \rightarrow H_2S + 2HCO_3^-$$

$$Me^{2+} + H_2S \rightarrow MeS\downarrow + 2H^+ \text{ (Me = Metal)}$$

Processes for the treatment of sulfate and metal-containing wastewaters can be categorized into passive and active methods.

4.1. Passive treatment methods (or lime neutralization)

The passive treatment, which is based on naturally occurring biological and physico-chemical processes such as oxidation, reduction, adsorption and precipitation to treat acid mine drainage (AMD) or other heavy metal containing wastewaters. Aerobic wetlands, compost wetlands and anoxic limestone drains are used for passive treatment of these wastewaters (118). Passive treatment methods require low-energy input, limited addition of reagents and low operation and maintenance costs. However, the process in general is very slow and cannot be controlled effectively. Large land requirements, build up of heavy metals in the wetland, formation of H_2S and sludge are some of the major drawbacks in the use of passive treatment (74, 83).

4.2. Active treatment methods as sulfidogenic bioreactors

Heavy metal treatment using anaerobic sulfate reduction is influenced by a variety of parameters such as electron donor, pH, temperature, sulfate concentration as well as species and concentration of heavy metal. Therefore, the use of active treatment method as sulfidogenic bioreactor for heavy metal removal is probably preferable to passive treatment methods due to careful control of the process conditions, resulting in the increase of heavy metal treatment efficiency. Heavy metal removal by sulfate reduction has been studied in various bioreactor-types such as continuously stirred tank reactor (CSTR) (82, 95), upflow anaerobic sludge blanket reactor (UASB) (61), off-line sulfidogenic bioreactor (34), fixed bed reactor (FBR) (48, 107), and permeable reactive barriers (PRB) (4).

5. Molecular biological approaches for analysis of microbial community in wastewater treatment.

Studies of microbial communities in wastewater treatment processes considered as a "black box" were limited for many years by the lack of methodological tools. The classical methods such as microscopic and phenotypic analysis commonly used to investigate microbial communities in wastewater have been known. Microscopic analysis is a cultivation-independent method based on morphotype and staining behavior by using transmission and phase contrast microscopy. Phenotypic analysis is a cultivation-dependent method based on morphological (cellular and colonial characters) and physiological and biochemical (growth conditions) features. Although phenotypic analysis is useful tool and widely used for the last decades, this method has three major disadvantages. First, they are time-consuming and laborious. Second, they might generate misleading results due to the variable alterations in gene expression (12, 94). Third, this method can not detect all organisms, for example only 0.1%, 0.25% and not more than 15% of the total bacteria were found to be cultivable in seawater (29), freshwater (44), and activated sludge (109), respectively. This is the main disadvantage of cultivated-dependent methods. Although the cell numbers determined by microscopic total direct cell counts are much higher than by cultivation-dependent method, it does not give any information about presentation of active organisms. In addition, the microscopic method failed to characterize non conspicuous organisms. Fortunately, the rapid development of the modern biological molecular approaches (Fig. 1-4) that use the gene sequence of the small subunit ribosomal rRNA as a molecular marker for phylogenetic identification have provide alternative approaches to overcome the problems associated with classical methods in recent years. They may help improving the knowledge of diversity and dynamic of microbial communities in wastewater treatment as well as microbial ecology in

generally. Currently, many molecular approaches including both PCR-based approaches (e.g. cloning and fingerprinting methods) and non-PCR based approaches (e.g. fluorescent *in situ* hybridization (FISH) and quantitative dot blot hybridization) have been developed. In this chapter, the role of rRNA genes that serve as phylogenetic molecular marker and the most powerful molecular tools used to characterize microbial communities, such as full-cycle rRNA approach, nucleic acid fingerprinting, and fluorescent *in situ* hybridization (FISH) with rRNA-targeted oligonucleotide probes are shown.

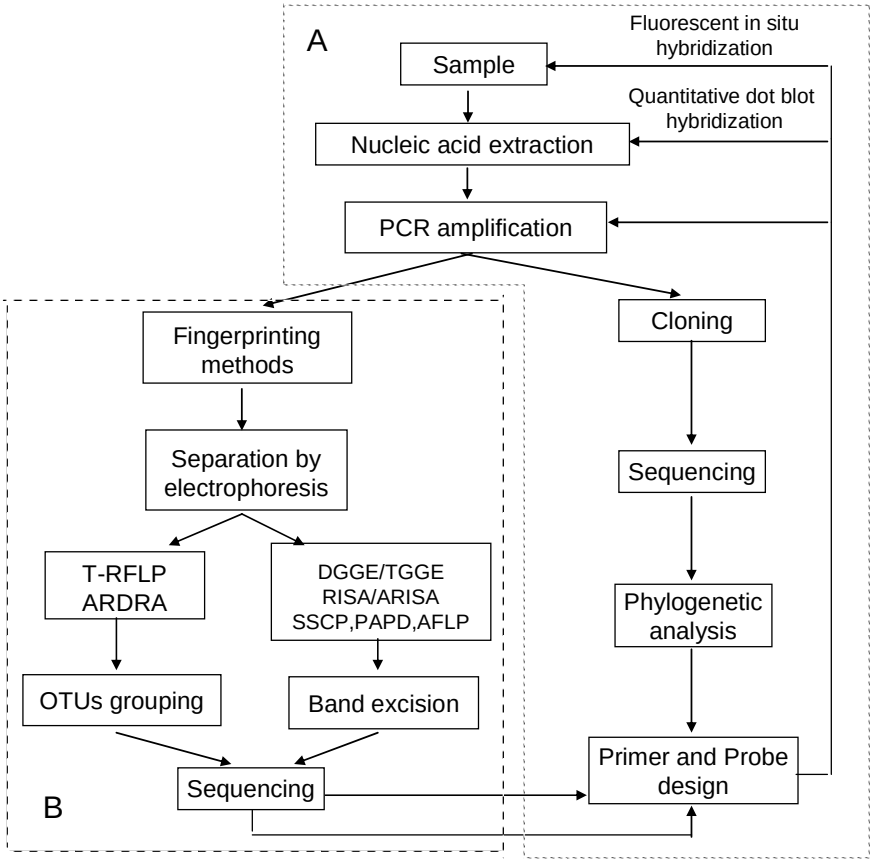


Fig. 1-4. Flow diagram of different molecular biological methods based on rRNA analysis for microbial ecology research. A/ Full-cycle rRNA approach. B/ Nucleic acid fingerprinting techniques.

5.1. Microbial community analysis using rRNA as a molecular marker

Molecular biological methods based on comparative analysis of small subunit ribosomal RNAs have been commonly used to investigate microbial communities in microbial ecology. They provided knowledge of the evolutionary relationship of microorganisms that allow grouping and identifying microorganisms through sequences of the obtained genes (114).

Among the three rRNA molecules of ribosomal RNAs (5S, 16S or 18S and 23S), 16S rRNA (Prokaryotes) or 18S rRNA (the counterpart to 16S rRNA in Eukaryotes) originates from the small subunit of the ribosome are the most used phylogenetic marker (68). Here we focus on 16S rRNA that used in prokaryotic taxonomy (*Archaea* and *Bacteria*). For the 16S rRNA gene (16S rDNA) more than 200,000 bacterial 16S rDNA sequences are available in GenBank, which comprises the DNA DataBank of Japan (DDBJ) (72), the European Molecular Biology Laboratory, UK (EMBL) (58), and GenBank at National Center for Biotechnology Information, USA (NCBI) (6). Sequence analysis is conducted by comparing the sequences with the available sequence in databases yield information about the identity or relatedness of the new sequences to known species. Several features of the 16S rRNA gene make it an important phylogenetic tool and hence a useful target for characterizing the genetic structure of microbial communities. First, this gene is present in all bacteria; thus it is a universal target for bacterial identification. Second, the function of 16S rRNA gene have remained constant over a long period, suggesting that sequence changes are more likely to reflect random changes (a more accurate measure of time) than selected changes. Third, the 16S rRNA genes (~ 1,500-bp) contain several regions of highly conserved sequence useful for obtaining proper sequence alignments, yet contain sufficient sequence variability in other regions of the molecule to serve as excellent phylogenetic molecular clocks. The 23S rRNA molecule (~ 2,900-bp) is a larger informative unit than the 16S rRNA, and in many cases has higher resolving power for phylogenetic reconstructions. However, due to its length, its sequencing has not been as popular as 16S rRNA. The 5S rRNA has also been used for phylogenetic measurements, but its small size (~ 120-bp) limits the phylogenetic information obtainable from this molecule. The described characteristics are the reasons why 16S rRNA has been referred as the “ultimate molecular chronometer” and widely used. Thus the number of 16S rRNA sequences in the databases is much greater than 23S and 5S (94, 14). Although 16S rRNA gene analysis provides a stable and quite satisfactory framework for prokaryotic classification, it has low phylogenetic power at the species level (37). Rosselló-Mora and Amann (94) reported that 16S rRNA gene sequence data cannot provide absolute resolution to taxonomic issues such as (i) different species with identical or nearly identical 16S rRNA sequences, (ii) micro heterogeneity of the 16S rRNA genes within a single species, or (iii) the occurrence of two or more 16S rRNA genes with relatively high sequence divergence in one organism. Therefore, the bacterial species definition can never be solely based on sequence similarity of rRNAs. However, comparative analysis of 16S rRNA is a very good method for a first phylogenetic affiliation of both potentially novel and poorly classified organisms (94, 40).

5.2. Full-cycle rRNA approach

The full cycle rRNA approach (Fig. 1-4A) includes DNA extraction, PCR amplification, cloning of rRNA sequences (Fig. 1-5), construction of a gene library, sequencing of clone inserts and phylogenetic analysis. Further steps are the design of new gene probes and finally the application of these newly-designed probes using fluorescent *in situ* hybridization (FISH) technique to identify and quantify *in situ* the mixed microbial population in the environmental samples.

PCR amplicons using universal primers for all bacteria and specific primers for groups or genera can be obtained by either DNA extracted from environmental mixed populations (environmental cultures) or cultured isolates (pure cultures). There are two corresponding procedures for each kind of generated amplicons: (i) DNA template extracted from pure cultures could be sequenced directly, so cloning step is not necessary; (ii) DNA template extracted from environmental cultures, the cloning step is necessary to separate rDNA fragments of different species which is required for sequence analysis. Cloning procedure shown in Fig. 1-5 includes five basic steps as follows (9). (i) A recombinant DNA molecule is produced by inserting a fragment of DNA, containing the gene of interest to be clone, into a circular DNA molecule called a vector. (ii) The vectors transport the gene into the host cell (e.g. *E. coli*). (iii) Multiplication of recombinant DNA molecule within the host cell. (iv) Division of host cell is performed by cultivation on selective medium. (v) The gene carried by the recombinant molecule is cloned after a large number of cell divisions (a colony or clone) of identical host cells. By cloning the DNA fragment containing the gene of interest is separated away from all the other genes in the original mixture of many different DNA fragments. Clone library is composed of high numbers of clones characterized by e.g. different 16S rRNA gene sequences. After cloning, sequencing of the 16S rRNA gene has served as an important tool for determining phylogenetic relationships which give information about the microbial diversity and might reveal the present of novel species (7). For phylogenetic sequence analysis the DNA fragment length is an important factor. Partial sequencing is only sufficient for known species, whereas a full gene sequence is essential for characterization of a novel species. Moreover, full gene sequence data is required in order to design new specific primers (PCR) and fluorescently labeled probes (FISH) for detection and/ or quantification of specific groups of microorganisms (1). However, cloning and sequencing of 16S rRNA gene for all clones within a library is very time consuming and expensive. Thus, screening by fingerprinting method ARDRA (amplified rDNA restriction analysis) (see 5.3.1) is necessary to ensure that only representative clones giving different OTUs (operational taxonomic units) will be sequenced (Fig. 1 - 4B and Fig. 1-5).

Full-cycle rRNA approach is the most powerful tools to explore microbial diversity and dynamic of mixed microbial population in environmental samples (15, 16, 98). By providing the precise taxonomical information about the species composition, these methods have revealed that microbial diversity actually is much higher than reported previously by using traditional cultivation-based methods. However, for microbial ecology analysis especially wastewater treatment process besides microbial diversity the information about the changes of microbial community structure after environmental perturbation with toxic compounds is also important. Information about the shifts of microbial populations could be useful for maintaining a stable prolonged reactor performance under fluctuating process conditions. For this purposed, sampling at different time points over a long periods is often required. Thus, full-cycle rRNA approach applied in combination with DGGE is necessary to obtain a complete picture of microbial ecology.

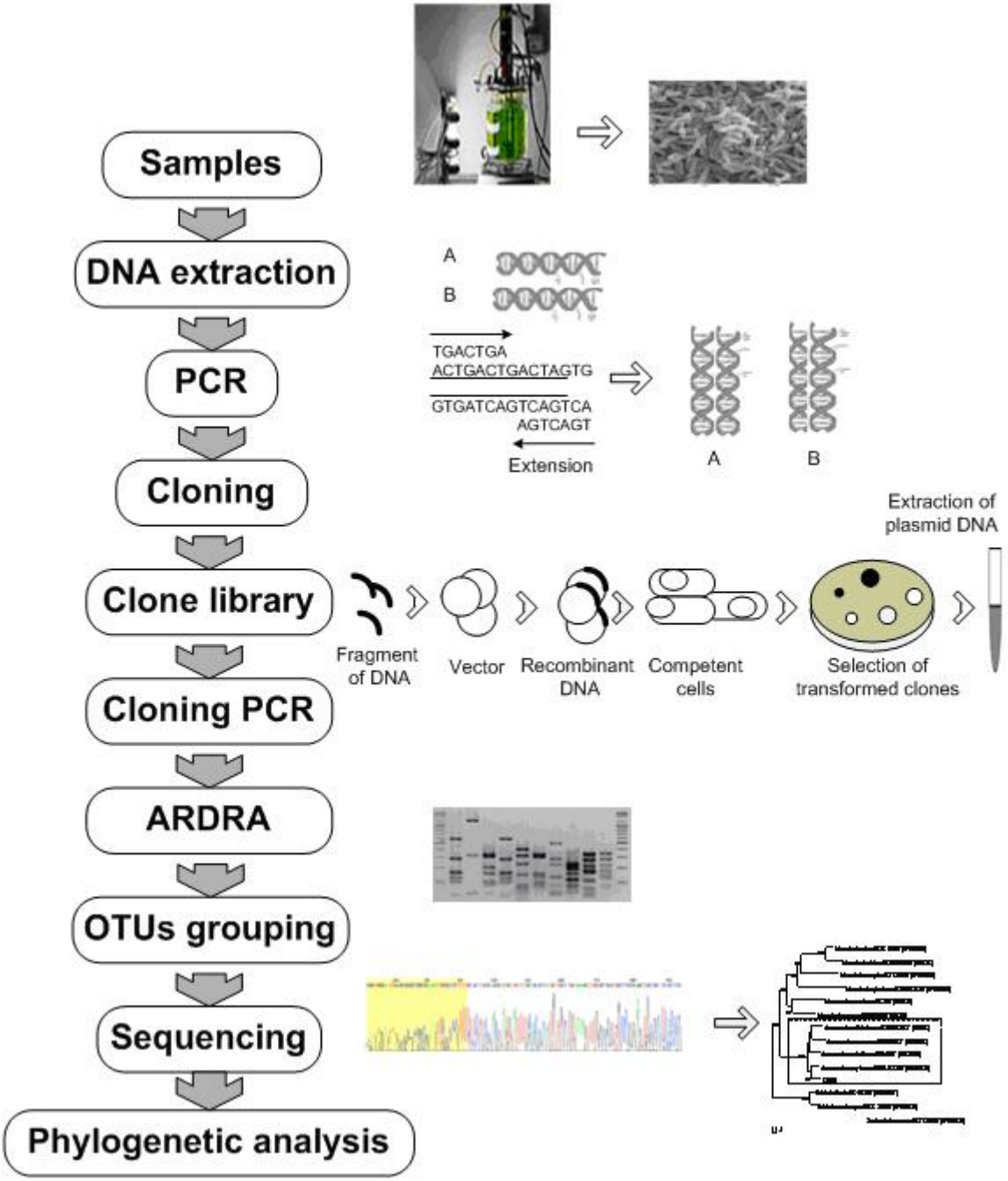


Fig. 1-5. Cloning and sequencing of 16S rRNA gene for studying an environmental microbial community

5.3. Nucleic acid fingerprinting

In contrast to cloning and sequencing of 16S rRNA gene, nucleic acid fingerprinting (Fig. 1-4B) is especially suited for screening of multiple samples in microbial ecology at the same time. The fingerprinting techniques produce characteristic band patterns that can be used for detection of diversity as well as comparison of microbial communities from different

environments and the changes in their composition overtime. Fingerprinting steps for analysis of microbial communities consists of (i) DNA extraction, (ii) PCR amplification, and (iii) analysis of PCR products by fingerprinting techniques, such as DGGE/TGGE, RISA/ARISA, SSCP, RAPD, AFLP, T-RFLP, ARDRA (Fig. 4B). In the present study, only two nucleic acid fingerprinting techniques ARDRA (Amplified 16S rDNA restriction analysis) and DGGE (Denaturing gradient gel electrophoresis) were applied (see chapter 3).

5.3.1. ARDRA (Amplified rDNA restriction analysis)

ARDRA, known as restriction fragment length polymorphism (RFLP) analysis of rRNA gene, is a suitable method to screen isolates and clones. The standard method for ARDRA is described by Heyndrickx et al. (38) (Fig. 1-4B and Fig. 1-5) as follows. The first step, e.g. a fragment of the rRNA gene of pure culture or clones is amplified using PCR and digested with restriction enzyme to produce DNA fragments of different length. In the second step, DNA fragments with different length are separated by gel electrophoresis. In the third step, numerical analysis of the band profiles allows the pure cultures or clones to be grouped according to the similarities in their combined ARDRA patterns. ARDRA is commonly carried out to screen the sequences separated by cloning (clone libraries) and then different OTUs (operational taxonomic units) were randomly selected for sequencing to avoid several identical 16S rDNA causing a waste of time and money. ARDRA was used by a significant number of studies to monitor the microbial community diversity (25, 41, 43). However, the estimation of microbial diversity by ARDRA is limited because the number of ARDRA patterns is higher than the number of amplified DNA fragments. Thus, some distinguishable ARDRA patterns may be derived from the same organism result in an overestimation of the community diversity (24). Therefore, ARDRA technique is more suitable to characterize pure cultures or to screen clone library into representative OTUs than to investigate the diversity of microbial communities (8, 11).

5.3.2. DGGE/TGGE (Denaturing gradient gel electrophoresis/Temperature gradient gel electrophoresis)

DGGE and TGGE methods described in detail by Muyzer et al. (80) (see Fig. 1-6) are usually employed to investigate the diversity of microbial communities in environmental samples as well as determine their dynamic changes in response to environmental variations through multiple sample analysis.

These methods enable detecting differences between DNA fragments of the same size but with different sequences based on electrophoresis of PCR-amplified 16S rDNA (or other

gene) fragments. Separation is based on the decreased electrophoretic mobility of a partially melted double-stranded DNA molecule in polyacrylamide gels containing a linear gradient of DNA denaturants (a mixture of urea and formamide) in DGGE or on a linear temperature gradient in TGGE. The specific melting temperature (*Tm*) (so-called melting domain) where half of the double-stranded DNA molecule is dissociated (melted) into single-stranded molecules. The different sequences of the DNA fragments will have melting domains with different *Tm* values that depend on percentage of the GC content of the sequence, the length of the sequence and concentration of DNA denaturants. Once a sequence reaches its melting domain at a particular position in the denaturing or temperature gradient gel, a transition of a helical to a partially melted molecule occurs, and migration of the molecule will stop. Molecules with different sequences will stop migrating at different positions in the gel and therefore are separated by generating band patterns.

The number of bands corresponds to the number of species present in the analyzed sample. Thus, microorganisms with different sequences in their 16S rRNA genes will produce unique gel band patterns that reflect the genetic biodiversity or the changes in species composition of a microbial community under different environmental conditions. Furthermore bands of interest can be excised from gels and the corresponding bacteria species can be identified by sequencing and phylogenetic analysis. Therefore, DGGE and TGGE techniques have become a power and routinely applied method particularly useful when examining microbial community in complex process such as wastewater treatment due to rapid perform and multiple sample throughput analysis. The main limitations of DGGE and TGGE include: (i) Intensities of DGGE/TGGE bands derived from different bacterial species do not allow quantitative conclusions about the *in situ* abundance of these bacteria due to potential PCR amplification bias of different templates. Thus, the presence and quantification of particular microbial populations should be verified by other methods such as FISH technique with specific fluorescently-labeled rRNA targeted oligonucleotide probes (published or newly designed probes based on sequence information). (ii) The short fragment length (ca. 500bp or less) used in DGGE and TGGE results in a less precise phylogenetic analysis in comparison with cloning of the whole 16S rRNA gene. (iii) Short sequences are less applicable for designing new specific primers and probes. Moreover, organisms in diverse microbial communities are not or difficult to be detected because DGGE patterns are complex with many faint bands. This might be explained that the organisms which contain low concentration of template DNA are probably outcompeted in the amplification process. Gich et al (33) reported that bacteria that constitute ≤ 9% of complex microbial communities may not be detected by DGGE. However, the sensitivity of detection can be increased by using group-specific primers or functional gene-specific primers for PCR amplification.

Heuer et al. (37) showed that only the application of *Actinobacteria*-specific primers resulted in distinct DGGE patterns representing the *Actinobacteria* diversity of the soil samples analyzed.

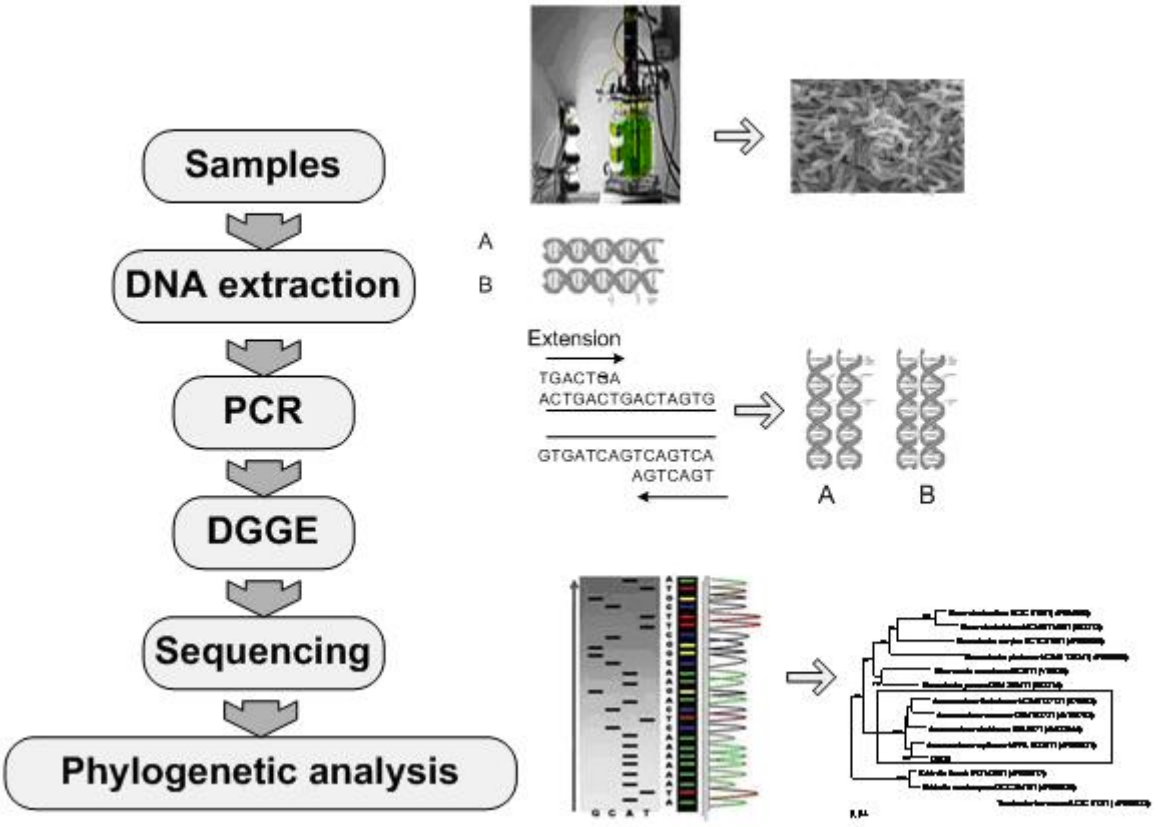


Fig. 1-6. PCR-DGGE technique for studying an environmental microbial community

5.3.3. Other fingerprinting techniques

*** RISA/ARISA** (Ribosomal rDNA internal spacer analysis/ automated rDNA internal spacer analysis)

The method involves PCR amplification of the spacer region located between the small (16S) and large (23S) subunit rRNA genes in the rRNA operon. This region is extremely variable in size (ranging from 50-bp to more than 1.5-kb) and nucleotide sequence. Primers are defined to target to conserved regions in the 16S and 23S genes. In RISA, the polymorphism revealed is linked to the length heterogeneity. Amplification products differing in length are separated on polyacrylamide gels on the basis of their size and visualized by silver staining. This tool has been used successfully to assess community fingerprints, each band corresponding to at least one organism. ARISA, the automated version of RISA is

developed in order to be able to assess community diversity more rapidly and more efficiently (69).

*** SSCP** (Single-strand conformation polymorphism)

In this technique, PCR amplicon is treated (i) with denatured substances e.g. formamide and (ii) with heat to create single-stranded fragments. Electrophoretic separation is then performed under non-denaturing conditions, allowing the fragments to partially re-nature and form folded conformations due to the different intramolecular interactions between the bases. These secondary structures result in different electrophoretic motilities, and hence separation of DNA strands differing by as little as a single base pair is possible. SSPC does not require complex apparatus for the preparation of gradient gels or the addition of GC clamps to the primers. However, it can be limited by the formation of single- and double-strand fragments, and multiple conformations for the same fragment (51).

*** RAPD** (Random amplified polymorphic DNA analysis)

RAPD strain identification is based on the PCR technique and the use of a single 10-base primer. Because the primer is short, there are usually many complementary sequences on the genomic DNA to which the primer will bind. DNA polymerase adds other bases to the primer, creating short pieces of double-stranded DNA. The PCR technique then creates millions of copies of these pieces. The various sizes of DNA pieces are then separated electrophoretically on an agarose gel and viewed by staining with Ethidium Bromide. This methods are less useful to differentiate small genomes (such as those of viruses), since the number of products is usually small (12).

*** AFLP** (Amplified fragment-length polymorphism)

AFLP is a restriction endonuclease and PCR based method that detects the polymorphism of the whole genome without prior knowledge of the nucleotide sequence. Advantages of AFLP are the high degree of reproducibility and small amount of template DNA needed. In the three-part technique, genomic DNA is double-digested with two restriction enzymes, resulting in three types of fragments. Adapters specific to the restriction sites are ligated to the fragment ends which serve as binding sites for selective primers in PCR amplification. PCR products are detected on denaturing polyacrylamide gels (71).

*** T-RFLP** (Terminal-restriction fragment length polymorphism)

T-RFLP analysis is a tool for assessing species richness and the population sizes of various species in a complex bacterial community. The concept was derived from traditional RFLP analysis. DNA is isolated from the bacterial community in a field sample and DNA coding for 16S rRNA is specifically amplified by PCR using primer pairs located in the conserved region of the gene. One primer is labeled with a fluorescent dye. The PCR product is then digested by a restriction enzyme and the length profile of terminal restriction fragment (TRF) labeled by the fluorescent dye is detected to identify species in the bacterial communities. The relative ratio of TRFs, estimated by measuring fluorescence emission intensity, indicates the relative abundance of bacterial species (116).

5.4. FISH (Fluorescent *in situ* hybridization)

PCR-based methods as DGGE fingerprinting and cloning for the analysis of microbial communities can be influenced by several pitfalls and potential biases (108) (see Chapter 3). PCR-based results might not reflect the real quantity of target sequence in the original samples. Therefore, the application of techniques that do not involve an amplification step are necessary to confirm the presence of bacterial species detected by using PCR-based methods and simultaneously qualify the abundance of these bacteria.

In contrast to DGGE fingerprinting and cloning, rRNA-based hybridization methods are able to quantify the abundance of microbes because of no need amplification step. There are two different types of rRNA-based hybridization methods that use phylogenetically based oligonucleotide probes for quantification: (i) Fluorescent *in situ* hybridization (FISH) (Fig. 1-7) and (ii) quantitative dot blot hybridization (1). FISH using fluorescently-labeled rRNA-targeted oligonucleotide probes have become a popular technique (26, 42, 54). The approach has been applied successfully for *in situ* studies of dynamic microbial population changes without cultivation in many ecosystems through analysis of phylogenetic identification and quantification. The basis of FISH is to detect rRNA sequence regions by a fluorescently-labeled probe that hybridizes specifically to its complementary target sequence within the intact cells. The standard procedure of this method was greatly described by Amann and Pernthaler (1, 90) (see Fig. 1-7). Oligonucleotide probes used by FISH techniques is a short DNA sequence (15-30 nucleotides) labeled with a fluorescent dye (77). The use of oligonucleotide probes targeting rRNA represents a revolution within wastewater treatment research and microbial ecology in generally. Quantitative dot blot hybridization that can quantify a specific rRNA region compared with total rRNA dot blot hybridizations of a directly isolated nucleic acid mixture with universal and specific oligonucleotide probes.

However, the limitation of this method is that the relative abundance of rRNA cannot be directly translated into cell numbers since ribosome contents of different bacterial species vary from about 10^3 to 10^5 ribosomes per cell and are a function of growth rate in many species (113). Thus, FISH is preferred method to quantify bacterial numbers *in situ* in the non destroyed environmental samples.

The main short comings of FISH are the lack of availability of probes targeting the bacteria of interest and the difficulty to select the suitable probes detecting organisms which might be involved in the process. Therefore, DGGE and cloning analysis might help to choose the suitable probes and even the obtained sequences from DGGE and cloning allow designing new probes.

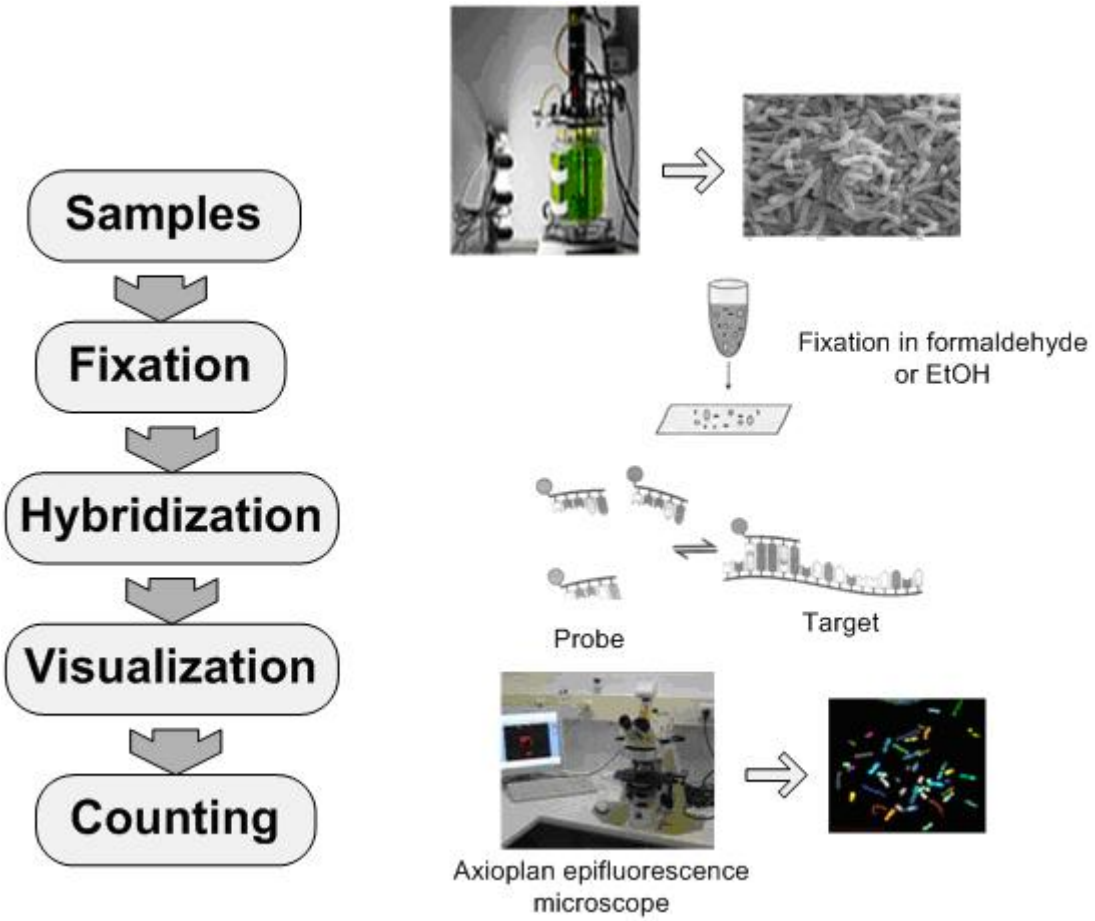


Fig. 1-7. FISH technique for studying an environmental microbial community

6. Objectives and content of this study

**** Objectives***

The studies of bacterial tolerance and removal to heavy metals and the effect of heavy metals on the shift of microbial structure are very important for the development of efficient treatment processes of heavy metal containing wastewaters based on the used of these bacteria. Therefore, this study was focused on the following objectives as follows.

- Selection of a high heavy metal tolerant sulfidogenic consortium by screening tests in test-tubes.
- Heavy metal removal in anaerobic batch conditions by the selected sulfidogenic consortium.
- Heavy metal removal in anaerobic semi-continuous stirred tank reactors (CSTR) by the selected sulfidogenic consortium.
- Application of different biomolecular techniques (cloning and sequencing, DGGE, and FISH) for assessing the effect of heavy metals (Cu^{2+}, Ni^{2+}, Zn^{2+}, and Cr^{6+}) on microbial community structure of the selected sulfidogenic consortium.

**** Content of this study***

This study consists of four chapters. **Chapter 1** is **state of knowledge** that can be divided into two main parts. **The first part** provides overviews of **heavy metal** pollution including toxicity of heavy metals, microbial resistance to toxic heavy metals, the classical methods that have been commonly applied for treatment of heavy metal containing wastewater, such as chemical, physico-chemical, and biological methods and then point out the importance of biological methods, especially metal sulfide precipitation by sulfate-reducing bacteria (SRB). Before introducing about principle of heavy metal removal by sulfate reduction, SRB with their taxonomy, phylogeny, biochemistry, and physiology are mentioned. **The second part** focuses on **molecular biological approaches** for analysis of microbial community in wastewater treatment. In this part, the role of rRNA genes and the most powerful molecular tools used to characterize microbial communities such as full-cycle rRNA approach, nucleic acid fingerprinting and fluorescent *in situ* hybridization (FISH) with rRNA-targeted oligonucleotide probes are shown. The importance of using a combination of different molecular techniques to minimize the limitations of each method in study of microbial community was revealed. The content of next two chapters, which are main subjects of this study, is summarized as follows. **Chapter 2** describes the **selection of a highest heavy metal**

tolerant sulfidogenic consortium by screening tests in test-tubes among 9 enriched consortia. Subsequently, the selected sulfidogenic consortium was further assessed on **heavy metal removal efficiency by anaerobic batch and semi-continuous stirred tank reactors (CSTR)**. **Chapter 3** presents **a combining application of cloning and sequencing, DGGE, and FISH for assessing the effect of heavy metals (Cu^{2+}, Ni^{2+}, Zn^{2+}, and Cr^{6+}) on microbial community structure of the selected sulfidogenic consortium**. In this chapter the importance of the use of a combination of different molecular techniques mentioned in Chapter 1 is discussed in detail through the obtained results from semi-continuous experiments. **Chapter 4 concludes** on the understanding of heavy metal removal by sulfate reduction and changes of microbial community structure of the selected sulfidogenic consortium obtained in this study, and furthermore, **suggestions for future research** are described.

7. References

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CHAPTER 2

Heavy metal removal in batch conditions and in anaerobic semi-continuous stirred tank reactors by a heavy metal tolerant sulfidogenic consortium

1. Introduction

The environmental pollution caused by wastewaters containing high concentrations of dissolved heavy metals and low pH from mining and industrial processing (e.g. metallurgical, electronic, electroplating and metal finishing industries) negatively impacts to living organisms as well as humans. The toxic effects of heavy metals include ion displacement and/or substitution of essential ions from cellular sites and blocking of functional groups of important molecules, e.g. enzymes, polynucleotides, and essential nutrient transport systems. This results in denaturation and inactivation of enzymes and disruption of cell organelle membrane integrity, as well as damage to the structure of DNA, nerves, livers and bones (28). Unlike organic contaminants, which can be degraded into harmless chemical species, heavy metals cannot be degraded. However, they can be transformed from mobile and toxic forms into their stable immobile and less toxic forms (8). Many methods have been used for treatment of heavy metal contaminated wastewaters. Among them, the classical physicochemical methods were widely applied (e.g. chemical precipitation, absorption, ion exchange, and electrochemical treatment, etc.). Despite effective treatment, these methods are expensive and generate large amounts of residual sludge (18). Therefore, immobilization of heavy metals through microbial mediated reduction and precipitation is now of considerable interest. Especially, metal sulfide precipitation by SRB has promise as an attractive alternative over physico-chemical and other methods. This method involves two stages: (i) Sulfate-reducing bacteria (SRB), under anaerobic conditions, oxidize simple organic compounds (e.g. lactate, acetate, propionate, butyrate, etc.) by utilizing sulfate as an electron acceptor and generating hydrogen sulfide and bicarbonate ion, and (ii) the biologically produced hydrogen sulfide reacts with dissolved heavy metals such as Cu, Zn, and Ni to form insoluble metal sulfide precipitates (10).

$$2CH_2O + SO_4^{2-} \rightarrow H_2S + 2HCO_3^-$$

$$Me^{2+} + H_2S \rightarrow MeS\downarrow + 2H^+ \text{ (Me = Metal)}$$

Hydrogen sulfide produced by SRB indirectly reduces highly soluble, toxic and mutagenic Cr^{6+} to the water-insoluble and significantly less toxic Cr^{3+} according to the following equation (9, 24).

$$3HS^- + 2Cr^{6+} \rightarrow 3S^o + 2Cr^{3+}\downarrow + 3H^+$$

In comparison with hydroxide and other precipitations, heavy metal precipitation by using biological sulfide has advantages which include low amounts of residual sludge, lower solubility even at acidic pH, highly reactive efficiency, and cost effectiveness (15, 29). This method not only eliminates toxic heavy metals, acidity and sulfate from heavy metal contaminated wastewater, but also enable the recovery of valuable metals as metallic sulfide (14).

The metal resistance of organisms varies with the species by developing a variety of specific resistance mechanisms such as metal exclusion by permeability barrier, active transport of the metal away from the cell, intracellular sequestration of the metal by protein binding, extracellular sequestration, enzymatic detoxification of the metal to a less toxic form, and reduction in metal sensitivity of cellular targets (7) (in detail in Chapter 1, section 1.1.2). In addition, the ability of heavy metal resistance of organisms is also dependent on the mobility, bioavailability, and toxicological effect of each heavy metal. For example, the Cr^{6+} is very soluble at all pH values and much more toxic than Cr^{3+}; As^{5+} is less mobile and toxic than As^{3+}. Studies that focus on searching for the sulfidogenic consortia with high heavy metal tolerance and removal are very important for development of heavy metal treatment by sulfate reduction.

Heavy metal treatment using anaerobic sulfate reduction is influenced by a variety of parameters such as electron donor, pH, temperature, sulfate concentration, and heavy metal species. Therefore, the use of an active treatment method as a sulfidogenic bioreactor for heavy metal removal is probably preferable to passive or other active treatment methods. The advantage of this method is the ease of control during the treatment process resulting in the permanent removal of heavy metals.

In recent years, heavy metal removal by sulfate reduction has been studied in various bioreactor-types such as continuous stirred tank reactor (CSTR) (23, 27), upflow anaerobic sludge blanket reactor (UASB) (20), off-line sulfidogenic bioreactor (13), fixed bed reactor (FBR) (19, 33), and permeable reactive barriers (PRB) (5).

The aim of the study presented in this chapter was to investigate the heavy metal removal efficiency of selected sulfidogenic consortium in five parallel semi-continuous stirred tank

reactors spiked with different concentrations of heavy metal mixtures (Cu^{2+}, Ni^{2+}, Zn^{2+}, and Cr^{6+}).

2. Materials and Methods

2.1. Sulfidogenic consortia

Nine sulfidogenic consortia (TX1-TX9) obtained form heavy metal contaminated sediments in Tong Xa, a settlement known for bronze, iron casting and electroplating in Nam Dinh, Vietnam. These cultures were cultivated and enriched under anaerobic conditions using modified Postgate medium B (25) (Table. 2-1). All procedures during preparation of the medium and cultivation were performed according to the modified Hungate's method for anaerobes (22) (Fig. 2-1). An array of 10ml-screw top test tubes containing a modification P_c medium were inoculated with 10% (v/v) diluted sediment, sparged with nitrogen gas, and incubated at 30^{o}C for 14 days. The growth of SRB was detected by the formation of ferrous sulfide (FeS) as a black precipitates at the bottom and the wall of the test tubes. The experiments were performed in triplicate.

Fig. 2-1. Gas station (left) and Anaerobic chamber (right)

2.2. Selection of a high heavy metal tolerance sulfidogenic consortium

To select a highest heavy metal tolerant consortium for the semi-continuous experiment, the heavy metal tolerance and removal ability of the obtained sulfidogenic consortia were assessed by screening tests in test-tubes and batch experiments in P_C medium spiked with different concentrations of Cu^{2+}, Zn^{2+}, Ni^{2+}, and Cr^{6+}. Heavy metal solutions were prepared from dissolution of chloride salt for Cu^{2+}, Zn^{2+}, Ni^{2+} and potassium dichromate ($K_2Cr_2O_7$) for Cr^{6+} then sterilized by membrane filtration (0.22μm).

Table 2-1. Composition of modified Postgate'B (P_B) and Postgate'C (Pc) medium (25)

Composition (g L^{-1})	Modified P_B	Modified Pc
KH_2PO_4	0.5	0.5
NH_4Cl	1.0	1.0
Na_2SO_4	1.0	4.5
$CaCl_2$. $6H_2O$	-	0.06
$MgSO_4$. $7H_2O$	2.0	0.06
Sodium lactate	3.5	4.42
Yeast extract	0.5	-
Ascobic acid	0.1	-
Thoglycolate Na	0.1	-
$FeSO_4$. $7H_2O$	0.5	-
Sodium citrate. $2H_2O$		0.3
pH	7.0-7.2	7.0-7.2

2.2.1. Screening tests in test tubes

Nine sulfidogenic consortia (TX1-TX9), obtained from heavy metal contaminated samples, were investigated preliminarily for their tolerance to individual heavy metals (Cu^{2+}, Zn^{2+}, Ni^{2+}, and Cr^{6+}). Array of 9 ml of modified P_C medium with 0.2 g L^{-1} $FeSO_4$. $7H_2O$ (Table 2-1) prepared in 10 ml-screw top test tubes was spiked with different initial concentrations of Cu^{2+} (0, 10, 25, and 50 mg L^{-1}), Zn^{2+} (0, 50, 100, and 150 mg L^{-1}), Ni^{2+} (0, 50, and 100 mg L^{-1}) and Cr^{6+} (0, 10, 25, and 50 mg L^{-1}). All cultivations were inoculated with 10% (v/v) of the enriched sulfidogenic consortia. The heavy metal tolerance was estimated by the growth of SRB through the formation of black precipitate (FeS) after 14 days of the incubation at 30^{o}C, pH6. Two consortia (TX1 and TX2) were selected and further assessed on their efficiency for heavy metal removal by measuring residual dissolved concentrations of Cu^{2+}, Zn^{2+}, Ni^{2+}, and Cr^{6+} after 14 days of experiment. The results indicated that TX2 is the highest heavy metal tolerant consortium.

2.2.2. Batch experiments

Removal efficiencies of individual heavy metals (Cu^{2+}, Zn^{2+}, Ni^{2+}, and Cr^{6+}) by the TX2 consortium was evaluated in the presence of different initial concentrations through heavy metal precipitation and sulfate reduction. Experiments were performed in identical anaerobic batch conditions using 1L-glass bottles containing 450 ml of modified Pc medium without $FeSO_4$. $7H_2O$ (Table 2-1) spiked with different concentrations of Cu^{2+} (0, 10, 25, 50, and 100 mg L^{-1}), Zn^{2+} (0, 25, 50, 100, and 150 mg L^{-1}), Ni^{2+} (0, 25, 50, 100, and 150 mg L^{-1}) and Cr^{6+}

(0, 10, 25, and 50 mg L^{-1}). All cultivations were inoculated with 10% (v/v) of the enriched TX2 consortium for 14 days at 30^{o}C, pH 6. Sampling was conducted at time intervals 0h, 2h, 6h, 12h, 24h, 72h, 168h, and 336h.

2.3. Semi-continuous experiments

2.3.1. Inoculum

A highest heavy metal tolerant sulfidogenic consortium (TX2) selected by screening tests in test tubes (Table 2-3) was applied for semi-continuous experiment as an inoculum. To enrich the bacteria number the cultivation step was repeated three times before inoculating the bioreactors.

2.3.2. Experimental set-up

The configuration and photograph of semi-continuous stirred tank reactor are present in Fig. 2-2

**** Bioreactors***

The experiment was carried out in five anaerobic semi-continuous stirred tank reactors (CSTR, V = 2 L) operated with a hydraulic retention time (HRT) of 20 days. The reactors were kept at 30^{o}C in a heated water bath and mixed by magnetic stirrers. All reactors were soaked in a 3M HNO_3 solution for 72h and rinsed with de-ionized before use to avoid metal contamination.

**** Feeding tanks***

Synthetic wastewater (see below) was prepared aseptically every week to avoid the contamination and then fed continuously at the top of the bioreactors by a peristaltic pump (Ismatec SA, Zuerich, Switzerland) with a volumetric flow rate of 100 mL d^{-1}.

To maintain the anaerobic condition, all bioreactors and feeding tanks were purged with filter sterilized nitrogen gas (0.22 μm). Gas produced during the treatment process was trapped by a gas collection system.

**** Synthetic wastewater composition***

The composition of the synthetic wastewater was prepared as follows: KH_2PO_4, 0.5 g l^{-1}; NH_4Cl, 1.0 g L^{-1}; Na_2SO_4, 3.7 g L^{-1}; Sodium lactate 4.42 g L^{-1}; and Trisodium citrate, 0.3 g L^{-1}. The synthetic wastewater was not supplemented with Fe^{2+} and reducing agents such as ascorbic acid, sodium thioglycolate, Na_2S to allow the valuation of the precipitation of other metals under investigation. Trisodium citrate was added to prevent any initial metal

precipitation. The pH was adjusted to 6.0 ± 0.2 using HCl and NaOH. For semi-continuous operation, synthetic wastewater was spiked with a mixture of Cu^{2+}, Ni^{2+}, Zn^{2+}, and Cr^{6+} in different concentrations as shown in Table 2-2. Solution for each heavy metal was prepared from the dissolution of chloride salt for Cu^{2+}, Ni^{2+}, Zn^{2+} and potassium dichromate ($K_2Cr_2O_7$) for Cr^{6+} and then sterilized by membrane filtration (0.22 μm).

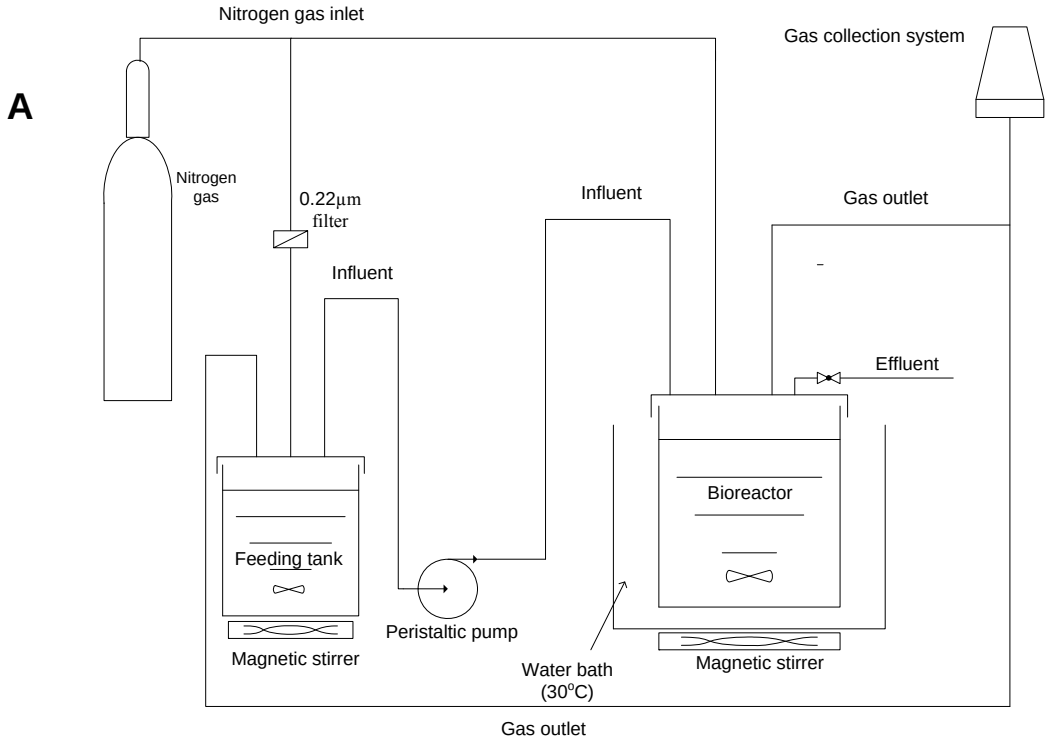

Fig. 2-2. Semi-continuous stirred tank reactor (A) configuration, (B) photograph

2.3.3 Experimental procedure

The bioreactors were first inoculated with 10% (v/v) of the enriched sulfidogenic consortium containing 1×10^8 cells mL^{-1} and incubated for 9 days at batch operating conditions using synthetic wastewater containing sulfate and sodium lactate as electron acceptor and donor, respectively, without heavy metals. After pre-incubation (start of the experiment), the reactors were fed with synthetic wastewater containing the desired concentrations of heavy metals. The reactors were operated semi-continuously for 12 weeks, with the exception of R5 which was only operated for 6 weeks (Table 2-2).

Table 2-2. Reactor operating characteristics

Reactor	Heavy metal* spiked in synthetic wastewater (mg L^{-1})	Heavy metal* loading rate (mg L^{-1} d^{-1})	Total time of operation (weeks)
R1	30	1.5	12
R2	60	3.0	12
R3	90	4.5	12
R4	120	6.0	12
R5	150	7.5	6

* Cu^{2+}, Zn^{2+}, Ni^{2+}, and Cr^{6+}

2.3.4. Analytical methods

Samples were taken weekly to measure sulfate, dissolve sulfide, dissolved heavy metals, and pH according to standard methods (2). Dissolved sulfide and pH were immediately measured after collection. For sulfate and dissolved heavy metal analyses the samples were filtered through 0.45 μm cellulose nitrate - membrane filters (Germany) before measuring. Dissolved sulfide, sulfate and Cr^{6+} were measured spectro-photometrically (Dr. Lange ISIS 6000). The concentrations of Cu^{2+}, Zn^{2+}, and Ni^{2+} were determined by an atomic absorption spectrophotometer (AAS) after acidifying with concentrated nitric acid (pH<2) to prevent metal precipitation and adsorption to surfaces.

**** EDS analysis***

Qualitative analysis of precipitates was realized by energy dispersive spectrometry (EDS) analysis using a Leica/ Cambridge, model StereoScan 360. The precipitates were obtained after filtering the effluent sample of R2 through 0.45 μm cellulose nitrate - membrane filters

(Germany). The filter paper was dried in an oven for 2h at 105^{o}C and then thin coated by Au for EDS analysis.

**** Total cell counts and fluorescent in situ hybridization (FISH)***

The abundance of SRB was estimated by total cell count using 4', 6' diamidino-2-phenylindole (DAPI) staining and FISH technique using 16S rRNA-target oligonucleotide probe (SRB385) labeled at the 5' end with indocarbocyanine (Cy3) reactive fluorescent dye. All procedures such as fixation of samples by paraformaldehyde and EtOH, DAPI staining, drying and washing were performed using modified standard procedure described previously (1). Hybridized and DAPI stained cells were visually detected by using Axioplan epifluorescence microscope (Carl Zeiss, Jena, Germany). Samples of R1-R4 were collected at the start, after two, six, and 11 weeks and of R5 at the start, after one and four weeks and immediately fixed with 4% (w/v) paraformaldehyde (PFA) and stored at -20^{o}C until analysis.

3. Results

3.1. Screening tests in test tubes

The effects of individual heavy metals (Cu^{2+}, Zn^{2+}, Ni^{2+}, and Cr^{6+}) with different initial concentrations on the growth of 9 sulfidogenic consortia (TX1-TX9) were investigated by screening tests in test-tubes. The results were shown in Table 2-3 indicated that two consortia TX1 and TX2 have highest tolerant capacities for all investigated heavy metals. The growth of TX1 consortium was possible with initial Cu^{2+}, Cr^{6+}, Ni^{2+} and Zn^{2+} concentrations of 50, 25, 50, and 100 mg L^{-1}, respectively, while TX2 consortium can growth at 50 mg L^{-1} Cu^{2+}, 50 mg L^{-1} Cr^{6+}, 100 mg L^{-1} Ni^{2+}, and 150 mg L^{-1} Zn^{2+}, respectively (Table 2-3a). To confirm the heavy metal tolerance of TX1 and TX2, residual heavy metal concentrations from the test tubes inoculated by these two consortia was measured at the end of experiment. The highest heavy metal removal efficiency was observed from the tubes inoculated with TX2 consortium, which was capable to remove 40% of 100 mg L^{-1} Ni^{2+}, 100% of 50 mg L^{-1} Cu^{2+}, 96% of 25 mg L^{-1} Cr^{6+}, and 100% of 100 mg L^{-1} Zn^{2+} (Table 2-3b).

B

50 mg/l:TX1:0.122 TX2: 0.05	Me.	Conc. (mg/l)	TX1	TX2	TX3	TX4	TX5	TX6	TX7	TX8	TX9
100 mg/l: TX1: 79 TX2: 60		0	+	+	+	+	+	+	+	+	+
	Ni (II)	50	+	+	(+)	(+)	+	+	-	±	±
		100	-/+	±	-	-	-	-	-	-	-
25mg/l: TX1: 0.2 TX2: 0.2			TX1	TX2	TX3	TX4	TX5	TX6	TX7	TX8	TX9
50mg/l: TX1: 0.2 TX2: 0.2		0	+	+	+	+	+	+	+	+	+
		10	+	+	+	+	(+)	+	+	+	+
	Cu (II)	25	+	+	+	+	±	+	-	±	+
		50	+	+	-	±	-	-	-	-	±
			TX1	TX2	TX3	TX4	TX5	TX6	TX7	TX8	TX9
25 mg/l: TX1: 4.3 TX2: <1.3		0	+	+	-	+	+	-	-	-	-
		10	+	+	-	+	±	-	-	-	-
	Cr (VI)	25	+	+	-	-	-	-	-	-	-
		50	-/+	±	-	-	-	-	-	-	-
			TX1	TX2	TX3	TX4	TX5	TX6	TX7	TX8	TX9
50mg/l: TX1:<0.05 TX2:<0.05		0	+	+	+	+	+	+	+	+	+
100mg/l: TX1:<0.05 TX2:<0.05		50	+	+	+	+	+	+	(+)	(+)	(+)
	Zn (II)	100	(+)	+	±	-	-/+	-	-	-	-
		150	-	+	-	-	-	-	-	-	-

+ positive; - negative; (+) weak positive

A

Table 2-3. (A) Heavy metal tolerance of 9 sulfidogenic consortia were detected by the formation of ferrous sulfide (FeS) as a black precipitate by screening tests in test tubes after 14 days; (B) Residual heavy metal concentrations from the test tubes inoculated by consortia TX1 and TX2 after 14 days.

3.2. Batch experiments

Batch experiments were carried out to evaluate the removal efficiencies of individual heavy metals (Cu^{2+}, Zn^{2+}, Ni^{2+}, and Cr^{6+}) by TX2, a heavy metal tolerant sulfidogenic consortium selected among 9 sulfidogenic consortia from screening tests in test tubes. All experiments were operated under identical conditions except the concentrations of heavy metals. The residual heavy metal and sulfate concentrations were estimated during the 14 day - batch experiment (~ 336h) are shown from Fig. 2-3 to 2-6. The results showed (Table 2-4) that significant differences in the behavior of TX2 consortium to each heavy metal were observed. An increase in the heavy metal concentration resulted in a decrease in the sulfate reduction and metal precipitation. The tolerance of TX2 consortium to each metal was relatively high with the inhibitory order was $Cr^{6+} > Ni^{2+} > Cu^{2+} > Zn^{2+}$.

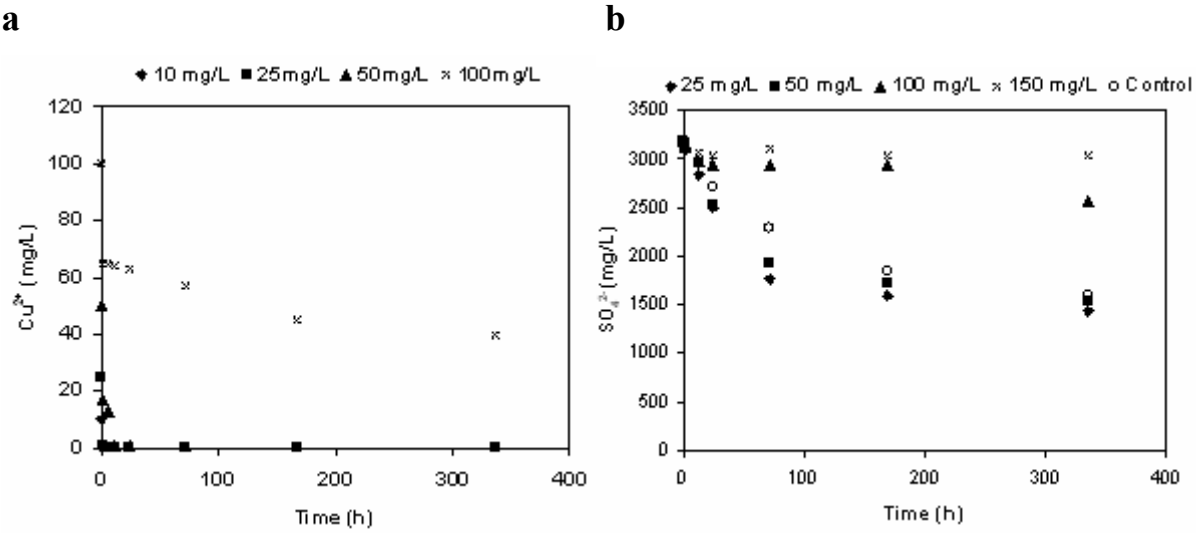

Fig. 2-3. (a) Residual concentrations of Cu^{2+} and (b) SO_4^{2-} by TX2 consortium in batch condition after 14 day-experimental period

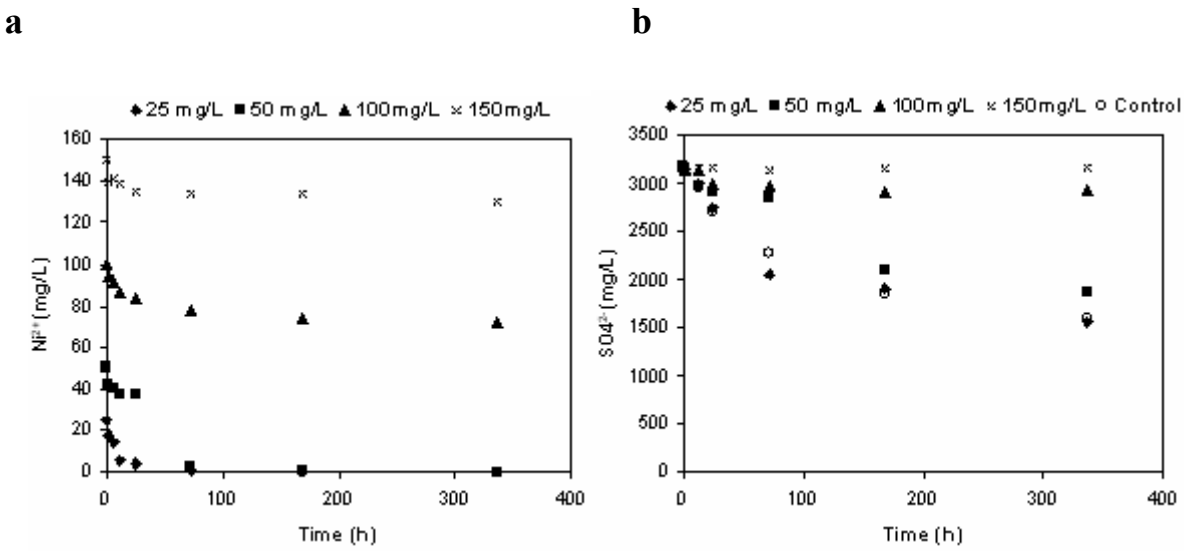

Fig. 2-4. (a) Residual concentrations of Ni^{2+} and (b) SO_4^{2-} (b) by TX2 consortium in batch condition after 14 day-experimental period

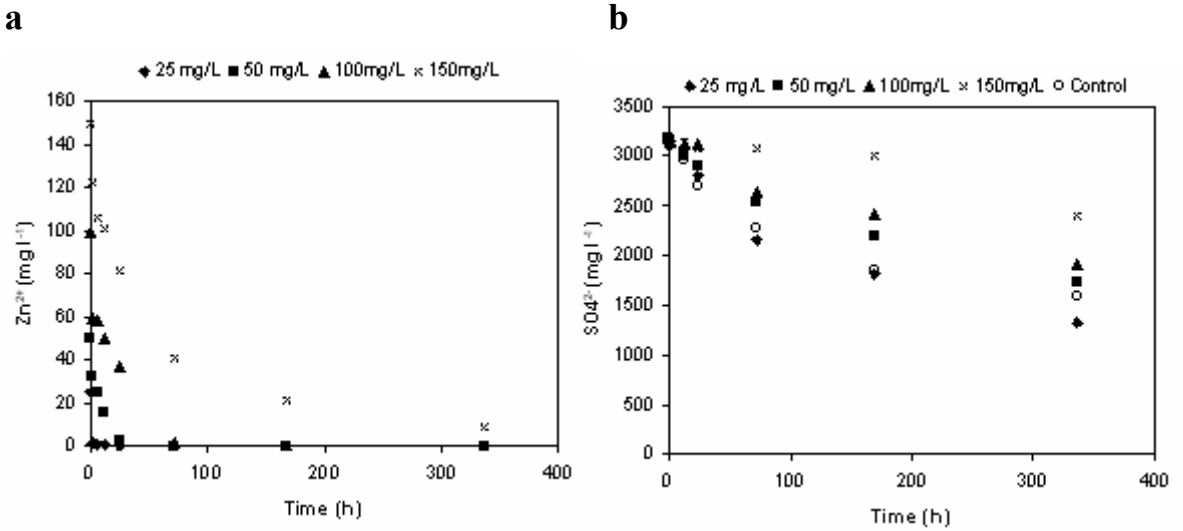

Fig. 2-5. (a) Residual concentrations of Zn^{2+} and (b) SO_4^{2-} by TX2 consortium in batch condition after 14 day-experimental period

a **b**

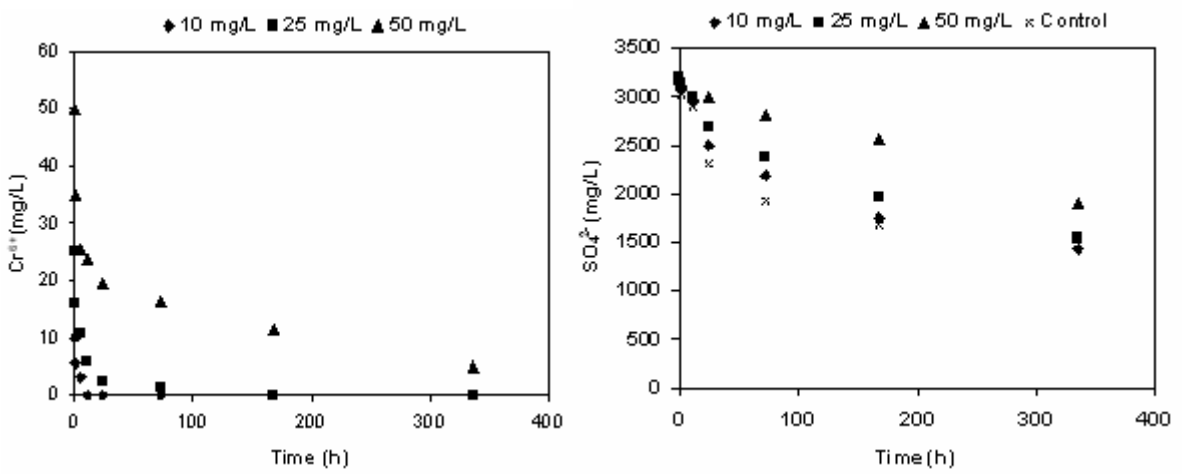

Fig. 2-6. (a) Residual concentrations of Cr^{6+} and (b) SO_4^{2-} by TX2 consortium in batch condition after 14 day-experimental period

Table 2-4. Removal efficiencies (%) of Cu^{2+}, Zn^{2+}, Ni^{2+}, and Cr^{6+} by TX2 consortium in batch condition after 14 days

Initial concentration (mg L^{-1})		**Removal efficiency (%)**
Cu^{2+}	10	100
	25	100
	50	100
	100	60
Zn^{2+}	25	100
	50	100
	100	100
	150	94
Ni^{2+}	25	100
	50	100
	100	28
	150	13
Cr^{6+}	10	100
	25	100
	50	90

3.3. Semi-continuous experiments

The obtained results showed that Cu^{2+}, Zn^{2+}, Ni^{2+}, and Cr^{6+} were removed effectively in R1 (1.5 mg L^{-1} d^{-1}), R2 (3 mg L^{-1} d^{-1}), and R3 (4.5 mg L^{-1} d^{-1}) throughout the experiment. The behavior of the sulfidogenic consortium in these three reactors was similar and marked by high levels of heavy metal removal, sulfate reduction, and sulfide production. However, the inhibition of the sulfidogenic consortium began to be detected in R4 (6 mg L^{-1} d^{-1}) from week 9 onwards. A toxic effect on the sulfidogenic consortium was observed in R5 (7.5 mg L^{-1} d^{-1}). No growth of SRB and almost no heavy metal precipitation were detected after four weeks of the experiment. Therefore, the results of sulfate reduction, sulfide production and heavy metal removal obtained from R2, R4, and R5 were shown representatively in Fig. 2-7 - Fig. 2-9.

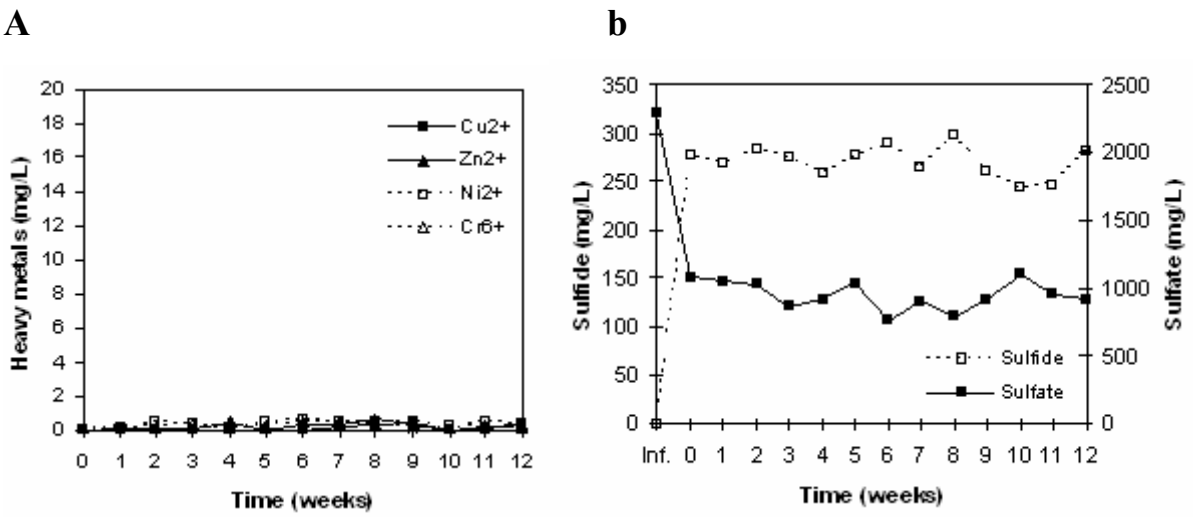

Fig. 2-7. (a) Concentrations of heavy metals (Cu^{2+}, Zn^{2+}, Ni^{2+}, and Cr^{6+}) and (b) sulfate and sulfide in R2 with time

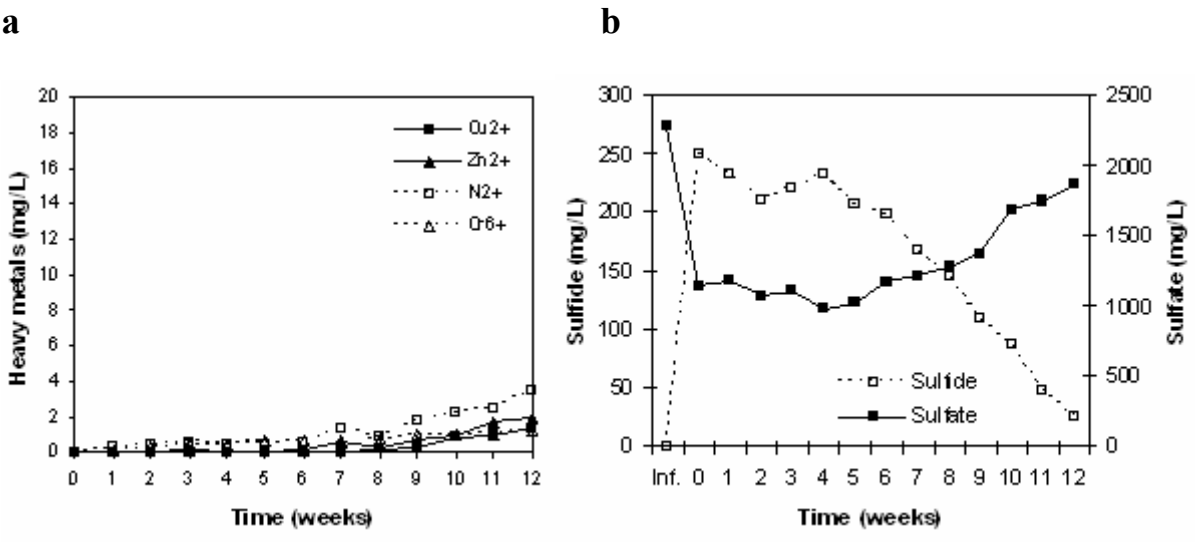

Fig. 2-8. (a) Concentrations of heavy metals (Cu^{2+}, Zn^{2+}, Ni^{2+}, and Cr^{6+}) and (b) sulfate and sulfide in R4 with time

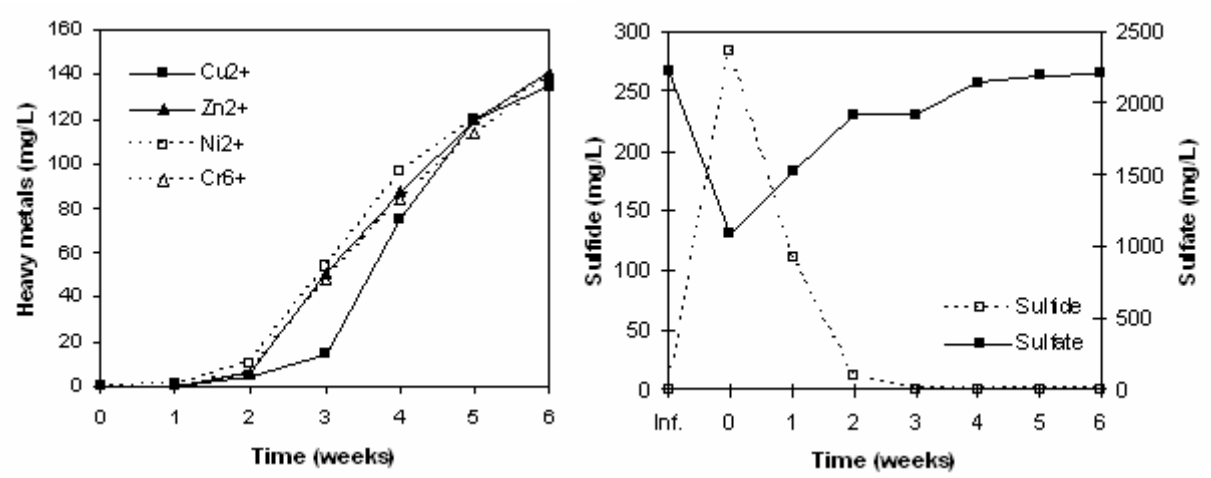

Fig. 2-9. (a) Concentrations of heavy metals (Cu^{2+}, Zn^{2+}, Ni^{2+}, and Cr^{6+}) and (b) sulfate and sulfide in R5 with time

3.3.1. Heavy metal removal

Heavy metal removal efficiencies of 96 - 100% for Cu^{2+}, 94 - 100% for Zn^{2+} and Ni^{2+}, and 96 - 100% for Cr^{6+} were achieved in R1 (1.5 mg L^{-1} d^{-1}), R2 (3 mg L^{-1} d^{-1}), and R3 (4.5 mg L^{-1} d^{-1}) during 12 weeks of operation. The residual heavy metal concentrations in R1-R3 throughout the experiment were low and ranged from < 0.2 to 0.74 mg L^{-1} of Cu^{2+}, < 0.05 to 1.8 mg L^{-1} of Zn^{2+}, < 0.2 to 1.5 mg L^{-1} of Ni^{2+}, and 0.06 to 0.7 of Cr^{6+} (Fig. 2-7a). Although Cu^{2+}, Zn^{2+}, Ni^{2+}, and Cr^{6+} were also removed effectively (98-100%) in R4 (6 mg L^{-1} d^{-1}) during the first 8 weeks, the decrease in heavy metal removal was observed from week 9 (91-

97%). The detected residual concentrations of Cu^{2+}, Zn^{2+}, Ni^{2+}, and Cr^{6+} in the effluent of R4 after 12 weeks were 1.3, 1.9, 3.5 and 1.25 mg L^{-1}, respectively (Fig. 2-8a). The removal of heavy metals in R5 (7.5 mg L^{-1} d^{-1}) was significantly lower than the 98-100% achieved during the first week to 78-91% at the second week and no heavy metal precipitation was detected in the effluent of R5 after four weeks of operation (Fig. 2-9a).

3.3.2. Effect of heavy metals on sulfate reduction and sulfide production

About 50% of the initial sulfate concentration (2250 ± 100 mg L^{-1}) was reduced and 280 ± 30 mg L^{-1} of dissolved sulfide was simultaneously produced at the start of semi-continuous operation (after 9 day pre-incubation). Sulfate was converted by about 43-67% of initial concentration to dissolved sulfide of 145-310 mg L^{-1} in R1-R3 (1.5-4.5 mg L^{-1} d^{-1}) throughout the experiment (Fig. 2-7b) and in R4 (6 mg L^{-1} d^{-1}) during the fist 8 weeks of experiment. However, only 17-39% of initial sulfate concentration was reduced in R4 from week 9 onwards (Fig. 2-8b). A gradual decrease of dissolved sulfide was also observed in the latter phase of R4 with the concentration of 110 mg L^{-1} at week 9 towards the end of experiment (after 12 weeks) with the low concentration of 26 mg L^{-1} (Fig. 2-8b). Only 33% of the initial sulfate concentration was reduced in R5 (7.5 mg L^{-1} d^{-1}) at the first week and this value significantly decreased between weeks 2 (15%) and 6 (2%). The corresponding concentration of dissolved sulfide at the first week in R5 was 111 mg l^{-1}, but no dissolved sulfide was detected from the third week onwards (Fig. 2-9b). The results indicated that the efficiency of sulfate reduction and sulfide production greatly decreased with the increase of heavy metal loading rate up to 6 (R4) and 7.5 mg L^{-1} d^{-1} (R5).

3.3.3. Sulfur balance

A sulfur balance was estimated from an average value of the obtained results of R2 (3 mg L^{-1} d^{-1}) throughout 12 weeks of operation. The calculations were based on elemental sulfur (S) by assuming that the total initial sulfur as sulfate (SO_4^{2-}) (~ 762 mg L^{-1}) was converted partly to total dissolved sulfide (S^{2-} + HS^- + H_2S). Therefore, the total final sulfur exiting in R2 effluent includes unconsumed sulfate (~ 305 mg L^{-1}) and generated sulfide (~ 269 mg L^{-1}). The percent ratio of total final sulfur to total initial sulfur was only 75%. The difference between total initial and final sulfur might be explained as follows: (i) The loss of ~ 48 mg L^{-1} (~ 6.2%) of sulfur for the precipitation of Cu^{2+}, Zn^{2+}, Ni^{2+}, and Cr^{6+} in the R2 (ii) The loss of sulfur as volatile sulfide through air oxidation in transferring samples and diffusion of H_2S gas.

3.3.4. Effect of heavy metals on sulfate-reducing bacteria population

The hybridized positive cells detected by FISH using the specific probe for SRB (SRB385) and total DAPI stained cells of the enriched sulfidogenic consortium were estimated in all five reactors. However, only three representative reactors (R2, R4, and R5) are shown in Fig. 2-10.

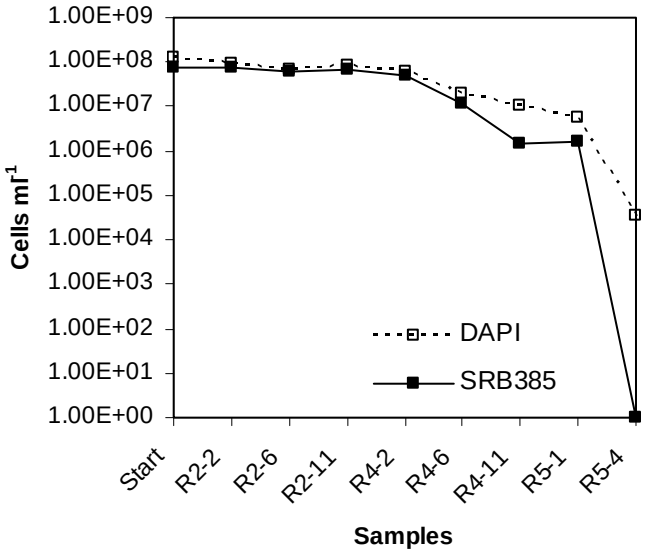

Fig. 2-10. Relative number of positive hybridized cells detected by specific probe for sulfate-reducing bacteria (SRB385) and total DAPI-stained cells in three representative anaerobic semi-continuous bioreactors (referred as R2, R4, and R5) with heavy metal (Cu^{2+}, Ni^{2+}, Zn^{2+}, and Cr^{6+}) loading rate of 3, 6, and 7.5 mg L^{-1} d^{-1}, respectively. Samples of R2 and R4 were analyzed after -2, -6, and -11 weeks and of R5 after -1 and -4 weeks of operation. Cell counts are on a logarithmic scale.

The abundance ratio of SRB detected by FISH to total cell counts at the start were quite similar in R1 (1.5 mg L^{-1} d^{-1}) with 58% (of 1.3×10^8 cell mL^{-1}) and 59% (of 1.0×10^8 to 1.3×10^8), respectively. The abundance ratio of SRB increased from 58% (of 1.3×10^8 cell mL^{-1}) at the start to 83-84% (of 6.9×10^7 to 9.2×10^7 cell mL^{-1}) in R2 (3 mg L^{-1} d^{-1}) and to 83-86% (of 3.9×10^7 to 7.4×10^7 cell mL^{-1}) in R3 (4.5 mg L^{-1} d^{-1}) throughout the experiment. A gradual decrease of SRB abundance was observed in R4 (6 mg l^{-1} d^{-1}) from 58% at the start to 63% (of 1.9×10^7 cell mL^{-1}) after 6 weeks and 14% (of 1.0×10^6 cell mL^{-1}) after 11 weeks of operation. The SRB abundance significantly decreased in R5 (7.5 mg L^{-1} d^{-1}) from 58% at the start to 30% (of 5.4×10^6 cell mL^{-1}) after one week; no positive hybridized cells was detected by specific probe for SRB (SRB385) after four weeks of operation. In addition, a distinct biomass loss through the significant decrease of the total cell counts from 1.3×10^8 cell mL^{-1}

at the start to 3.5 × 10^4 cell mL^{-1} after four weeks of experiment was observed in R5. FISH results are consistent with the obtained results of sulfate reduction, dissolve sulfide production and heavy metal removal, indicated that SRB played a key role in heavy metal removal.

3.3.5. Effect of heavy metals on pH value

The effects of heavy metals on the sulfidogenic consortium were also observed by the change of pH value (Fig. 2-11). The pH increased from an initial value of 6 in the influent to more than 7 at the start of semi-continuous operation (after 9 day pre-incubation) and in the effluent of R1-R3 (1.5-4.5 mg L^{-1} d^{-1}) throughout the experiment as well as in the effluent of R4 (6 mg L^{-1} d^{-1}) during the first seven weeks. However, the effluent pH of R4 dropped gradually to between 6.91 and 6.58 towards the end of the experiment. The effluent pHs of R5 (7.5 mg L^{-1} d^{-1}) were more than 7 at only during the first two weeks and dropped gradually to 6.88 after three weeks and to 6.55 after six weeks of operation.

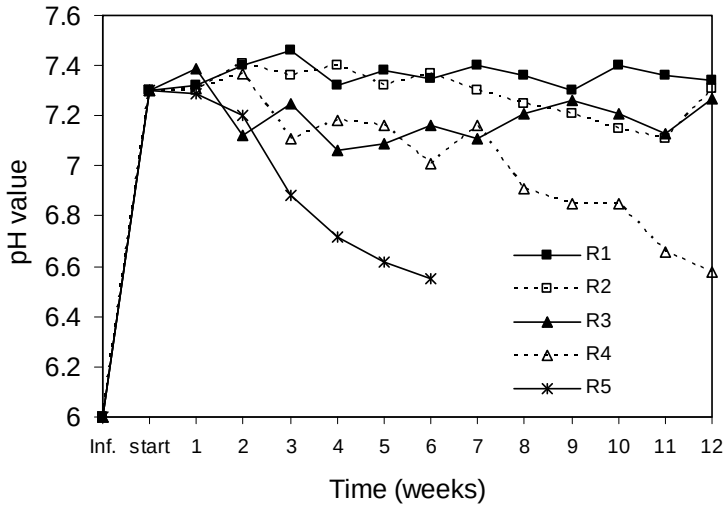

Fig. 2-11. The changes of pH value in the effluent of R1-R5 with time.

3.3.6. Qualitative EDS analysis

Qualitative EDS analysis of the precipitate of R2 (3 mg L^{-1} d^{-1}) experiment was performed. The precipitates as CuS, ZnS, and NiS were confirmed by the EDS spectrum with strong peaks of copper, zinc, nickel and sulfur shown in Fig. 2-12.

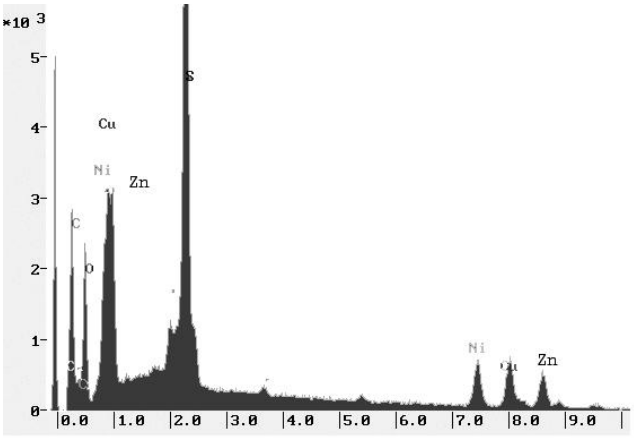

Fig. 2-12. EDS spectrum of the precipitate of R2 experiment

4. Discussion

The impact of heavy metals on microorganisms is classified as toxic (causing death) and/or inhibitory (causing a reduction in metabolic activity). Toxic concentration is the lowest initial dissolved metal concentrations at which no bacterial growth is observed. The obtained results of semi-continuous experiment in this study showed that heavy metal mixtures (Cu^{2+}, Zn^{2+}, Ni^{2+} and Cr^{6+}) were removed effectively (94-100%) without any inhibition to the growth of SRB throughout the experiment in R1-R3 with heavy metal loading rate ranging from 1.5 - 4.5 mg L^{-1} d^{-1}. However, SRB abundance and heavy metal removal began to decrease at heavy loading rate of 6 mg L^{-1} d^{-1}after 8 weeks of experiment (91-97%). A toxic effect on the growth of SRB that resulted in failure of hybridized positive cell detection and heavy metal precipitation was observed at a heavy metal loading rate of 7.5 mg L^{-1} d^{-1} (R5). The heavy metal loading rate of 6 (R4) and 7.5 mg L^{-1} d^{-1} (R5) therefore are considered to be the inhibitory and toxic concentrations to the sulfidogenic consortium in the present study, respectively. The toxic concentrations of heavy metals to single SRB species or SRB consortia ranging from a few mg L^{-1} to as much as one hundred mg L^{-1} were reported by other authors. A Cu^{2+} concentration of 1.92 mg L^{-1} causing death of organism was shown by Sani et al. (28) who used a single SRB species (*Desulfovibrio desulfuricans*) and a specific medium to prevent metal ions from abiotic precipitation. Lack of growth of SRB consortium at 12 mg L^{-1} of Cu^{2+} and 20 mg L^{-1} of Zn^{2+} was shown by Utgikar et al. (31). Hao et al. (16) reported that the toxic concentrations of individual heavy metal for SRB consortium in batch test were 20 mg L^{-1} Cd^{2+}, 20 mg L^{-1} Cu^{2+}, 20 mg L^{-1} Ni^{2+}, 25 mg L^{-1} Zn^{2+}, and 60 mg L^{-1} Cr^{3+}, 75 mg L^{-1} Pb^{2+}, but only 10 mg L^{-1} heavy metal mixture. Azabou et al. (4) who studied heavy metal inhibition and precipitation by a mixture of SRB in batch condition showed that

more than 72 mg L^{-1} of Zn^{2+} caused death of organisms. The results obtained from different studies were not similar, suggesting that the toxic and inhibitory effect of heavy metals on SRB cultures are influenced by many factors such as the chemical and physiochemical properties of the surrounding SRB environment and the species composition of microbial community. Moreover, the simultaneous presence of heavy metals could induce synergistic or cumulative toxic effects. Utgikar et al. (30) reported that the toxic effects of binary mixtures of Cu and Zn were significantly higher than the toxic effect of individual heavy metal. This was also demonstrated by the study of Hao et al. (16) mentioned above. The removal efficiency of the heavy metals in the present study is relatively high. 94-100% of about 10-30 mg L^{-1} heavy metal mixture (Cu^{2+}, Zn^{2+}, Ni^{2+} and Cr^{6+}) was removed by sulfidogenic consortium weekly throughout 12- week operational period in semi-continuous stirred tank reactors. The obtained results might be due to the use of an indigenous sulfidogenic consortium isolated from heavy metal contaminated sediment, resulting in a high heavy metal tolerance and removal. Possible explanations for why the use of indigenous consortia may be more advantageous than the use of single species could be: (i) indigenous consortia containing multi-species have adapted to a heavy metal polluted environment by developing a variety of resistance mechanisms (see introduction). They are less liable to mutate and to be contaminated from other microorganisms, and (ii) they contain more than one kind of organism that facilitate the formation of reducing conditions by completely oxidizing completely carbon sources (7). In addition, it is difficult to maintain culture purity due to the ubiquity of microorganisms in the environment. Therefore, using a consortium instead of single species is an optimum option and widely applied for heavy metal treatment bioreactors.

An increase of heavy metal loading rate of up to 6 and 7.5 mg L^{-1} d^{-1} in this study resulted in a decrease or even no detection of hybridized positive cells, sulfate reduction, sulfide production, and heavy metal precipitation. Contrary to common belief that only soluble metallic ion can be toxic or inhibitory, the insoluble metallic compounds, especially metal sulfides, could affect the activity of SRB by deposition on the surface of the cells and blocking the access to the substrate and other nutrients (32). At low levels of sulfide precipitate, the bacteria themselves may directly accelerate metal sulfide precipitation and facilitate settling of the solids by binding the metal in their cell walls and extracellular polymeric substances (EPS) (6). This was demonstrated by Jalali (17) and Azabou et al. (3), who evaluated the influence on copper and zinc removal, respectively. The precipitation of copper and zinc was detected more quickly in the presence of bacteria cells than without bacteria cells in these both studies. Thus, association of copper and zinc with the bacterial cells could promote the precipitation rate. Although the presence of bacterial cells may facilitate metal precipitation, the high level of sulfide precipitate can act as a barrier between

the cells and their essential growth nutrients. Therefore, the influent with heavy metal concentrations below the inhibitory level to maintain a maximum rate of sulphidogenesis are required to have a successful operation for heavy metal sulfidogenic bioreactors.

Copper removal was faster and more efficient than zinc and nickel. Especially, this was revealed clearly in batch experiment (Fig. 2-3). The difference in removal of these heavy metals can be explained by the solubility products of CuS, ZnS, and NiS, which are respectively 4×10^{-38}, 4.5×10^{-24}, and 3×10^{-21} mol L^{-1} (12). As mentioned in the introduction, soluble Cr^{6+} can be reduced into much less toxic and insoluble Cr^{3+} by reacting with bacterially produced hydrogen sulfide (9, 24). However, precipitation of Cr as metal sulfide is not stable in aqueous medium in comparison with Cu, Zn, and Ni. Thus, the reduction to Cr^{3+} is likely to be followed by rapid deposition as hydroxides with the concentration of soluble Cr^{3+} in equilibrium with $Cr(OH)_3$ is ~ 6.3×10^{-31} mol L^{-1} (11).

Metal sulfides have been attributed to the major precipitation of Cu, Zn, and Ni by biological activity of SRB in the present study. This is confirmed by a simultaneous decrease of sulfate and heavy metal concentrations in the effluent and EDS spectrum with strong peaks of copper, zinc, nickel and sulfur. This is in agreement with Azabou et al. (3), who showed that zinc removal was possible only in the presence of sulfate. This suggests that sulfide produced from sulfate reduction by activity of SRB is responsible for zinc removal as ZnS. However, in addition to precipitation with sulfide, heavy metals may also have been removed through sorption to the biomass or by other precipitation mechanisms i.e., hydroxide and carbonate precipitation, as well as generated alkalinity (13, 24). However, sulfide precipitation is the dominant mechanism, whereas other mechanisms play only a minor role for the removal of heavy metals in anaerobic sulfidogenic bioreactors (21, 26).

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CHAPTER 3

Application of different biomolecular techniques for assessing the effect of heavy metals on microbial community structure in a heavy metal tolerant sulfidogenic consortium

1. Introduction

Wastewater from mining and industrial processing (e.g. metallurgical, electronic, electroplating and metal finishing industries) normally contains high concentrations of heavy metals. Therefore, immobilization of heavy metals through microbial mediated reduction and precipitation is now of considerable interest. Especially, metal sulfide precipitation by sulfate-reducing bacteria (SRB) has become an attractive research field in recent years. Under anaerobic conditions, SRB oxidize simple organic compounds by utilizing sulfate as an electron acceptor and generate hydrogen sulfide and alkalinity. This hydrogen sulfide reacts with dissolved divalent metal ion, such as Fe^{2+}, Ni^{2+}, Cu^{2+}, and Zn^{2+} to form heavy metal sulfides (20), while SRB reduce Cr^{6+} indirectly by production of hydrogen sulfide (7, 41). The activity of the SRB results in a decrease of sulfate and heavy metal concentrations and an increase of alkalinity due to the production of bicarbonate (20). The potential advantages of heavy metal sulfide precipitation include the production of denser sludge, lower sludge volume and lower solubility products as compared to hydroxide precipitation produced in classical chemical treatment processes. Moreover, valuable metals from biologically precipitated metal sulfide can be recovered and recycled (6, 19).

Although the mechanism of anaerobic heavy metal bioremediation by SRB is well understood, relatively little is still known about changes in microbial community structure and abundance after environmental perturbation with toxic substances, such as heavy metals (16).

Recent developments in biomolecular approaches, such as cloning and sequence analysis (24), denaturing gradient gel electrophoresis (DGGE) (31), fluorescent *in situ* hybridization (FISH) (3, 35), quantitative real-time polymerase chain reaction (qPCR) (48, 56) and microarrays (29) have opened up new perspectives for the study of microbial communities. These techniques enable researchers to overcome the problems that are difficult challenges through use of conventional microbiological techniques based on isolation of pure cultures

and morphological, metabolic, and biochemical analyses (39). To minimize the limitations of each method, only a combination of different biomolecular techniques can help us to obtain a better understanding of the microbial community in anaerobic bioreactors that has been often been treated as a 'black box' (49). Knowledge of bacterial composition in sulfidogenic consortia and chemical parameters relevant for the heavy metal wastewater treatment process, such as sulfide, sulfate, heavy metal concentration and pH, might help for the better control of operation and performance improvements of sulfidogenic reactors (40, 49).

So far, most studies have focused on the diversity of microbial communities from heavy metal-contaminated sites (8, 21, 32) or toxic effect of heavy metals on pure cultures (47, 44). Studies of the effect of combined heavy metals in different concentrations on microbial community structure and abundance of indigenous sulfidogenic consortia are limited (24, 45, 46), and most lack information about the quantitative *in situ* abundance of microbial populations.

The objective of the study presented in this chapter was to investigate the effect of Cu^{2+}, Ni^{2+}, Zn^{2+}, and Cr^{6+} on the structure of a sulfidogenic consortium originated from a heavy metal contaminated sediment from Vietnam. A combination of different biomolecular approaches, such as cloning and sequencing of 16S rRNA gene, DGGE of 16S rRNA and functional gene-*dsrB* encoding dissimilatory sulfite reductase and FISH was used to analyze the microbial populations from four anaerobic semi-continuous bioreactors operated with different heavy metal concentrations throughout 12 weeks of operation.

2. Materials and Methods

2.1. Samples

Inoculum

A highest heavy metal tolerant sulfidogenic consortium (TX2) selected by screening tests in test tubes (chapter 2 and Table 2-3) was applied for semi-continuous experiment as an inoculum. To enrich the bacteria number the cultivation step was repeated three times before inoculating the bioreactors. A part of this enriched sulfidogenic consortium was collected and immediately frozen at -20^{o}C for 16S rDNA cloning analysis.

Reactor samples

The experiment was carried out in four anaerobic semi-continuous stirred tank reactors (CSTR, V = 2 L) (referred as R1, R2, R3, and R5) operated with a hydraulic retention time (HRT) of 20 days (Fig. 2-2). The bioreactors were first inoculated with 10% (v/v) of the enriched sulfidogenic consortium (see inoculum) containing 1×10^8 cells mL^{-1} and incubated

for 9 days at batch operating condition using synthetic wastewater without heavy metals. After pre-incubation (start of the experiment), the reactors were operated semi-continuously for 12 weeks and fed with synthetic wastewater containing 26 mM sulfate and 39.5 mM sodium lactate as electron acceptor and donor, respectively, and the desired concentrations of heavy metals. Reactor 1 (R1) was spiked with a mixture of Cu^{2+}, Ni^{2+}, Zn^{2+}, and Cr^{6+} in loading rate of 1.5 mg L^{-1} d^{-1} each. The loading rate of each metal was increased up to 3, 4.5, and 7.5 mg L^{-1} d^{-1} for reactor 2 (R2), 3 (R3), and 5 (R5), respectively (Table 2-2). Samples of R1, R2 and R3 were collected at the start, after two, six, and 11 weeks and of R5 at the start, after one and four weeks for DGGE and FISH analysis. Samples used for FISH were immediately fixed with 4% (w/v) paraformaldehyde (PFA) and stored at -20^{o}C until further analysis. For PCR-DGGE, samples were frozen at -20^{o}C.

2.2. DNA extraction

Genomic DNA were extracted from inoculum and reactor samples by using Fast DNA SPIN kit for soil (MP biomedical, Heidelberg, Germany) according to the manufacturer's instructions. The extracted DNA was diluted to an adequate concentration with sterile water for subsequent PCR reactions to minimize the formation of hetroduplex molecules and stored at -20 ^{o}C until further use.

2.3. PCR amplification of 16S rRNA and *dsrB* genes

To amplify the nearly complete 16S rRNA gene for cloning analysis, a universal primer set of 27F (5' AGA GTT TAG TCC TGG CTC AG 3') and 1492R (5' GGT TAC CTT GTT ACG ACT T 3') for domain the *Bacteria* was used (23). Vector primers M13F (5' GTA AAA CGA CGG CCA G 3') and M13R (5' CAG GAA ACA GCT ATG AC 3') were used for amplification of the clone inserts.

For DGGE analysis, a 350 bp-fragment of dissimilatory sulfite reductase (*dsrB*) gene and a 550 bp-fragment of 16S rRNA gene of the domain *Bacteria* were amplified by using two sets of primer as follows: DSRp2060F (5' CAA CAT CGT YCA CCA GGG 3') (17, 33)/DSR4R (5' GTG TAG CAG TTA CCG CA 3') (47) and 341F (5' CCT ACG GGA GGC AGC AG 3') (23)/907R (5' CCG TAC ATT CCT TTR AGT TT 3') (23) , respectively (primers were purchased from Eurofins MWG Operon, Ebersberg, Germany). A 40-base GC clamp (GCCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG) was attached to the end 5' of each forward primer to obtain a stable melting behavior of the DNA fragments in the DGGE (31).

PCR amplification was carried out in a Primus 96 advanced Thermocycler (Peqlab Biotechnologie GmbH, Erlangen, Germany). The reaction mixture (50 µl) contained 25 µl of

a HotStartTag Master Mix (2.5 units HotstarTag DNA Polymerase; 15 mM $MgCl_2$ and 200 µM of each dNTP, QIAGEN GmbH, Hilden, Germany), 0.3 µM of each primer and 10-50 ng of template DNA, RNase-free water added to the final volume of 50 µl reaction.

The following cycling program was used (i) for the 16S rDNA primer pair 27F and 1492R, and (ii) the vector primers M13F and M13R: Initial heat activation of HotstarTag at 95^{o}C for 15 min, 35 cycles of denaturation at 95^{o}C for 45 s, primer annealing at 53^{o}C for 45 s and primer extension at 72^{o}C for 90 s, and a final extension at 72^{o}C for 10 min.

For DGGE, a "touchdown" PCR program (14, 31) was used for amplification of *dsrB* and 16S rRNA gene fragments to reduce the formation of spurious by-products and increase the specificity of the amplification. The annealing temperature was set 10^{o}C higher than the expected annealing (65^{o}C for DSRp2060F/DSR4R and 60^{o}C for 341F/907R), and decreased 1^{o}C every second cycle until a 'touch down' of 55 and 50^{o}C, respectively. The following cycling program was used for primer DSRp2060F/DSR4R: 95^{o}C for 15 min, followed by 20 cycles (95^{o}C for 45 s; a 'touchdown'-annealing step: 65^{o}C to 55^{o}C for 30 s; 72^{o}C for 30 s), another 15 cycles (annealing: 55^{o}C), 72^{o}C for 20 min; for primer 341F/907R: 95^{o}C for 15 min, followed by 20 cycles (94^{o}C for 1 min; a 'touchdown'-annealing step: 60^{o}C to 50^{o}C for 1 min; 72^{o}C for 1 min), another 15 cycles (annealing: 50^{o}C), 72^{o}C for 20 min.

The PCR products of 16S rRNA and *dsrB* gene fragments were purified with Qiaquick PCR purification kit (QIAGEN GmbH, Hilden, Germany) after checking by agarose electrophoresis in Tris-Acetate EDTA (TAE) buffer with Ethidium Bromide (EtBr) and then applied for cloning, DGGE, and sequencing.

2.4. Clone library analysis

To remove smearing, inappropriate bands, and primer-dimers, a distinct band of expected length (ca. 1500 bp) from the PCR product of DNA extracted from the inoculum encoding the 16S rRNA gene was excised from agarose gel. PCR clean-up Gel extraction kit (Macherey-Naglel GmbH and Co. KG, Germany) was used to extract the excised bands. The eluted PCR product was ligated into the pCR -®4-TOPO ® plasmid vector and transformed into competent *E. coli* TOP 10 cells using TOPO-TA cloning kit according to the manufacturer's instructions (Invitrogen, CA, USA). The colonies were randomly selected and examined for the presence of the correct insert size by PCR with primers M13F and M13R. Ninety positive clones were screened by amplified ribosomal DNA restriction analysis (ARDRA), using enzyme *Hae*III (GG↓CC, Fermentas, USA) according to the manufacturer's protocol. Clones were grouped according to their restriction patterns defining different operational taxonomic units (OTUs). Subsequently, four clones of each OTU were randomly selected for sequencing

with the vector-specific primers M13F and M13R (Eurofins MWG Operon, Ebersberg, Germany).

2.5. DGGE of *dsrB* and 16S rRNA gene fragments

DGGE was performed according to the manufacturer instructions of D Code system (Bio-Rad Laboratories, Hercules, CA, USA). After purification, about 300 ng of PCR products were applied to 6% (w/v) polyacrylamide (40% Acrylamide/Bisacrylamid (37:1) gels containing denaturant gradients of 30-60% (16S rRNA gene) and 8% (w/v) polyacrylamide gels containing denaturant gradients of 40-70% (dissimilatory sulfite reductase (*dsrB*) gene). Hundred percent denaturing solution contained 7 M urea and 40% formamide. Electrophoresis was run in 1 x TAE buffer (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA, pH 8) at 60^{o}C for 16 h at 100 V. The gels were stained for 30 min in 1 x TAE containing SYBR Gold nucleic acid stain (Molecular Probes, USA).

A DGGE marker was made by a mixture of PCR-amplified 16S rRNA gene products of uncultured clones (provided by E. Müller, Institute of Water Quality Control, Faculty of Civil Engineering and Geodesy, Technische Universität München, Germany) and pure strains from the German collection of microorganisms and cell cultures (DSMZ). Representative bands were excised, re-amplified using the corresponding primer pairs and sequenced (Eurofins MWG Operon, Ebersberg, Germany).

2.6. Phylogenetic analysis

Nucleotide sequences obtained from DGGE bands of 16S rRNA genes and functional *dsrB* genes as well as clone library were compared to public databases by using the program Basic Local Alignment Search Tool (BLAST) search program (2, NCBI http://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify the nearest phylogenetic neighbor sequences. For similarity, sequence data were aligned and analyzed with the ARB software package (26). Three sequences of 16S rRNA gene of the clones (VN_TX2-2, VN_TX2-12, and VN_TX2-19) and one sequence of *dsrB* genes (VN_TX2-5S) of DGGE band were deposited in the GenBank database (NCBI) under the accession number HQ108123, HQ108124, HQ108125, and HQ144227, respectively.

2.7. Total cell counts and Fluorescent *in situ* hybridization (FISH)

Total cell count using 4', 6' diamidino-2-phenylindole (DAPI) staining and FISH technique using different 16S rRNA-target oligonucleotide probes labeled at the 5' end with indocarbocyanine (Cy3) reactive fluorescent dye (Table 3-1) were performed using the

modified standard procedure described previously (3, 35). Oligonucleotide probes were purchased from Eurofins MWG Operon. The EUB338 mix represents equal concentration of probe EUB388, EUB338-II and EUB338-III. The probe NON338, which is complementary to probe EUB338, was used to determine nonspecific probe binding. Briefly, samples were fixed by 4 % (w/v) paraformaldehyde in phosphate-buffered saline (PBS) at 4^{o}C for 2 h. Samples were hybridized at 46^{o}C for 2 h in mixture of oligonucleotide probes and hybridization buffer with different formamide concentrations as described in Table 3-1. DAPI staining, drying and washing were carried out under standard conditions. Hybridized and DAPI stained cells were visually detected by using axioplan epifluorescence microscope (Carl Zeiss, Jena, Germany). For quantification a manual counting of individual bacteria cells was carried out. Ten to twenty microscopic fields were investigated to obtain (i) total cell counts per ml (DAPI positive cells) and (ii) percentages of positive hybridized cells by specific probe to total DAPI stained cells.

Table 3-1. rRNA-targeted oligonucleotide probes with corresponding specificity, target region, sequence and recommended formamide concentration (FA)

Probe	Specificity	Target [b]	Sequence (5'-3')	%FA[a]	Reference
EUB338	Domain *Bacteria*	388-355	GCT GCC TCC CGT AGG AGT	0-50	(4)
EUB338-II	*Planctomycetales*	388-355	GCA GCC ACC CGT AGG TGT		(11)
EUB338-III	*Verrucomicrobiales*	388-355	GCT GCC ACC CGT AGG TGT		(11)
EUB388 mix	Domain *Bacteria*	388-355			(4, 11)
NON338	Negative control	388-355	ACT CCT ACG GGA GGC AGC	0	(48)
Arch915	*Archaea*	915-934	GTG CTC CCC CGC CAA TTC CT	35	(42)
SRB385	SRB	385-402	CGG CGT CGC TGC GTC AGG	30	(4)
DSB129	*Desulfobacter*	129-146	CAG GCT TGA AGG CAG ATT	15	(13)
DBM221	*Desulfobacterium*	221-240	TGC GCG GAC TCA TCT TCA AA	35	(13)
DBB660	*Desulfobulbus*	660-679	GAA TTC CAC TTT CCC CTC TG	60	(13)
DSV1292	Some *Desulfovibrio*	1292-1309	CAA TCC GGA CTG GGA CGC	35	(28)

a. Percentage of formamide (FA) in hybridization buffer
b. The number corresponds to the *E. coli* position (5)

3. Results

3.1. Clone library analysis

A 16S rDNA clone library was constructed from the inoculum with the universal primer set 27F and 1492R for the domain *Bacteria* to investigate the microbial community structure.

A total of 90 clones containing the correct insert size were screened into four different OTUs based on *Hae*III restriction fragment banding patterns. Four representatives of each OTU were randomly selected for sequencing. The first OTU was composed of sixty-eight clone sequences (76% of the clone library, representative clone VN_TX2-2). The representative clone sequences of this OTU were closely related to *Desulfovibrio vulgaris* (AccNr. AF418179, DSM 644, 99% similarity) affiliated within the family *Desulfovibrionaceae* of the class *Deltaproteobacteria.* The representative clone sequences of the second and third OTUs were both affiliated within the family *Enterobacteriaceae* of the class *Gammaproteobacteria*, in which the second OTU (20 clones; 22% of the clone library) (representative clone VN_TX2-12) were closely related to *Citrobacter amalonaticus* (AccNr. AF025370, 99% similarity), and the third OTU (only one clone; 1% of the clone library) was related to *Klebsiella* sp. (AccNr. AY941831, 98% similarity). The fourth OTU was composed of one clone (1% of the clone library, representative clone VN_TX2-19) related to *Clostridium sartagoforme* (AccNr. NR026490, DSM 1292, 99% similarity).

3.2. DGGE of 16S rRNA and *dsrB* gene fragments

For large numbers of samples the cloning technique is not suitable. The analysis is too laborious and time consuming. DGGE is the more powerful tool for monitoring community behavior after the exposure to different dosages of toxic heavy metals. Therefore samples were collected from the four reactors at three different incubation times for DGGE analyses of the (i) 16S rRNA gene and (ii) the functional gene-*dsrB* (see 3.2.2).

3.2.1. DGGE analysis of 16S rRNA gene fragments

16S rRNA based DGGE was used to investigate the combined effect of Cu^{2+}, Ni^{2+}, Zn^{2+}, and Cr^{6+} on the changes of microbial community structure in the sulfidogenic consortium (Fig. 3-1). Four bands A, B, C and D representing a low bacterial diversity were detected in DGGE profiles not only at the start, but also in R1 (1.5 mg L^{-1} d^{-1}) during the 12- week experiment (Fig. 3-1, lanes 1, 2, 3, and 4). A total of four representative bands, denoted with arrows on the gel, were excised, re-amplified, purified and sequenced (Fig. 3-1, lane 1). The phylogenetic analysis of the partial sequence (550 bp) of the DGGE bands showed that the bands A, B, C and D were classified to different species *Pertrimonas sulfuriphila* (AccNr. AY570690, similarity 99%), *Clostridium* sp. (AccNr. EU862317, similarity 99%), *Clostridium indolis* (AccNr. NR026493, similarity 99%) and *Desulfovibrio vulgaris* (AccNr. AF418179, DSM 644, similarity 100%), respectively. Fig. 3-1 shows the position band D coincides with band no.7 of the DGGE marker corresponding to *Desulfovibrio vulgaris* (DSM type strain 644). The intensity of DGGE bands A and D indicated that *Pertrimonas*

sulfuriphila and *Desulfovibrio vulgaris* were dominant at the start and in R1, while *Clostridium* sp. and *Clostridium indolis* corresponding to bands B and C were not. A shift of microbial community structure was observed in R2 (3 mg L^{-1} d^{-1}), R3 (4.5 mg L^{-1} d^{-1}), and R5 (7.5 mg L^{-1} d^{-1}) in comparison with the start and R1 (1.5 mg L^{-1} d^{-1}). The bands B and C corresponding to *Clostridium* sp. and *Clostridium indolis* disappeared in R2, R3 and R5 throughout the experiment. In R2 after two weeks and R5 after one week of operation, both *Pertrimonas sulfuriphila* and *Desulfovibrio vulgaris* were present. However, the comparison of the signal intensity of the DGGE bands A and D indicates that *D. vulgaris* had become more dominant than *Pertrimonas sulfuriphila* (Fig. 3-1, lane 6). In contrast, in R2 after six and 11 weeks of operation and in R3 throughout the experiment only one distinct DGGE band (D) identified as *Desulfovibrio vulgaris* was visualized. The signal intensity of band D was similar in all reactors during the whole incubation with the exception of R5 after four weeks of operation. The band D detected weakly in R5 after four weeks indicating that the abundance of *Desulfovibrio vulgaris* was probably decreased. The DGGE profiles of all reactors demonstrated that the increase of heavy metal dosage had a great effect on the microbial community structure and composition of sulfidogenic consortium. Especially in R5 with a heavy metal loading rate of 7.5 mg L^{-1} d^{-1}, only one weak band corresponding to *D. vulgaris* was detected after four weeks of operation.

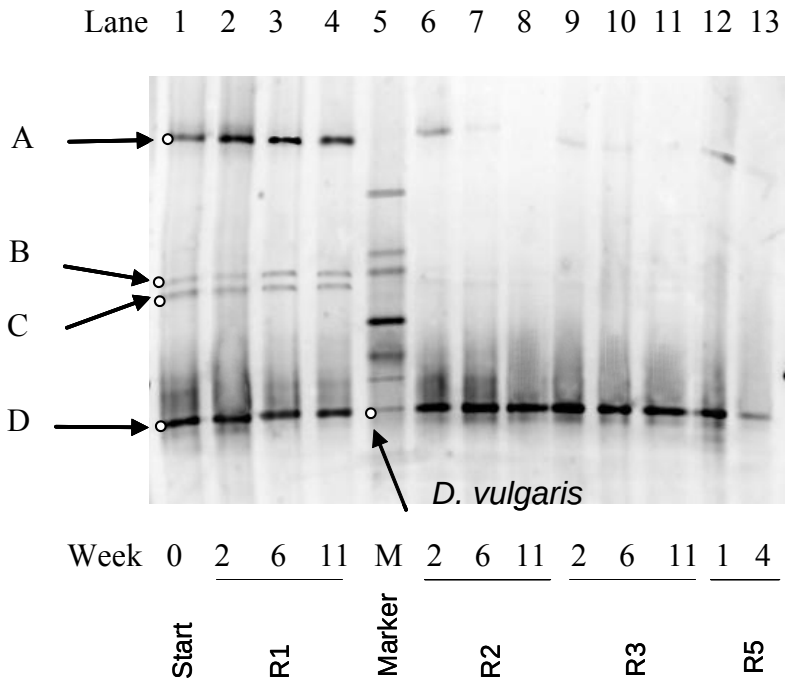

Fig. 3-1. DGGE band profiles of the samples exposed to the mixtures of Cu^{2+}, Ni^{2+}, Zn^{2+}, and Cr^{6+} taken from R1 (1.5 mg L^{-1} d^{-1}), R2 (3 mg L^{-1} d^{-1}), R3 (4.5 mg L^{-1} d^{-1}), and R5 (7.5 mg L^{-1} d^{-1}) during 12 week-operation. PCR products amplified with a primer set for domain *Bacteria*

GC-341F/907R. 16S rRNA gene fragments were analyzed on gels containing denaturant gradients of 30-60%. Marker (M), DGGE profile of 16S rRNA gene fragments from a DNA mixture of 5 pure strains and 2 clones, in which the last band indicated by white dots belonging to *Desulfovibrio vulgaris* (DSM 644). Bands indicated by A-D were excised and sequenced.

3.2.2. DGGE analysis of *dsrB* gene fragments

The functional *dsrB*-gene encodes β subunits of a key enzyme (dissimilatory sulfite reductase) that catalyzes the reduction of sulfite to sulfide by all dissimilatory sulfate-reducing prokaryotes (SRPs) (22, 47). *DsrB* was used as a molecular marker to rapidly assess the community composition of sulfate-reducing *Bacteria* and *Archaea* (22, 47, 29). With this approach it is possible to differentiate between SRPs at the genus and species level and might give more detailed information of sulfate-reducing communities (17). One sole band was visualized in all DGGE profiles (Fig. 3-2) verifying that SRB are really represented by only one species at the start and in all reactor samples. Three representative bands, marked by a white circle were excised and sequenced. The phylogenetic analysis of the partial sequence (350 bp) of the DGGE bands (representative VN_TX2-5S) showed that they all are related to *Desulfovibrio vulgaris* (AccNr. ABH06952, 99% similarity). The DGGE band signal intensity indicates that *Desulfovibrio vulgaris* was constantly dominant at the start, in R1 (1.5 mg L^{-1} d^{-1}), and also in the reactors containing higher dosages of heavy metals as R2 (3 mg L^{-1} d^{-1}), R3 (4.5 mg L^{-1} d^{-1}), from the beginning to the end of the experiment, and R5 (7.5 mg L^{-1} d^{-1}) after one week of operation. However after four weeks of operation, the population in R5 was characterized by a weak DGGE band signal of the *dsrB* gene which was in agreement with the results of 16S rRNA gene based DGGE.

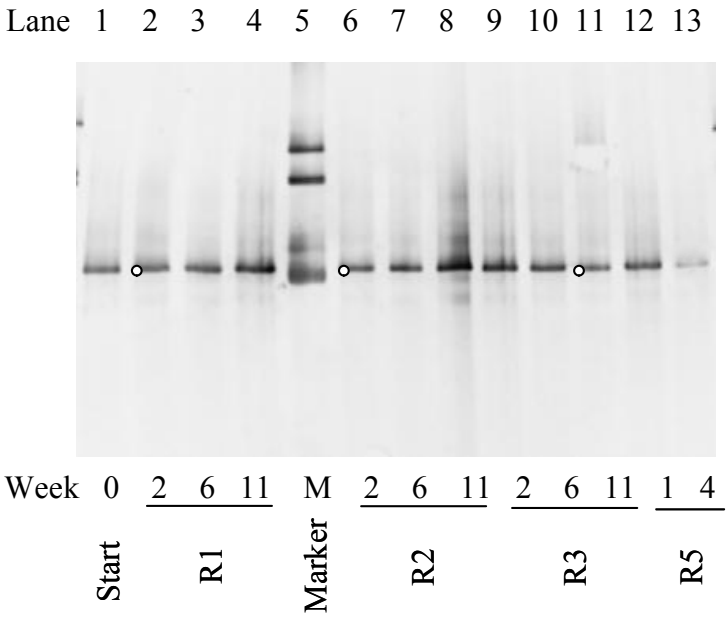

Fig. 3-2. DGGE band profiles of the samples exposed to the mixtures of Cu^{2+}, Ni^{2+}, Zn^{2+}, and Cr^{6+} taken from R1 (1.5 mg L^{-1} d^{-1}), R2 (3 mg L^{-1} d^{-1}), R3 (4.5 mg L^{-1} d^{-1}), and R5 (7.5 mg L^{-1} d^{-1}) during 12 week-operation. PCR products were amplified with a functional gene (*dsrB*) primer set for sulfate-reducing *Bacteria* and *Archaea*, GC-DSRp2060F/DSR4R. *DsrB* gene fragments were analyzed on gels containing denaturant gradients of 40-70%. Marker (M), 100bp-DNA ladder. Bands indicated by white dots were excised and sequenced.

3.3. Total cell counts and Fluorescent *in situ* hybridization (FISH)

The enriched sulfidogenic consortium used as inoculum for all reactors was first analyzed with probes (i) for domain *Bacteria* (EUB338 mix), (ii) *Archaea* (Arch915), (iii) sulfate-reducing bacteria [SRB385, four genus-specific probes; *Desulfobacterium* (DBM221), *Desulfobacte*r (DSB129), *Desulfobulbus* (DBB660), *Desulfovibrio* (DSV1292) (Table 3-1). Positive FISH signals were only detected with the probes EUB338 mix, SRB385, and DSV1292 in this consortium. Therefore, these three probes were applied to confirm *in situ* the presence and to quantify the abundances of the *Desulfovibrio vulgaris* identified by DGGE and cloning in the four reactors (R1, R2, R3, and R5). No unspecific hybridization was detected by probe NON338. The percent abundance of hybridized positive cells with the specific oligonucleotide probes EUB338 mix, SRB385, and DSV1292 to total DAPI stained cells are shown in Fig. 3-4. At the start, the relative percentages of positive cells that hybridized with EUB338 mix, SRB385 and DSV1292 to total DAPI counts (1.3×10^8 DAPI stained cells mL^{-1}) were 80%, 59% and 46%, respectively. In R1 (1.5 mg L^{-1} d^{-1}) after two, six and 11 weeks, probes EUB388 mix, SRB385 and DSV1292 detected 79 to 83%, 58 to

61%, and 47 to 49% of total cells (1.0×10^8 to 1.3×10^8 DAPI stained cells mL^{-1}). Probe DSV1292 and SRB385 gave strong positive hybridization signals and a high percentage of cells in R2 and R3 throughout the experiment (see Fig. 3-3, A and B). In R2 (3 mg L^{-1} d^{-1}) after two, six and 11 weeks, compared to total cells (6.9×10^7 to 9.2×10^7 DAPI stained cells mL^{-1}), the ratios were ranged from 85 to 88% of EUB338-positive cells, 78 to 86% of SRB385-positive cells and 71 to 83% of DSV1292-positive cells. In R3 (4.5 mg L^{-1} d^{-1}) after two, six and 11 weeks, EUB388 mix detected 87% to 89% of total cells (3.9×10^7 to 7.4×10^7 DAPI stained cells mL^{-1}), 84 to 86% and 80 to 82% of cells recognized by SRB385 and DSV1292, respectively (Fig. 3-4). In R5 (7.5 mg L^{-1} d^{-1}) after one week, EUB388 mix detected 50% of total cells (5.4×10^6 DAPI stained cells mL^{-1}) and only 30% of SRB385-positive cells and 22% of DSV1292-positive cells. In general FISH signal intensity was weaker in comparison with R2 and R3 (see Fig. 3-3, C and Fig. 3-4). No positive signal could be detected with EUB338-mix probes in R5 after the four-week- operational period. In addition a significant decrease of the total cell counts from 1.3×10^8 cell mL^{-1} at the start to 3.5×10^4 DAPI cells mL^{-1} after four weeks of operation was observed in R5 (Fig. 3-3, D and Fig. 3-4)

The results of FISH, consistent with 16S rRNA and *dsrB* gene based DGGE, indicated that *Desulfovibrio vulgaris* made up a significant part of the microbial community and represents the only SRB in R1, R2, and R3. The high percent abundance ratio of DSV1292 positive cells to total cells increased in R2 and R3; thereby implying that *Desulfovibrio vulgaris* was nearly a pure culture.

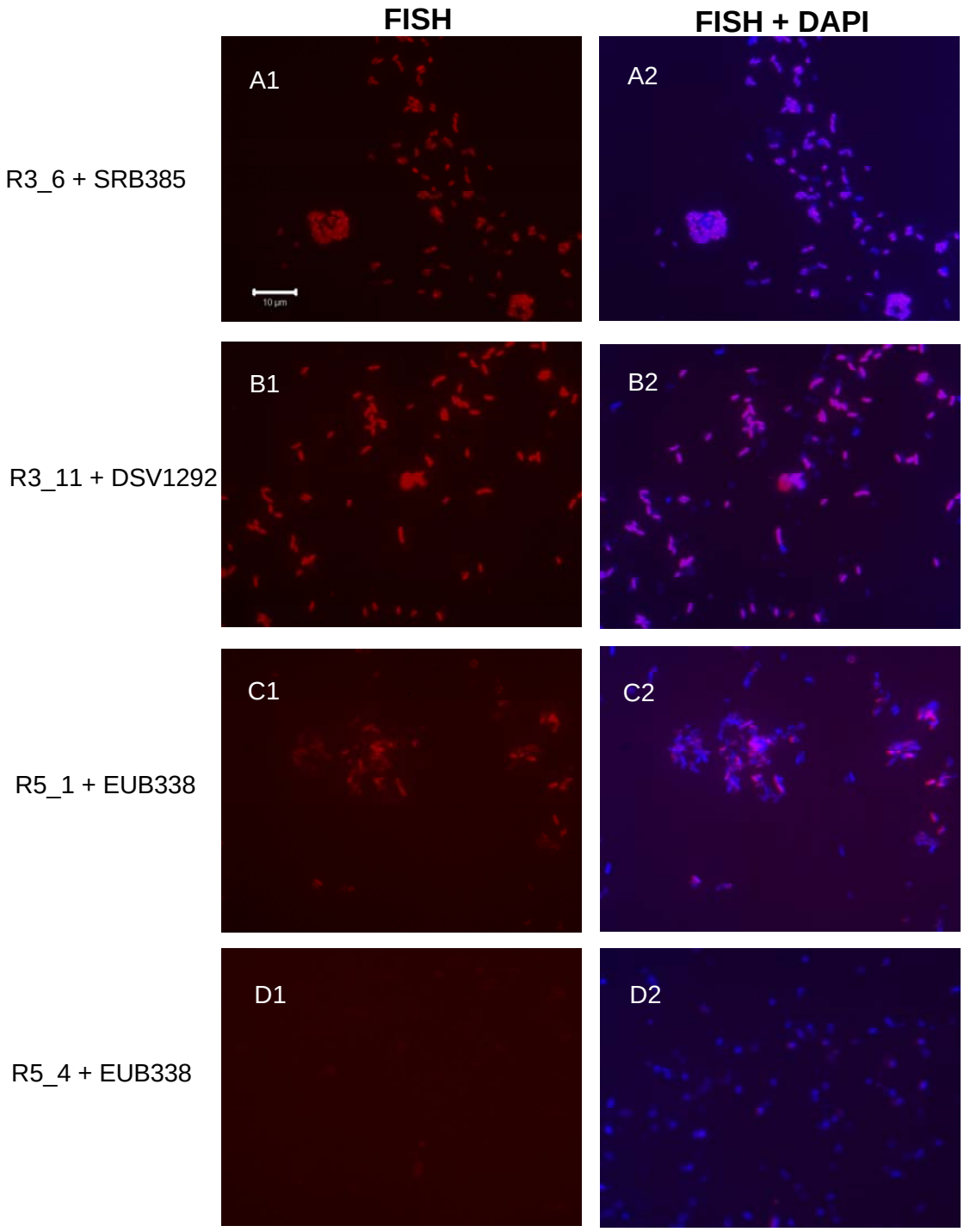

Fig. 3-3. Epifluorescence microscope images of bacteria populations in R3 and R5 with the influent heavy metal (Cu^{2+}, Ni^{2+}, Zn^{2+}, and Cr^{6+}) loading rates of each metal of 4.5 and 7.5 mg L^{-1} d^{-1}, respectively. FISH signal with specific probes labeled with Cy3 (red, left) and overlap of hybridized cells with Cy3 labeled probe and DAPI positive cells (magenta, right). A/ Hybridization with probe SRB385, specific for sulfate-reducing bacteria in R3 after six weeks; B/ Hybridization with probe DSV1292, specific for *Desulfovibrio* in R3 after 11 weeks; C/ Hybridization with probe EUB338 mix, specific for domain *Bacteria* in R5 after one week; D/ Hybridization with probe EUB338 mix, specific for domain *Bacteria* in R5 after four week; The scale bar (10 µm) applies to all of the images.

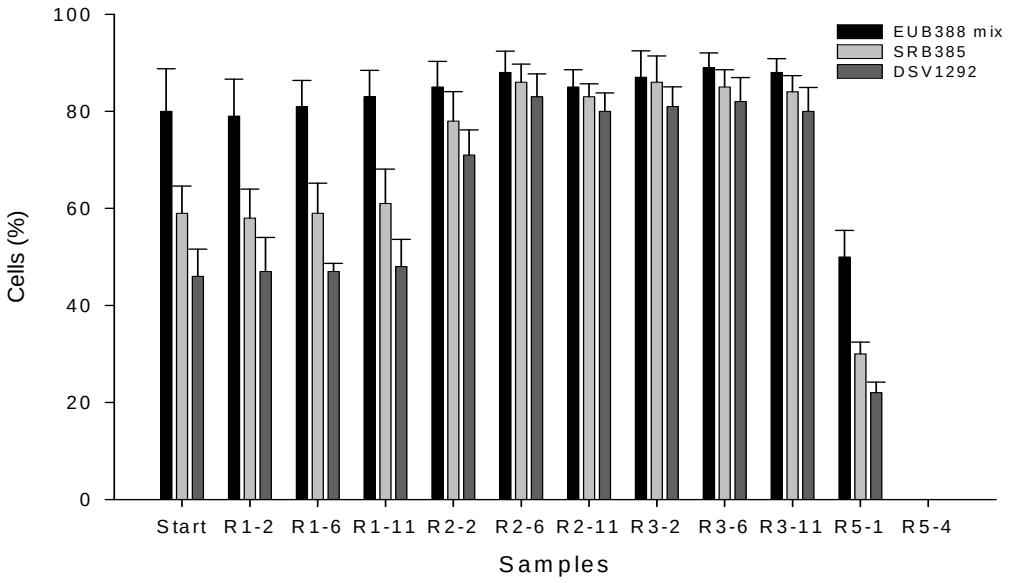

Fig. 3-4. Relative abundances (%) of positive hybridized cell detected by probes EUB388 mix (specific for domain *Bacteria*), SRB385 (specific for sulfate-reducing bacteria), and DSV1292 (specific for *Desulfovibrio*) to total DAPI-stained cells in four anaerobic semi-semi-continuous bioreactors (referred as R1, R2, R3, and R5). The reactors were operated with influent heavy metal (Cu^{2+}, Ni^{2+}, Zn^{2+}, and Cr^{6+}) loading rates of 1.5 mg L^{-1} d^{-1} (R1), 3 mg L^{-1} d^{-1} (R2) , 4.5 mg L^{-1} d^{-1} (R3), and 7.5 mg L^{-1} d^{-1} (R5) for each metal, respectively. Samples of R1, R2, and R3 were analyzed after -2, -6, and -11 weeks and of R5 after -1 and -four weeks of operation. Shown are mean values of 10 to 20 microscopic fields with corresponding standard deviations.

4. Discussion

The investigated heavy metal tolerant sulfidogenic consortium showed high efficiencies of heavy metal removal for Cu^{2+}, Ni^{2+}, Zn^{2+}, and Cr^{6+}, spiked with loading rates of 1.5, 3, and 4.5 mg L^{-1} d^{-1} each. The concentration of each metal was steadily reduced by 96-100% for Cu^{2+}, 94-100% for Zn^{2+} and Ni^{2+}, and 96-100% for Cr^{6+} during 12 weeks of operation. The removal efficiency of heavy metals with loading rate of 7.5 mg L^{-1} d^{-1} each of Cu^{2+}, Ni^{2+}, Zn^{2+}, and Cr^{6+} was significantly decreased from 98 - 100% during the first week and almost no heavy metal removal was observed at the fourth week.

The low bacterial diversity detected in the enriched inoculum as well as in the reactor start population resulted in a low microbial diversity in the reactor samples with one dominant SRB (*Desulfovibrio vulgaris*) and three other non-SRB species. This might be due to isolation from heavy metal-contaminated sediment by selective enrichment culture medium, and

consequently only few bacterial species might be present in the original consortium. The loading rate of 1.5 mg L^{-1} d^{-1} had no effect on the microbial community structure of the inoculated sulfidogenic consortium. In contrast, an increasing heavy metal loading rate from 1.5 mg L^{-1} d^{-1} to 3 or 4.5 mg L^{-1} d^{-1} caused the changes in microbial community structure, but the high heavy metal removal efficiencies (96 - 99%) were not influenced. *D. vulgaris* was present as the only species and a significant increase of its cell number at heavy metal loading rate of 3 and 4.5 mg L^{-1} d^{-1} was observed, whereas the disappearance of non-SRB species was most likely due to the toxic effect of high heavy metal concentrations. This indicated that *D.vulgaris* is the key population in heavy metal removal of the analyzed consortium. The cell number increased in comparison with the reactor start and consequently a stable biomass concentration was determined during the whole experiment. A toxic impact even on the growth of the key bacterial population (*D. vulgaris*) was observed at heavy metal loading rate of 7.5 mg L^{-1} d^{-1} resulting in the significant decrease of total cell numbers and cell activity indicating no bacteria could survive under this concentration. These findings were consistent with the decrease of heavy metal removal efficiencies after four weeks and almost no precipitation of heavy metals after six weeks exposure to heavy metal loading rate of 7.5 mg L^{-1} d^{-1}. Therefore, this concentration is extremely toxic to the growth of all bacteria even the key organism *D. vulgaris*.

Although *Desulfovibrio vulgaris* was the most abundant organism and played a key role in the heavy metal removal, information about the presence and potential role of non-SRB are necessary. The question arises whether the heavy metal tolerance and removal of a consortium is due to only one SRB species (e.g. *Desulfovibrio vulgaris*) or if other organisms in the sulfidogenic consortium moderated the toxicity of heavy metal by their metabolic products. In this study, the capacity for removal and tolerance of Cu^{2+}, Ni^{2+}, Zn^{2+}, and Cr^{6+} by *Pertrimonas sulfuriphil*, *Clostridium* sp., *Citrobacter amalonaticus*, and *Klebsiella* sp., has not been investigated in detail. However, the ability of heavy metal tolerance of these organisms was revealed by their presence in the DGGE profile at loading rate of 1.5 mg L^{-1} d^{-1}. In addition, the higher number detected with the specific probe for the domain *Bacteria* in comparison with the abundance of *D. vulgaris* verified that *D. vulgaris* was not the only organism present at 1.5 mg L^{-1} d^{-1} heavy metal loading rate. The tolerance of the described secondary population might be due to (i) the adaptation after long exposure time in heavy metal-contaminated sediment or (ii) heavy metal removal ability by an indirect process through metabolic production or by a direct enzymatic process. Recent studies have investigated heavy metal tolerance abilities of these organisms. For instance, Cunningham et al. (10) demonstrated that *Clostridium thermoaceticum* had the capacity to precipitate cadmium. Macaskie et al. (27) described enzymatically mediated bioprecipitation of uranium

by a *Citrobacter* sp. In addition, Qiu et al. (38) reported the effective precipitation of copper with the formation of copper sulfide by this species. The mechanism of cadmium precipitation utilized by *Clostridium thermoaaceticum* appears to be very similar to those of *Klebsiella aerogenes* (1), which utilize the reduction of sulfate to sulfide. To date, heavy metal resistance or reduction of *Pertrimonas sulfuriphil*, a mesophilic anaerobic fermentative bacterium (18), have not yet been documented and thus, require further investigation.

The activity of the described secondary bacterial population might also have a syntrophic relationship with sulfate-reducing bacteria (SRB). They might be initial degraders of macromolecules, providing easily degradable carbon sources as electron donors for SRB. Therefore, the knowledge of the microbial community composition of both SRB and non - SRB populations during heavy metal reduction facilitates the understanding of the microbial processes in bioreactors. This could be useful for designing sulfidogenic bioreactors or bioremediation strategies for heavy metal-contaminated sites, maintaining a stable prolonged reactor performance under fluctuating process conditions. Furthermore, the malfunctions may be detected at a very early stage of the removal process so that a complete collapse of anaerobic heavy metal treatment reactors can be avoided.

The application of different biological molecular tools to study microbial community structure is necessary in order to avoid the biases and limitations of each method. Cloning of full 16S rRNA gene provides more exact phylogenetic information than DGGE which is suitable for only short sequence fragments (200-600 bp) (40). However, cloning is a time consuming and laborious method and consequently it is unpractical for high sample throughput monitoring changes in microbial communities over time without cultivation. For this DGGE is a more powerful tool. Therefore the application of cloning was suitable to investigate the microbial community structure of the enriched inoculum. PCR-DGGE was the more favorable technique to monitor the community behavior in response to different heavy metal concentrations. The comparison of cloning and DGGE results showed that the bacteria species detected by DGGE identified as *Desulfovibrio vulgaris*, *Clostridium sp*., and *Clostridium indolis* coincides with the dominant clone species. However, one sequence presented in high number in the clone library (*Citrobacter amalonaticus*) was not detected by DGGE, which most likely is due to a change of the secondary population in the inoculum during the reactor performance.

16S rRNA gene based analysis now provides the most general framework for studies of natural microbial diversity including both domain *Bacteria* and *Archaea*. However, its limitation is that it does not provide a direct link to physiology. Thus, novel lineages of sulfate-reducing prokaryotes (SRPs) with distant relatives which share metabolic features

such as sulfate reduction can not be identified by rRNA analysis alone (33). Recent studies indicated that sequence analysis of dissimilatory sulfite reductase (*dsr*) genes that catalyzes the reduction of sulfite to sulfide (22) is successfully used for the detection of SRPs in different metal contaminated environment, such as Cu-Pb-Zn mine (32), and uranium tailing site (8). However, as mentioned above, beside sulfate reducers that play a key role in heavy metal removal the potential role of non-SRPs that are only detected by 16S rRNA analysis in the sulfidogenic consortium are necessary. They might not only involve tolerance and removal of heavy metals but also provide a syntrophic relationship with SRPs. In the present study, no *Archaea* was detected by both techniques PCR-DGGE and FISH using the functional *dsrB* gene and specific probe for *Archaea*. Therefore, a combination of universal primers for 16S rRNA gene specific for the domain *Bacteria* and specific primers for functional *dsr* genes of sulfate-reducing prokaryotes (SRPs) was effectively applied to precisely investigate the changes of these microbial communities.

Although the result of cloning and DGGE reflected the composition of microbial community and its changes over time they did not give information of the actual abundance of microbial populations. Both of these PCR-derived methods are not intended to be quantitative because of the influence of several pitfalls, such as DNA extraction efficiency for different bacterial species, and bias of PCR amplification (50). In contrast to cloning and DGGE, the FISH technique is able to quantify *in situ* the abundance of microbes with fluorescently labeled rRNA targeted oligonucleotide probes because there is no need for DNA extraction and PCR amplification steps (12). However, the main shortcoming of the FISH technique is the selection of suitable genus- and species-specific probes which might be involved in the process. Therefore, cloning and PCR-DGGE are helpful techniques to find the dominant organisms and to choose the precise probes for FISH as shown in the present study. FISH was used in this study to verify the presence of dominant SRB species *D. vulgaris* retrieved by cloning and DGGE and to simultaneously assess their role in heavy metal removal by *in situ* detection and quantification. Moreover, it is difficult to confirm if the obtained results from DGGE and cloning represented active heavy metal resistant species or their killed or dormant organisms because of the stability of DNA from dead or dormant cells in the samples. In contrast the FISH technique only detects actively growing organisms with a sufficient content of target molecules. Positive hybridized cells are metabolically active possessing large numbers of ribosomes including the target rRNA molecules (3, 9). The target rRNA molecule is degraded within hours of cell death and, therefore, it is assumed that only those cells which were alive during the sampling time will be detected. Moreover, inactive cells (e.g. spores) were characterized by a low cell wall permeability inhibiting the penetration of probes and consequently failed to be detected by FISH (3, 15). Nielsen et al.

(33) showed that only 35% of the microbial population was estimated by FISH in comparison with 75% based on DGGE band intensities in a deteriorated biological phosphorus removal reactor. For example, in this study, the results obtained by FISH at a heavy metal loading rate of 7.5 mg L^{-1} d^{-1} after four weeks were not in agreement with DGGE results. Despite the fact that no positive hybridized cells were detected by FISH, a weak band D was still observed in both DGGE gels of 16S rRNA and *dsrB* gene. Hence, DGGE can be used to short-cut the more laborious clone approach but it should always be verified by FISH. Overall, combining cloning, DGGE, and FISH are necessary to minimize the biases and errors of each method and to give a better understanding of how the bacterial community structure changes.

5. References

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Chapter 4

Conclusions and suggestions for future research

1. Conclusions

Removal of individual (in bath reactors) or mixtures of Cu^{2+}, Zn^{2+}, Ni^{2+}, and Cr^{6+} (in semi-continuous reactors) can be achieved effectively by the selected sulfidogenic consortium. The selection of the most suitable sulfidogenic consortium has been done by screening tests in test tubes among different sulfidogenic consortia isolated from heavy metal contaminated sites in Vietnam.

In batch experiment, the complete removal 100% of 50 mg L^{-1} Cu^{2+} and Ni^{2+} were observed in 72h and 336h, respectively. The efficiencies of removal were 100 and 90% after 168 and 336h respectively when 25 and 50 mg L^{-1} of Cr^{6+} were present initially. Higher removal efficiency was achieved in reactors spiked with Zn^{2+}, in which 100% of 100 mg L^{-1} and 94% of 150 mg L^{-1} Zn^{2+} were removed after 168 and 336h, respectively. Copper removal was faster and more efficient than zinc and nickel. Especially, this was revealed clearly in batch experiment. The difference in removal of these heavy metals can be explained by the solubility products of each metal.

The most important results with respect to an effective removal of heavy metal mixtures from synthetic wastewater have been achieved in semi-continuous operated stirred tank reactors at lab scale. These experiments have been accompanied by both chemical and molecular biological analyses.

Results obtained from chemical analysis showed that the effective removal (94-100%) of a heavy metal mixture (Cu^{2+}, Zn^{2+}, Ni^{2+}, and Cr^{6+}) can be achieved in semi-continuous operated reactors with heavy metal loading rates ranging from 1.5 to 4.5 mg L^{-1} d^{-1}. The reactors have been operated stable for a time period of 12 weeks. The dissolved heavy metal (Cu^{2+}, Zn^{2+}, Ni^{2+}, and Cr^{6+}) concentrations could be kept below 2 mg L^{-1}, which has to be regarded as unproblematic for the microorganisms. At a higher loading rate of 6 mg L^{-1} d^{-1} the heavy metal removal efficiency (91-97%) was reduced from week 9 onwards which indicated a disturbance of the involved micro organisms. The dissolved heavy metal concentrations quickly exceeded 2 mg L^{-1} within the last three weeks. No heavy metal

precipitation was detected at a heavy metal loading rate of 7.5 mg L^{-1} d^{-1} after four weeks of operation.

The effects of heavy metals on the sulfidogenic consortium were also observed by the change of pH value. The value of pH will affect the efficiency of sulfidogenic bioreactors, and a source of alkalinity obtained from sulfate reduction process may be necessary to raise the influent pH.

In this study, precipitation of Cu, Zn, and Ni as sulphides was the main removal mechanism. This is confirmed by a simultaneous decrease of sulfate and heavy metal concentrations in the effluent and by EDS analysis of the precipitate.

The combination of FISH, DGGE and cloning proved to be an effective tool to monitor the changes of microbial community structure in all five reactors operated continuously. FISH confirmed the results obtained by DGGE and cloning showed that the loading rate of 1.5 mg L^{-1} d^{-1} had no effect on the microbial community structure of the inoculated sulfidogenic consortium. Therefore, *Desulfovibrio. vulgaris*, *Pertrimonas sulfuriphila*, *Clostridium* sp., *Citrobacter amalonaticus* and *Klebsiella* sp. were also hypothesized as heavy metal tolerance and/or removal bacteria at loading rate of 1.5 mg L^{-1} d^{-1}. The changes in microbial community structure were observed at higher loading rate; 3 and 4.5 mg L^{-1} d^{-1}. *D. vulgaris* was present as the only species with a significant increase of its cell number and high heavy metal removal efficiencies (96-99%) were still achieved at these both loading rates. From these results it is concluded that *D. vulgaris* play a key role in heavy metal removal. Extremely toxic to the growth of all bacteria even the key organism *D. vulgaris* was observed at heavy metal loading rate of 7.5 mg L^{-1} d^{-1}. The results indicated that the efficiency of heavy metal removal, sulfate reduction and sulfide production decreased with the increase of heavy metal loading rate up to 6 and 7.5 mg L^{-1} d^{-1}.

The total cell count in the semi-continuous operated reactors has been 10^8 cells mL^{-1}. This is not very high for a technical reactor. Nevertheless, even with higher biomass concentrations the toxic effect of the heavy metal mixture should be kept in mind. Especially, in the treatment systems where the metal precipitation and sulfate reducing processes are established in the same reactor, resulting in the activity of sulfidogenic consortia are affected and the precipitated metals and biomass are lost due to washing out. As the lab system has been operated of optimum conditions it cannot directly be concluded that the heavy metal load can easily be increased with increasing biomass concentration as a real reactor might have a different microbial consortia. The experiments proved that by choosing the right

retention time the designed system is able to treat wastewater streams with up to 90 mg L^{-1} of heavy metal concentration.

2. Suggestions for future research

Further research should focus on a suitable separation of the biomass and heavy metal precipitation from the treated wastewater stream by using an ultra filtration membrane system or application of a two-stage process in which the metal precipitation is separated from the biological sulfate reduction step to improve the treatment efficiency. Moreover, excess sulfide in effluent can be recycled.