Metal supplementation to anaerobic granular sludge bed reactors: An environmental engineering approach

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Metal supplementation to anaerobic granular sludge bed reactors: An environmental engineering approach

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ABSTRACT

Metal deficiencies of anaerobic and aerobic wastewater treatment systems are not a rare phenomenon. A rational trace metal supply is thus required in wastewater treatment systems in order to achieve maximal substrate conversion capacities.

The objective of this thesis is to optimize essential metal dosing in granular sludge bed based reactors like the Upflow Anaerobic Sludge Bed (UASB) and Expanded Granular Sludge Bed (EGSB) reactors, which are used for the anaerobic treatment of organically polluted wastewater. Optimization of essential metal dosing in sludge bed based reactors is a compromise between achieving the maximum biological activity of the biomass present in the reactor, while minimizing the costs of the supplied metal and metal losses into the environment.

In this thesis, the fate of metals in the anaerobic granular sludge is studied. The boundary conditions for a stable reactor operation are evaluated. On the one hand, nutrient deficiency is studied by cobalt, nickel and zinc deprivation of methanol-fed UASB reactors and by molybdenum, selenium and tungsten deprivation of propionate fed UASB reactors. On the another hand, toxicity of cobalt to methanol fed methanogenic granular sludge is studied. Also, metal dosing strategies in UASB reactors are evaluated.

In continuous methanol fed UASB reactors, cobalt supplementation is required to maintain a high specific methanogenic activity (SMA), preventing reactor acidification. Repeated pulse additions of low cobalt concentrations to the influent are shown to minimize metal losses from the sludge and at the same time overcome cobalt limitation.

This thesis shows how the retention of cobalt in the sludge during 24 hours following a cobalt pulse at low cobalt concentrations correlates with the decline of the sludge SMA with methanol in methanol fed UASB reactors during 16 and 35 days following the pulse addition. This demonstrates that not a single parameter but a combination of different parameters affects and correlates with the reactor performance upon the cobalt pulse addition.

The correlation between metal retention and reactor performance does not indicate that metal retention is the cause of different reactor performances. For that reason, the predictions of the reactor operation should not be overstretched, but it is the basis for further research on the quantification of metal bioavailability. Moreover, the correlation between the initial metal retention and subsequent metal bioavailability can be used as a tool for metal dosing strategies, not only for UASB reactors but also for other bioprocesses using biofilms.

TABLE OF CONTENTS

1. General introduction 9

2. Bonding strength analysis of metals and sulfur fractioning in methanol-grown anaerobic granular sludge 33

3. Acidification of methanol fed anaerobic granular sludge bioreactors by cobalt deprivation: Induction and microbial community dynamics 55

4. Role of nickel in high rate methanol degradation in anaerobic granular sludge bioreactors 72

5. Zinc deprivation of methanol fed anaerobic granular sludge bioreactors 95

6. Effect of tungsten, molybdenum and selenium limitation on propionate degradation in anaerobic methanogenic granular sludge 119

7. Cobalt Toxicity in Anaerobic Granular Sludge: Influence of Chemical Speciation 137

8. Effect of chelation on cobalt dosing strategy in methanol fed anaerobic granular sludge bioreactors 157

9. Cobalt dosing to anaerobic granular sludge bioreactors: Quantification of metal retention to predict methanogenic activity 181

10. Summary and general discussion 203

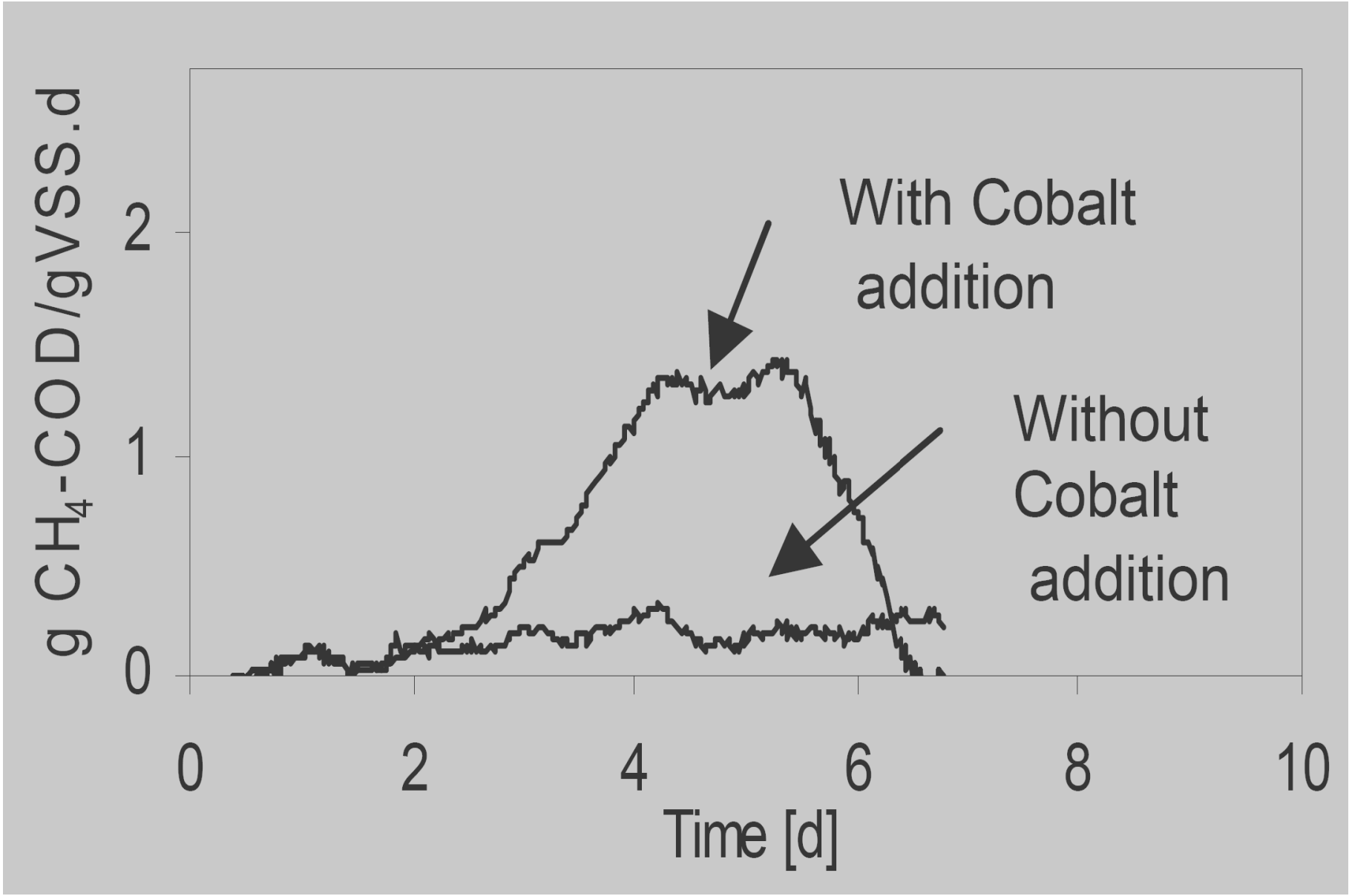
10 NL. Samenvatting en algemene discussie 223

List of abbreviations 242

List of publications 243

Acknowledgments 244

Training and supervision plan 246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GENERAL INTRODUCTION

1

Chapter 1

1.1. GENERAL INTRODUCTION

1.1.1. Background

Sludge bed processes like the Upflow Anaerobic Sludge Bed (UASB) and Expanded Granular Sludge Bed (EGSB) are the mostly applied processes for the anaerobic treatment of industrial effluents (Van Lier, 2007). The success of these sludge bed reactors is based on the so-called three-phase separator, which enables the reactor to separate a gas, water and granular sludge mixture under high turbulence conditions, which allows for compact, cheaper reactor designs (Mulder et al., 2001). The microecosystem present in the granular sludge requires nutrients for anabolic and catabolic metabolism. Metals are important micronutrients. They are crucial constituents in enzymes and enzymatic cofactors and they play an important role in many enzymatic reactions. Absence of sufficient quantities of metals will limit growth and catabolic activities of the microbial population present in the UASB reactor. However, a metal excess will be also harmful for the microbial population. Many metals are classified as priority pollutants and many metals are important constituents of industrial effluents (Upadhyay, 2006; Gazea et al., 1996).

The exact amounts of trace metals needed in a sludge bed process will vary depending in the wastewater treated. Trial approaches are used nowadays to asses their benefit for anaerobic processes (Opure BV, personal communication).

In order to develop optimal operational practice of anaerobic granular sludge bioreactors regarding metal supplementation, an accurate understanding of the interaction between metals and the UASB granules as well as of the effect and efficiency of metal supply are prerequisites to maximise the methanogenic activity, to reduce the costs of metal supply and to minimize its losses into the environment.

1.1.2. Scope of this thesis

The metal nutrient requirements and proper design of metal supplementation in UASB reactors needs to set off from the description of the interactions between metals and microbes. This allows to understand the role that metals have in microbial systems. This includes the effects that metals cause in microbial biofilms, more specific in the granular sludge present in UASB reactors and the role of metals in the enzymatic functioning of the anaerobic microbes.

In order to gain a deeper knowledge on trace metal requirements in UASB reactors, the possible interactions between trace metals and UASB granules were studied. This includes liquid and solid phase speciation of metals, limitation of microbial activity in anaerobic granular sludge reactors because of lack of essential metals and the toxic effects caused by an excess of them.

Metal dosing strategies have been limitedly studied in UASB processes. Past studies dealing with metal supplementation strategies in UASB reactors give an overview of what

already has been done and provide hints on possible routes to follow in order to design optimal metal supplementation strategies (Zandvoort et al., 2004).

1.2. METALS IN MICROBIAL SYSTEMS

1.2.1. Metals and biofilms

A biofilm is defined as a sessile community of microorganisms characterized by cells attached to a substratum or to each other, which are embedded in a matrix of extracellular polymeric substances that they have produced (Donlan and Costerton, 2002). The cells can exhibit an altered phenotype with respect to growth rate or gene transcription (Donlan and Costerton, 2002). Over the past 40 years, understanding of microbial biofilms has increased (Hall-Stoodley et al., 2004; Battin et al., 2007). Biofilms are regarded as a prominent mode of microbial life in nature (Hall-Stoodley et al., 2004). Mounting evidence indicates that biofilm microorganisms can undergo a process of cell specialization that is analogous to the differentiation in multicellular organisms (Purevdorj-Gage et al., 2005; Lewis, 2007). The best-known example of this is the innate resistance and/or tolerance that biofilms, unlike planktonic cell populations, have to antibiotics, surface disinfectants and metal ions (Spoering and Lewis, 2001; Teitzel and Parsek, 2003).

What makes biofilms less susceptible to metal toxicity than exponentially growing planktonic cell populations? Trace metals exist in wastewater in a variety of chemical species, most of them as cations that are either complexed, to varying degrees, by inorganic and organic ligands or are adsorbed on or bound within particles (Hassler and Wilkinson, 2003). Metals can cycle between different oxidation states (Frankenberger Jr and Arshad, 2001; Strigul et al., 2005). Both complexation and redox cycling affect the behavior of these metals in biofilm systems because of the large differences in the reactivity, kinetic lability, solubility or volatility of different metal oxidation states and metal complexes that can occur in such heterogeneous system as the microbial biofilm (Sunda and Huntsman, 1998). Time dependent and dose-respond toxicity assays have shown that the various microbial subpopulations present in biofilms react differently upon exposure of the entire population to metal ions (Harrison et al., 2005; Davies et al., 2007).

Microbial biofilms, natural or engineered, can be used to remediate heavy metal pollution by biochemical modification and/or the accumulation of toxic metal ions (Muñoz et al., 2006; Singh et al., 2006). An understanding of the metal fate in biofilms is crucial for the successful design of biological wastewater treatment systems that are used for biodegradation of organic contaminants, which are frequently intermingled with metal pollution (Singh et al., 2006). The different metal species have a distinct chemistry and function through diverse biochemical routes of toxicity.

1.2.2. Metals and microbes

Metals are very important in all biological processes of any kind of living organism. Of the 95 naturally occurring elements of the periodic table, no less than 25 perform essential biological functions. Some of these, e.g. zinc, nickel, copper, selenium, cobalt, chromium, molybdenum, tungsten, manganese or iodine, are required in small amounts and are termed essential trace elements. Most of them are part at the active site of enzymes. Table 1.1 lists the role of some essential trace elements in various enzymes involved in anaerobic reactions and transformations (Oleszkiewicz and Sharma, 1990; Zandvoort et al., 2006).

Table 1.1. Role of some essential trace elements in various enzymes involved in anaerobic reactions and transformation.

Element	Functions	Element	Functions
Cu	• Superoxide dismutase • Hydrogenase (facultative anaerobes) • Nitrite reductase • Acetyl–CoA synthase	Ni	• CO–dehydrogenase • Acetyl–CoA synthase • Methyl–CoM reductase (F_{430}) • Urease • Stabilizes DNA, RNA • Hydrogenase
Co	• B_{12}–enzymes • CO–dehydrogenase • Methyltransferase	Se	• Hydrogenase • Formate dehydrogenase • Glycin reductase
Fe	• Hydrogenase • CO–dehydrogenase • Methane monooxygenase • NO–reductase • Superoxide dismutase • Nitrite and nitrate reductase • Ntrogenase	W	• Formate dehydrogenase • Formylmethanofuran–dehydrogenase • Aldehyde–oxydoreductase • Antagonist of Mo
Mn	• Stabilizes methyltransferase in methane–producing bacteria • Redox reactions	Zn	• Hydrogenase • Formate dehydrogenase • Superoxide dismutase
Mo	• Formate dehydrogenase • Nitrate reductase • Nitrogenase	V	• Nitrogenase • Chloroperoxydase • Bromineperoxydase

The relation between metal concentrations in the system and its presence in the catalytic centers of the intracellular enzymes is not direct (Ermler, 2005). The presence of high metal concentrations in a bulk liquid does not mean that the bacteria or archaea present take up the metal and incorporate it into the catalytic center of its enzyme (Jansen, 2004). Jansen (2004) studied metal transport from the bulk liquid to *Methanosarcina barkeri* cells assuming that the driving force of metal uptake is the flux originated by the metal gradient between the metal concentration in the bulk liquid and the metal content of the cell. Jansen (2004) developed a mechanistic model able to quantify the cobalt and nickel uptake, cofactor content and methanogenic activity of pure cultures of *Methanosarcina barkeri* under well-defined conditions of metal speciation in the medium.

1.2.3. Metal toxicity concepts in microbial systems

At high concentrations, heavy metals may act as inhibitors by blocking of enzyme functions. The toxic effect of heavy metals is primarily non–specific and reversible (Nies, 1999; Toes, 2008). This type of inhibition is characterized by the reversible binding of the inhibitor with either the enzyme or the enzyme-substrate complex. Less frequently, metals act as competitive inhibitors (they compete with the substrate). This type of inhibition depends on the affinities of the metal and enzyme, as well as on the relative metal concentrations of the competing metals (Oleszkiewicz and Sharma, 1990). Toxic effects are exerted at concentrations that can easily be found in natural and wastewaters (He et al., 2005). Metals, such as Fe(II), Ni(II) and Co(II), are non–biodegradable and the effects on microorganisms and other aquatic fauna are ecologically significant (Ram et al., 2000).

Several models have been developed in the last decades to help to explain the effect of trace metals on fish, with applications to other organisms in aquatic media and/or soils. The basic theory started in the ‘70s with the realization that: (i) Metal toxicity could be largely explained by the toxicity of the free ion, (ii) Complexation by inorganic and organic ligands would decrease metal toxicity by decreasing the free ion activity, (iii) A variety of water quality parameters affected metal toxicity by effects on both the organism and metal speciation and (iv) Hardness cations were specifically protective because they competed with free metal ions for key binding sites on the organisms.

Pagenkopf (1983) proposed the Gill Surface Interaction Model (GSIM), while Morel (1983) formulated the Free–Ion Activity Model (FIAM), both of which brought together these ideas in a single conceptual framework (Fig. 1.1) (Hassler et al., 2004). Both the GSIM and FIAM are the theoretical predecessors of the Biotic Ligand Model (BLM) (Paquin et al., 2002a). The BLM has been proposed as a tool to quantitatively evaluate the manner in which water chemistry affects the speciation and biological availability of metals in aquatic organisms. The BLM is fundamentally a chemical equilibrium-based model. One of the components of this

model is the site of action of toxicity of the organism. The BLM is used to predict the degree of metal binding at this site of action, and this level of accumulation is in turn related to a toxicological response (Paquin et al., 2002a)..

The role of metal complexation is critical because formation of organic and inorganic metal complexes renders a significant fraction of the total non–bioavailable metal. This model should be applicable to any organism for which the site of action is directly in contact with the external aqueous environment. In fact, this modeling framework defines the bioavailability of metals (Di Toro et al., 2001).

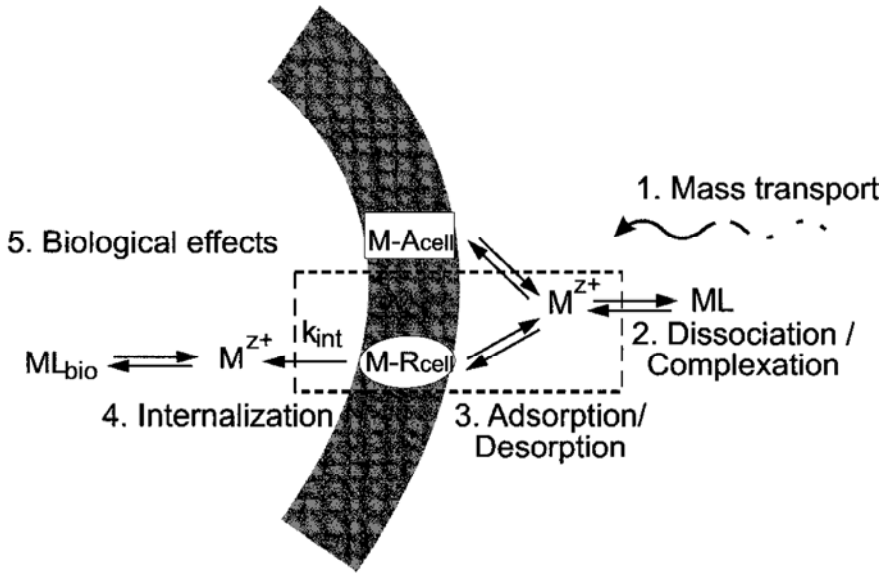

Figure 1.1. Conceptual framework of the FIAM and BLM, including (1) mass transport of the free metal (M^{z+}) and a hydrophilic complex (ML) in solution; (2) dissociation/complexation with a ligand in solution; (3) specific ($M - R_{cell}$) or non–specific ($M - A_{cell}$) adsorption of the metal to the surface of the organism; (4) metal transport into the organism characterized by an internalization rate constant (k_{int}) and a subsequent reaction with an intracellular ligand (ML_{bio}); (5) expression of the biological effect. The box identifies the reactions that are taken into account in the FIAM and BLM (After Hassler et al., 2004).

As shown in Fig. 1.1, dissolved metal exists in solution as partially free metal ion. This species is hypothesized to be the bioavailable species (Jansen, 2004). The rest of the metal exists as non–bioavailable metal complexes that result from reactions of the metal with organic and inorganic ligands. The toxicity of metals to organisms is assumed to occur as the result of the free metal ion reacting with the physiologically active binding sites (Heijerick et al., 2002; Paquin et al., 2002b; Lock et al., 2007).

1.3. METALS IN ANAEROBIC DIGESTION TECHNOLOGY

1.3.1. Granular sludge bioreactors

Anaerobic digestion was one of the first biological unit-processes used for the treatment of highly concentrated and complex wastewater (Lettinga et al., 1979). Over the decades, several anaerobic reactor configurations have been developed (Seghezzo et al., 1998; Qureshi et al., 2005). From the available anaerobic technologies, granular sludge bed reactors are by far mostly applied. The granular sludge consists of spherical biofilms without support material. Different types of granular sludge based reactors are nowadays available. The UASB reactor, the expanded granular sludge bed (EGSB) reactor, and the internal circulation (IC) reactor are the most common examples (Mulder et al., 2001). At present, 90 % of all industrial anaerobic wastewater installations are sludge bed reactors in which biomass retention is attained by the formation of sludge granules (Van Lier, 2007).

In order to sustain growth and to carry out biochemical transformations, the UASB process requires macronutrients such as phosphorus, nitrogen, potassium, etc. In addition to the fundamental requirement of macronutrients, anaerobic microorganisms require essential metals for biomass metabolism, growth, activity and stability of the UASB process (Speece et al., 1983; Singh et al., 1999; Aquino and Stuckey, 2007). Not only the distribution of metals in the granular sludge present in the UASB reactor exists in a high variety of chemical species, but they are also not always bioavailable for the microorganism (Fig. 1.2).

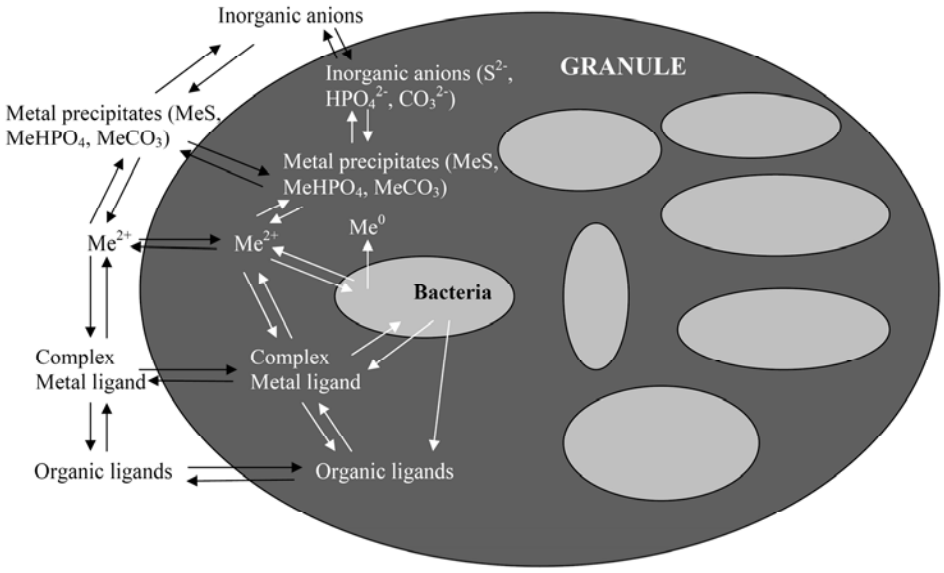

Figure 1.2. Fate of metals in the granular sludge matrix.

The minimum level of essential metals, which would support a desired growth rate, must be maintained (Jansen et al., 2007). However, also a maximum level of nutrient requirement exists above which many nutrients are inhibitory, rather than stimulatory (Lin and Chen, 1999). Different metal mixtures have been used in studies on UASB reactors, but they are found to differ in composition, concentration and the forms in which they are supplemented to the feed (Singh et al., 1999; Zandvoort et al., 2004; Tiwari et al., 2006). Also, the appropriate quantification and suitable ranges in which nutrient dosing is required during the entire operational period are basically unknown. Thus, knowledge about the effect of metals in the biomass and the metal distribution through the UASB system is necessary in order to obtain a proper strategy for metal supplementation (Fig. 1.3).

1.3.2. Metal limitation in bioreactors

The role of different metabolic active metals can be studied by investigation of metal limitations of the methanogenic degradation of the model substrates methanol and propionate. The degradation pathway of methanol is well known to be cobalt dependent and previously used in similar studies (Florencio et al., 1993; Florencio et al., 1994; Zandvoort et al., 2002a), but so far efficient cobalt dosing strategies in methanol fed UASB reactors have not been described. Propionate is a key intermediate in the conversion of complex organic matter under methanogenic conditions. It may account for a large fraction of the methane produced in UASB reactors (De Bok et al., 2004). Propionate accumulation in anaerobic reactors occurs in case of process failure, e.g. overloading, temperature decrease, and can further increase the thus induced negative effects, such as pH drop, toxicity (Pereira et al., 2003). Limitation of propionate degradation may occur in case one essential metal for the microorganisms involved in the propionate degradation is missed in the reactor.

1.3.2.1. Methanol: model substrate to study the impact of metal limitation in bioreactors

Methanol is a colorless, water-soluble simple alcohol containing one carbon atom. Methanol has been used in industry for more than 100 years. It is a feedstock for chemical syntheses, e.g. for formaldehyde, acetic acid and methyl tertiary-butyl ether and is a solvent in a variety of consumer products, e.g. paints and varnishes, antifreeze, windshield washers, cleansing solutions and adhesives (World Health Organization, 1997). The world methanol production in 2006 amounted up to 42839 million metric tons (www.methanol.org). Methanol is an important constituent of wastewater generated in the petrochemical industry and in the widely used “kraft” process for wood pulping in the paper industry.

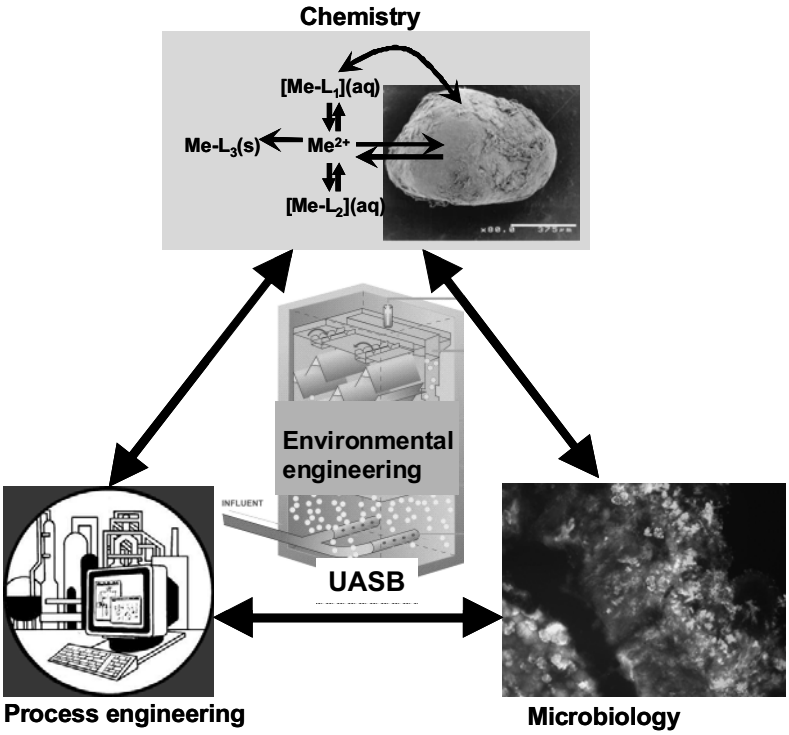

Figure 1.3. Multidisciplinary approach required for determining the interactions of metals with the solid phase, liquid phase and the microorganisms present in anaerobic bioreactors, which will lead to a more rational and efficient metal supply to bioreactors.

Methanolic wastewater is commonly treated at full-scale in UASB reactors (Weijma and Stams, 2001). Methanol is converted to methane via several pathways, i.e. direct conversion to methane by methylotrophic methanogens (Nishio et al., 1992), indirect conversion to acetate by acetogens (Van der Meijden et al., 1984) coupled to acetoclastic methanogenesis (Huser et al., 1982) or indirect conversion to H_2 and CO_2 (Cord-Ruwisch et al., 1988) coupled to hydrogenotrophic methanogenesis (Whitman et al., 1982).

Acetogens and methanogens are the main trophic groups involved in mesophilic methanogenic methanol conversion (Florencio et al., 1993; Florencio et al., 1994). Acetogens have a lower substrate affinity for methanol (K_s = 16 mM) compared to methanogens (K_s = 0.25 mM) (Florencio et al., 1995). Thus, acetogens can only outcompete methanogens at high methanol concentrations. Acidification of upflow anaerobic sludge bed (UASB) bioreactors is the main concern for stable operation of methanol-fed anaerobic wastewater treatment bioreactors. This is due to an imbalance between acetate formation from methanol by acetogenic

bacteria and acetate consumption by methanogenic bacteria (Fig. 1.4). Previous research has suggested that UASB reactor acidification during treatment of methanolic wastewater is related to elevated methanol concentrations in the bioreactor, which can be induced by methanogenic activity decrease due to depletion of the sludge by cobalt (Zandvoort et al., 2002a), nickel (Zandvoort et al., 2002b) and iron (Zandvoort et al., 2003).

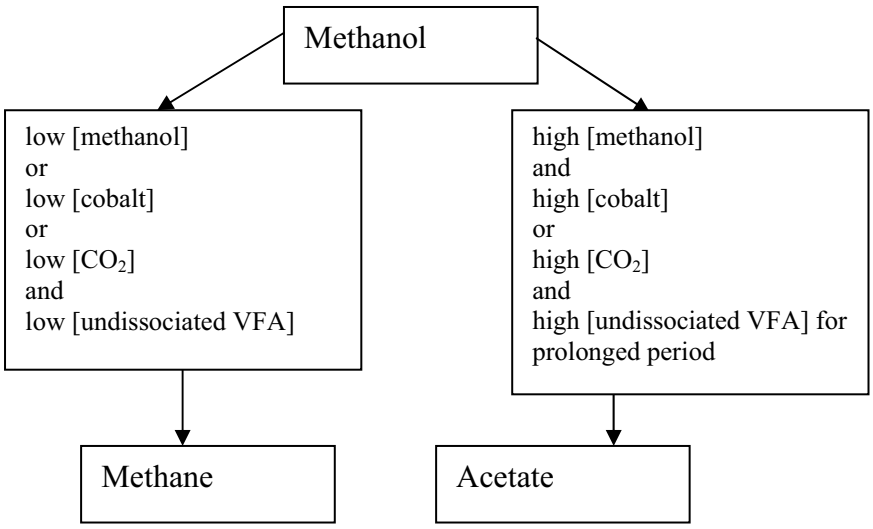

Figure 1.4. The fate of methanol in anaerobic bioreactors after Florencio (1994).

Role of cobalt in methanol degradation

Cobalt plays an important role in the direct conversion of methanol to methane, as it forms the metallocenter of corrinoid compounds that catalyze the methyl transfer from methanol to methyl-coenzyme M, the common precursor of methane for all methanogenic substrates (Thauer, 1998; Jiang, 2006). The corrinoid compound is vitamin B_{12} (5´-deoxyadenosylcobalamin). This molecule has three parts: a central ring, an adenosyl moiety and a nucleotide loop. As can be seen in Fig. 1.5, the central atom in this molecule is cobalt (Roth et al., 1996).

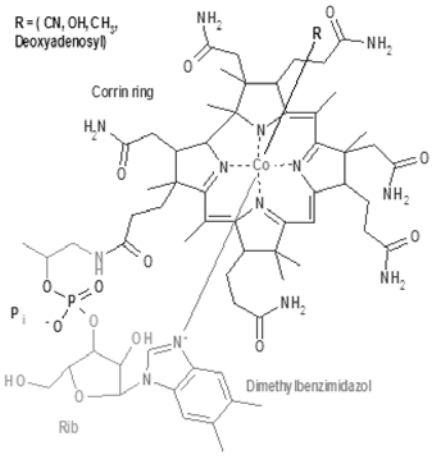

Figure 1.5. Structure of coenzyme B_{12}.

Role of nickel in methanol degradation

Nickel plays a key role in the methanogenic conversion of methanol: one mol of methyl-S-CoM reductase contains 2 mol of tightly, but not covalently, bound coenzyme F_{430} (Ellefson et al., 1982). F_{430} is a nickel porphinoid (Fig. 1.6). In addition, many hydrogenase enzymes involved in hydrogen formation possess nickel (Deppenmeier et al., 1992; Kemner and Zeikus, 1994; Jiang, 2006). For example, carbon monoxide dehydrogenase (CODH), which possesses two nickel-containing metallocentres, is present in both acetoclastic methanogens and acetogenic microorganisms (Hausinger, 1987).

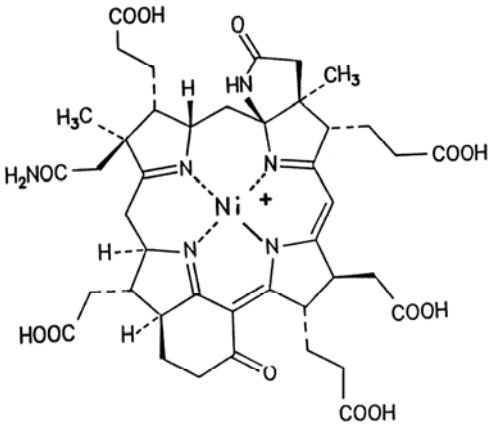

Figure 1.6. Structure of coenzyme F_{430} in the Ni^{2+} oxidation state (Thauer, 1998).

Role of zinc in methanol degradation

Zinc is another key element in methanogenesis (Fig. 1.7) and its potential deprivation from anaerobic granular sludge has thus far not been investigated. Zinc is involved in coenzyme M activation during the formation of methyl-coenzyme M (Sauer and Thauer, 1997). MT2 (Co-methyl-5-hydroxybenzimidazolylcobamide (CH_3-B_{12}-HBI): HS-CoM methyltransferase) is a zinc containing monomeric protein which catalyzes methyl transfer from methylated MT1 (methanol: 5-hydroxybenzimidazolylcobamide (B_{12}-HBI) methyl-transferase) to coenzyme M (Fig. 1.7) (Harms and Thauer, 1996). The methylation of coenzyme M to methyl-coenzyme M is a reaction in which a thiol group is alkylated and all enzymes catalyzing alkyl transfer to thiols have been found to be zinc proteins (Matthews and Goulding, 1997; Penner-Hahn, 2007). The postulated role of zinc in these enzymes is that of a Lewis acid that activates the thiol groups to be alkylated (Goulding and Matthews, 1997).

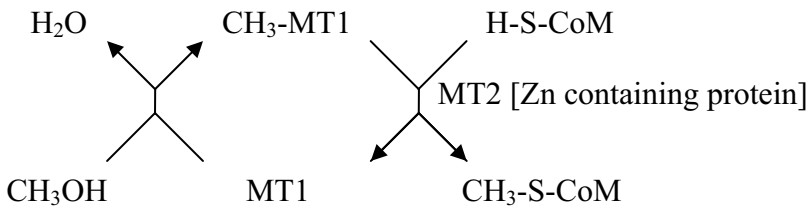

Figure 1.7. Methyl-coenzyme M formation from methanol and coenzyme M MT1: methanol: 5-hydroxybenzimidazolylcobamide (B_{12}-HBI) methyl-transferase. MT2: Co-methyl-5-hydroxybenzimidazolylcobamide (CH_3-B_{12}-HBI): HS-CoM methyltransferase. (From Sauer and Thauer, 1997).

1.3.2.2. Role of oxyanions in propionate degradation

Oxidation of propionate to acetate, carbon dioxide, hydrogen and formate, can only proceed if the products hydrogen and formate are removed by methanogens or other hydrogen utilizing bacteria (Stams, 1994; Schink, 1997). To make the energy yield from propionate oxidation energetically feasible for the bacteria and archaea involved, a very low concentrations of hydrogen, i.e. H_2 partial pressures lower than 10 Pa (Kaspar and Wuhrmann, 1978; Schink, 1997), and formate (Jia and Fang, 1999) are nedded.

Molybdenum, tungsten and selenium are essential elements, usually present as oxyanions, for the growth of a variety of anaerobic microorganisms, including propionate oxidisers (Jiang, 2006). Enzymes containing these elements usually catalyze redox reactions, such as formate dehydrogenase (FDH). FDH catalyzes the first reaction of carbon dioxide

reduction to formate in the acetyl-CoA pathway (Gollin et al., 1998), FDH also involves energy generation and carbon-assimilation and is highly important for syntrophic propionate oxidation (Plugge et al., 1993). The stimulating effect of tungsten and molybdenum on the growth rate of propionate degrading bacteria is mainly related to FDH (Kletzin and Adams, 1996). *Syntrophobacter fumaroxidans* is a common propionate degrading bacterium in UASB granules (Harmsen et al., 1998; Fernandez et al., 2007). The *Syntrophobacter fumaroxidans* genome sequence includes four gene clusters coding for FDH which contain molybdenum, tungsten and/or selenium (Doe Joint Genome Institute, 2007). Deprivation of these oxyanions will thus also affect the activity in the propionate degrading bacterium present in the UASB reactor (Jiang, 2006).

1.3.3. Metal toxicity in bioreactors

Trace metals a too high concentration can cause inhibition of the methanogenic process (Ram et al., 2000). Although heavy metal toxicity in UASB reactors has been studied abundantly, reported values of toxic concentrations vary considerably between different authors (e.g. Fang, 1997; Lin and Chen, 1997; Karri et al., 2006). In view of the importance of nickel and cobalt in methanogenic process, toxicity studies of these metals are of high importance. The inhibition by nickel and cobalt of the methanogenic activity of anaerobic sludge has been studied abundantly and high differences in toxic concentrations were found (Table 1.2). This is due to the fact that most authors report the total metal concentration in the liquid phase, and do not consider metal speciation. Therefore, the observed toxic concentrations are affected by differences in medium composition, which causes changes in metal speciation (Worms et al., 2006; Jansen et al., 2007).

Free metal ion bioavailability models, like the BLM (Hassler et al., 2004), have been used most often as the base of metal toxicity studies. The presence of organic complexing ligands, such as citrate, that decreases the free metal concentration, clearly influences the cobalt and nickel toxicity to bacteria, as to e.g. *Pseudomonas aeruginosa* (Chen et al., 2006) or *Bacillus subtilis* (Krom, 2002). However, these toxicity models were established and verified for natural waters and higher organisms such as freshwater algae *Chlorella kesslerii* (Hassler et al., 2004), *Urchin larvae* (Lorenzo et al., 2006) or mussels (*Mytilus galloprovincialis*) (Beiras et al., 2003). Thus far, this type of model has never been applied to the anaerobic consortia present in biofilms or granules from wastewater treatment reactors.

Table 1.2. Toxic concentrations of cobalt, nickel and zinc in methanogenesis processes.

Metal	Organism	Reactor /technique	Toxic concentration	Conditions	Reference
Cobalt	Mixed anaerobic sludge	Anaerobic toxicity bioassays	• Up to 35 - 400 mg·L^{-1} (no detectable inhibition) • 600 – 800 mg·L^{-1} (7-17% inhibition) • 950 mg·L^{-1} (100% inhibition)	The sludge was fed with nutrients and acetate or glucose as the sole carbon source under oxygen-free condition at 35±1ºC.	Bhattacharya et al., 1995
	Wet slurry from digested cattle manure	Batch bottles	• 120 µg·g dry matter^{-1}	The experiment was performed at 37°C	Jain et al. 1992
Nickel	Anaerobic granular sludge	UASB reactor	• 81 mg·L^{-1} (50% inhibition of VFA degradation) • 440 mg·L^{-1} (50% inhibition of VFA degradation)	The digesters were acclimated in a 13.5-liter UASB reactor at 35±1 °C. The HRT was 1 and 2 days.	Lin and Chen, 1999
	Anaerobic starch-degrading granules	UASB reactor	• 118 mg·L^{-1} (IC_{50} or 50% inhibition of SMA)	The reactor was continuously fed with synthetic wastewater composing starch as the sole organic carbon source.	Fang and Hui, 1994
	Anaerobic granular sludge	UASB reactor	• 81 mg·L^{-1} (50% inhibition, bed sludge) • 78 mg·L^{-1} (50% inhibition, blanket sludge)	The reactors were treating winery wastewater and were acclimated in a 13.5-liters UASB reactor at 35±1 ºC. 1 day HRT and the acclimation period for the seed sludge was six months.	Lin and Chen, 1999
	Anaerobic granular sludge	UASB reactor	• 118 mg·L^{-1}L (IC_{50})	Granules were sample from four UASB reactors at COD loading rate of 10 g COD·l^{-1}·d^{-1} for over six months at 37°C.	Fang, 1997
Zinc	Anaerobic granular sludge f	Batch bottles	• 690 mg·L^{-1} (50% inhibition of methanogenic activity with sludge operated at HRT 1 day) • 270 mg·L^{-1} (50% inhibition of methanogenic activity with sludge operated at HRT 2 day)	The experiment was performed at 35 ± 1 °C. Winery wastewater as substrate.	Lin and Chen, 1999
	Anaerobic granular sludge	Batch bottles	• 96 mg·L^{-1} (50% inhibition of methanogenic activity)	The experiment was performed at 37 ± 1 °C Starch synthetic wastewater as substrate.	Fang, 1997

UASB reactor: Upflow anaerobic sludge bed reactor
CSTR: Completely stirred tank reactor

1.4 METAL DOSING TO BIOREACTORS

The supply of metals to bioreactors has received less attention than the inhibiting effects of metals on the microbial activity (Lin and Chen, 1999). Metal deficiencies of aerobic (Clark and Stephenson, 1998; Burgess et al., 1999a; Burgess et al., 1999b; Philips et al., 2003) and anaerobic (Speece et al., 1983; Takashima and Speece, 1989; Patidar and Tare, 2004; Zitomer et al., 2008) waste water treatment systems are not a rare phenomenon and a rational trace metal supply is required in wastewater treatment systems in order to achieve maximal substrate conversion rates.

No extensive research about metal supplementation to anaerobic wastewater treatment systems has been carried out in the last decades (Table 1.3). Moreover, the approaches used to study metal supplementation are hardly comparable. Different reactor configurations have been used, such as UASB reactors (Shen et al., 1993; Espinosa et al., 1995), Fed-batch UASB sludges (Sharma and Singh, 2001), anaerobic download fixed film reactors (Murray and Van den Berg, 1981), anaerobic film expanded bed reactors (Kelly and Switzenbaum, 1984), upflow flocculent sludge reactors (Callander and Barford, 1983) or continuous stirred tank reactors (Percheron et al., 1997). Different substrates have been fed to these metal supplementation studies, including volatile fatty acids (Shen et al., 1993), sulphate laden organics (Patidar and Tare, 2006), cane molasses stillage (Espinosa et al., 1995), methanol (Florencio et al., 1993), food industry wastewater (Oleszkiewicz and Romanek, 1989), distillery wastewater (Sharma and Singh, 2001), bean wastewater (Murray and Van den Berg, 1981), whey (Kelly and Switzenbaum, 1984), sugar cane fermentation waste (Callander and Barford, 1983) or molasses wastewater (Percheron et al., 1997). Nutrient supply has also been studied at different temperatures, ranging from 7 °C (Li et al., 2007) up to 55 °C (Paulo et al., 2004). These studies indicate that metal supplementation improves in most of the cases the reactor efficiency, mainly measured as the increase in methane production.

In continuous flow methanol fed UASB reactors, cobalt supplementation is required to maintain a high SMA, and therefore, prevent reactor acidification, which could also occur in case of excess of cobalt dose (Zandvoort et al., 2003). Different cobalt dosing strategies have been studied in methanol fed UASB reactors: continuous addition of a low $CoCl_2$ concentration to a methanol fed UASB reactor only slightly enhanced the SMA of the sludge (Zandvoort et al., 2002a). The same author studied the possibility of pre-loading sludge by pre-incubating the inoculum in a 1 mM $CoCl_2$ solution for 24 hours. Pre-loading clearly overcame cobalt limitation in the methanol fed UASB reactor (Zandvoort et al., 2004). Pulse dosing of cobalt increased the SMA with methanol as the substrate 4 times, clearly overcoming cobalt limitation (Zandvoort et al., 2004). A drawback of these dosing protocols is that high amounts of cobalt are lost with the effluent. Obviously, there is a need for other kinds of metal dosing strategies that minimize the metal losses by manipulating the metal dissolution rate.

Table 1.3. Stimulation of biological conversions in anaerobic bioreactors by metal supply.

Supplied metal	Reactor	Temperature (°C)	pH	Substrate	OLR g COD·L^{-1}·d^{-1}	Effect upon metal addition	Reference
Fe	CSTR	35		Acetate	0.25	Increase acetate removal	Hoban and Van Den Berg, 1979
Fe	upflow flocculent sludge reactor	36	7.55	Cane fermentation waste	0.7-50	Increase acetate removal	Callander and Barford, 1983
Ni, Co and Fe	Fed-Batch pH-stat		6.6	Acetate	4.3	Increase acetate removal	Speece et al., 1983
Trace metals	anaerobic film expanded bed reactor	20, 25, 30 and 35	6.9	Whey		Increase COD removal	Kelly and Switzenbaum, 1984
Ni	Poultry waste digester	50	7.5	Chicken manure		Increase biogas formation	Williams et al., 1986
Ni, Co and Fe	UASB reactor	35	6.9-7.3	Food industry wastewater	15	Faster sludge growth and better sludge retention. Increase in COD removal	Oleszkiewicz and Romanek, 1989
Co	UASB reactor	30	6.8	Methanol	8.5	Increase COD removal	Florencio et al., 1993
Ni, Co and Fe	UASB reactor	35	6.8	Volatile fatty acids	11	Increase COD removal	Shen et al., 1993
Ni, Co and Mo	UASB reactor	35	7.8	Cane molasses stillage	21.5	Increase COD removal	Espinosa et al., 1995
Co and Fe	CSTR	35	7.8	Molasses wastewater	2	Improve anaerobic digestion	Percheron et al., 1997
Ni, Co and Fe	Fed-batch granular sludge	35	6.7-7.5	Distillery wastewater	5.9	Improve methanogenic activity	Sharma and Singh, 2001
Co	UASB reactor	30	7.0	Methanol	20	Increase SMA and methanol removal	Zandvoort et al., 2004
Co	UASB reactor	55		Methanol	4.3-8.9	High activity of methanogens	Paulo et al., 2004
Trace metals	UASB reactor	7 - 25		High concentrated organic wastewater		Increase VFA removal	Li et al., 2007

UASB reactor: Upflow anaerobic sludge bed reactor
CSTR: Completely stirred tank reactor

1.5 OUTLINE OF THIS THESIS

The main objective of this thesis is to answer the following question: "What is the optimal metal dosage strategy in anaerobic granular sludge bioreactors?".

Chapter 2 focuses on the fractionation and bioavailability of heavy metal in anaerobic granular sludge during operation of UASB reactors. This is achieved by combining the results from the bonding form analyses of metals and sulfur.

Metal supplementation is studied through focusing on the boundary situation that inadequate metal supplementation strategies could induce, which is either metal limitation and/or metal toxicity. Chapters 3 to 6 focus on the deprivation of the essential trace elements, cobalt, nickel, zinc, molybdenum, tungsten and/or selenium from the influent of methanol and propionate fed UASB reactors. Chapter 7 focus on cobalt toxicity for methanol fed anaerobic granular sludge. The influence of cobalt speciation in toxicity assays is evaluated. The experiences and data obtained in the previous chapters are used to design an optimal dosing metal strategy, i.e. pulse addition of low amounts of cobalt. In Chapter 8 a comparison is made between the pulse addition of low amounts of cobalt bound to EDTA and cobalt as $CoCl_2$. In Chapter 9 is studied whether the metal retention upon pulse dosing to UASB reactors can be correlated to the reactor performance in the period following the dosing. Such a correlation will facilitate implementation of a metal addition strategy, which will minimize metal losses from the wastewater treatment system while the sludge SMA will be sufficient for a certain conversion capacity.

Finally, Chapter 10 summarizes the main results of this thesis and list recommendations for future research.

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BONDING FORM ANALYSIS OF METALS AND SULFUR FRACTIONATION IN METHANOL-GROWN ANAEROBIC GRANULAR SLUDGE

ABSTRACT

This study investigated the metal and sulfur bonding form distribution in mesophilic (30 °C, pH 7) methanol-grown anaerobic granular sludge from upflow anaerobic sludge bed reactors operating at an organic loading rate of 3.8 g CH_3OH-$COD \cdot L^{-1} \cdot d^{-1}$. This was achieved by applying a modified Tessier sequential extraction scheme to investigate the metal bonding forms and a sequential extraction scheme for sulfur and simultaneously extracted metals to granular sludge samples of the reactors after 0, 22, 35 and 43 days of operation. Metals were also determined in the sulfur extracts. Co and Ni were predominated by oxidizable bonding forms, which increased together with the pseudo-total content during reactor operation. Omission of Co and Ni from the influent led to only a minor decline of the pseudo-total content in the sludge, mainly from the acid-soluble fraction. The ratio of simultaneously extracted metals (Co, Fe, Mn, Ni) to acid-volatile sulfides was lower than 1, indicating that the sludge contained sufficient sulfide to bind the metals as metal monosulfides. The bioavailability of metals in the methanol-grown anaerobic granular sludge investigated is therefore mainly controlled by sulfide formation/dissolution.

2

2.1. INTRODUCTION

The use of anaerobic bioreactors for wastewater treatment depends on the optimal functioning of the microbial population and their retention within the bioreactor. Besides macronutrients, micronutrients are also required (Takashima and Speece, 1989; Goodwin et al., 1990), *e.g.* Co, Ni and Zn are contained in cofactors and enzymes involved in methanogenesis (Kida et al., 2001). The micronutrients concentration can fall below a critical value in some industrial wastewaters, which can cause a limitation of the microbial activity in bioreactors. Therefore, a mixture of low concentrated essential micronutrients is dosed to the influent of these full-scale anaerobic bioreactors (Lettinga and Hulshoff Pol, 1991). The dosed micronutrients interact with the granular sludge matrix in anaerobic reactors, which can function as a sink or a source of the essential elements (Osuna et al., 2004; van Hullebusch et al., 2005a; van Hullebusch et al., 2005b). In anaerobic systems, the bioavailability of micronutrients is affected by the precipitation of sulfides (Jong and Parry, 2003; La, 2003), phosphates (van Hullebusch et al., 2003), or carbonates. Sulfide formation as a result of biological sulfate reduction is very important in anaerobic reactors, which can remove over 99 % of heavy metals, *e.g.* Fe, Cu and Cd, from acid mine drainage (La, 2003).

The fate of micronutrients in anaerobic reactors has been studied and metal fractionation (Osuna et al., 2004; van Hullebusch et al., 2005b), metal accumulation (van Hullebusch et al., 2003; Willow and Cohen, 2003; van Hullebusch et al., 2005b), dosing strategies (Zandvoort et al., 2004) as well as metal dynamics have been quantified during reactor operation (Zandvoort et al., 2005a; Zandvoort et al., 2005b). These studies indicated the importance of metal sulfides in anaerobic bioreactors. High fractions of the total metal content were retrieved in oxidizable forms (bound as sulfide or in organic matter). Although sulfides play an important role in metal bioavailability, only few data have been published on the sulfur chemistry in anaerobic wastewater treatment bioreactors. Most investigations deal with systems treating wastewaters containing high metal concentrations, *e.g.* acid mine drainage (Jong and Parry, 2004a). Other studies present data on sulfur fractionation based on results from sequential extraction schemes that are not specific to sulfur (Osuna et al., 2004; van Hullebusch et al., 2005b; Zandvoort et al., 2005a).

In sediment geochemistry, the ratio between HCl-extractable metals (simultaneously extracted metals, SEM) and acid-volatile sulfides (AVS) is used to assess toxic effects of metals in anaerobic systems (Di Toro et al., 1990; Di Toro et al., 1992; Leonard et al., 1993; Zaggia and Zonta, 1997). A ratio below 1 indicates the presence of sufficient sulfide in the system to bind added metals as relatively insoluble metal sulfides, which reduces the toxic potential compared to situations with a lower sulfide content (SEM/AVS > 1) (Hare et al., 1994). Although this theory does not take the toxicity of uncomplexed sulfide into account (Morse and Rickard, 2004), it can also be used to describe the potential availability of metals in anaerobic wastewater

reactors.

The aim of this study was to gain a deeper insight into the heavy metal fractionation and bioavailability in anaerobic granular sludge during operation of upflow anaerobic granular sludge bed (UASB) reactors. This was achieved by combining the results from the bonding form analyses of metals and sulfur.

2.2. MATERIALS AND METHODS

2.2.1. Bioreactor setup

Two UASB reactors were operated for 43 days at 30 (± 2) °C at an organic loading rate of 3.8 g CH_3OH-COD·L^{-1}·d^{-1}. The UASB reactor setup has been described in detail by Zandvoort et al. (2002a). Operating conditions different from that publication are listed in Table 2.1. Both reactors received the same influent except for Co and Ni (Table 2.2). Co was omitted in the influent to R2, while Ni was omitted in the influent to R1. To avoid precipitation in the storage vessels, each influent was composed of three streams: (1) macronutrients (NH_4Cl, $MgSO_4 \cdot 7\ H_2O$, $CaCl_2 \cdot 2H_2O$) and micronutrients (Table 2.2); (2) methanol with $NaHCO_3$ and K_2HPO_4; and (3) dilution water. Tap water (Co, Ni: < 1 µg·L^{-1}) was used to prepare the influent and as dilution water. The pH of the reactor liquid was set to pH 7.0 (± 0.2) by the addition of 2.52 g $NaHCO_3$ per liter of influent.

Table 2.1. Operating conditions of the UASB reactors used in this study.

Condition	Value
Hydraulic retention time	8 h
Organic loading rate	3.8 g CH_3OH-COD·L^{-1}·d^{-1}
Superficial upflow velocity	0.1 m·h^{-1}

Table 2.2. Composition trace element solution added to influent.

Compound	Metal	Conc. [µM]
$FeCl_2 \cdot 4H_2O$	Fe (II)	5
$CuCl_2 \cdot 2H_2O$	Cu (II)	0.5
$ZnCl_2$	Zn (II)	0.5
$MnCl_2 \cdot 4H_2O$	Mn (II)	0.5
$NiCl_2 \cdot 6H_2O$[a]	Ni (II)	0.5
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	Mo (VI)	0.5
$Na_2SeO_4 \cdot 5H_2O$	Se (VI)	0.5
$Na_2WO_4 \cdot 2H_2O$	W (VI)	0.5
$CoCl_2 \cdot 6H_2O$[b]	Co (II)	0.5

a: Ni was omitted in the influent to R1.
b: Co was omitted in the influent to R2.

2.2.2. Source of biomass

The reactors were inoculated with methanogenic granular sludge from two full-scale wastewater reactors. One reactor (R1) was inoculated with sludge (8 g volatile suspended solids (VSS) of reactor) taken from a UASB reactor treating paper-mill wastewater (Industrie-water Eerbeek B.V., Eerbeek, The Netherlands). The second reactor (R2) was inoculated with sludge (15 g $VSS \cdot L^{-1}$ of reactor) from an expanded granular sludge bed (EGSB) reactor treating alcohol distillery wastewater (Nedalco, Bergen op Zoom, The Netherlands). These sludges were selected based on previous research, which showed that Eerbeek and Nedalco sludges become deprived of Ni and Co, respectively, when treating synthetic Co (Zandvoort et al., 2002a) or Ni (Zandvoort et al., 2002b) free methanol-based wastewater.

The initial composition of the total element content of the two sludges deviates from previously published data of material from the same origin (Zandvoort et al., 2003; Osuna et al., 2004; van Hullebusch et al., 2005b). The sludge of R1 has a higher content of almost all of the selected elements, with the exception of Co and S, *e.g.* Ni is approximately 50 %, P 100 % and Mn 300 % higher than the values given in Zandvoort et al. (2003). On the other hand, Nedalco sludge (R2) shows lower metal concentrations than those given by Zandvoort et al. (2003), *e.g.* Fe corresponds to only 50 %. These differences are due to changes in the operating conditions and wastewater composition due to changes in the production processes at the full-scale reactors.

2.2.3. Biomass sampling

Sampling took place on startup of the reactors (day 0) and after 22, 35 and 43 days of operation. Sludge was taken from the middle of the sludge bed of the reactor with a syringe and an attached polyethylene tube. During each sampling, sludge was removed from about the same height of the reactor. To prevent oxidation of oxidizable components in the sludge, *e.g.* sulfides (Lasorsa and Casas, 1996), the sludge was transferred for further manipulation to an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI, USA) equipped with Pd catalyst and N_2 / H_2 (95 / 5 vol.-%) as forming gas (Indugas, The Netherlands).

To remove the reactor liquid and the fines from the sludge samples, the material was put in plastic sieves and rinsed with anaerobic ultrapure water (UPW; Milli-RO system, Millipore, Bedford, MA, USA) until the runoff stayed clear. Excess water was removed by gently pressing the sieve on lab/household paper.

2.2.4. Total element contents

Total element contents were determined at least in duplicate by aqua regia digestion of a fresh, wet sludge sample (1 g wet sludge, 7.5 mL HCl, 2.5 mL HNO_3). The digestions were performed in Teflon vessels in a temperature and pressure controlled microwave oven

(Milestone Ethos E, Milestone S.r.l., Sorisole, Italy) with the following settings (max.): 1000 W; 170 °C and 30 bar. Chemicals were of analytical reagent grade or better. Vessels used for metal analysis were cleaned by rinsing in a 4 M HNO_3 acid bath for at least 12 h prior to use. After cooling, the digests were paper filtered (589[1] Black ribbon ashless, Schleicher & Schuell) and diluted with UPW to a final volume of 100 mL. Yttrium (Y: 1 mg·L^{-1}) was added as an internal standard for the element determinations by inductively coupled plasma-optical emission spectroscopy (ICP-OES; Vista-MPX CCD, Varian, Australia). Element concentrations of all analyses are given based on the total solids (TS) content (Table 2.3), which was determined in triplicate by drying 1–2 g of sludge in Al pans at 105 °C until a constant weight was reached.

Table 2.3. Total solids content in anaerobic granular sludge from the methanol-fed UASB reactors (R1, R2; n = 3).

Days of operation	Total solids [%]	
	R1	R2
0	18.0±0.5	6.3±0.1
22	15.9±0.3	6.3±0.2
35	14.6±0.2	5.9±0.0
43	15.3±0.1	6.1±0.1

2.2.5. Sulfur fractionation and simultaneously extracted metals

The sulfur pool of the sludge was separated into three operationally defined fractions: acid-volatile sulfides (AVS), acid-soluble sulfur (ASS) and residual sulfur (S_{res}). Berner (1964) gave the first definition of AVS. The method is based on the formation of hydrogen sulfide from metal monosulfides after the addition of hydrochloric acid (1 M, analytical reagent grade; Eq. 2.1).

$$MeS + HCl \rightarrow H_2S\uparrow + Me^{2+} + 2\,Cl^- \quad (2.1)$$

Me = metal, *e.g.* Fe

The extraction setup consisted of gas washing bottles (100 mL; Duran®, Schott, Germany) (Fig. 2.1). Reaction vessel A was filled with 2 g of wet sludge and 40 mL of 1 M HCl in an anaerobic chamber. Vessel A was connected to the trapping vessel B (Fig. 2.1). Up to six samples were digested at one time using hose clamps to attain a similar gas flow in all vessels. The evolved H_2S was stripped from A by purging with inert gas (Ar, Indugas, The Netherlands) and trapped in B in approximately 40 mL 0.3 M NaOH (Nriagu and Soon, 1985) (Eq. 2.2).

$$H_2S + NaOH \rightarrow HS^- + Na^+ + H_2O \quad (2.2)$$

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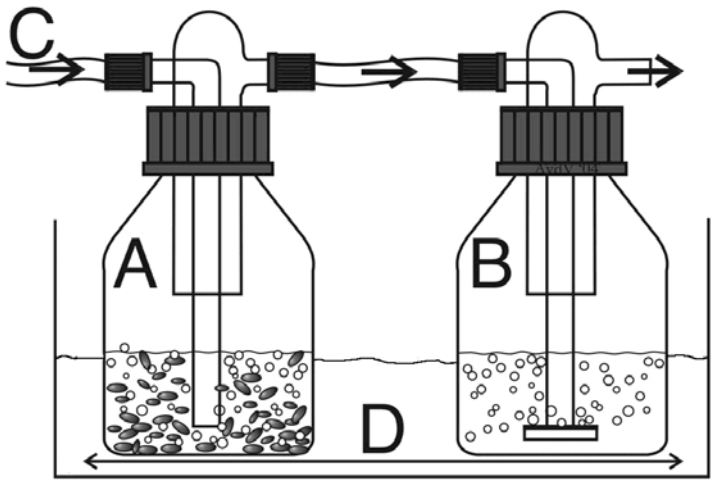

Figure 2.1. Setup for the extraction of acid-volatile sulfides (A – 100 mL reaction vessel; B – 100 mL trap for H_2S; C – inert gas stream (Ar); D – water bath with shaker).

The solution from A, which contains the ASS fraction, was filtered (589[1] Black ribbon ashless) in a 100-mL volumetric flask. The solid residue, containing S_{res}, was treated according to the determination of the total element content. The solution from B, containing AVS, was transferred to a 100-mL volumetric flask. Each of the three extracts (AVS, ASS, S_{res}) was filled with UPW to a final volume of 100 mL after the addition of an internal standard (Y: 1 mg·L^{-1}) for determination by ICP-OES. Three standard sets were used to match the matrix of the three extracts (AVS: 50 % 1 M HCl; ASS: 50 % 0.3 M NaOH; S_{res}: 10 % aqua regia). In solution A, the simultaneously extracted metals (SEM) (Di Toro et al., 1992) were also analyzed by ICP-OES. As a control, the sum (S_{sum}) of the three S fractions (AVS, ASS, S_{res}) is compared to the total S content (S_{tot}; Eq. 2.3):

$$[S_{sum}] = [AVS] + [ASS] + [S_{res}] \approx [S_{tot}] \pm 20\,\% \qquad (2.3)$$

Deviations are likely to occur due to inhomogeneities of the granular sludge and the use of wet sludge for the analyses (Cutter and Oatts, 1987). Regarding the potential errors, a deviation of ± 20 % was regarded as plausible. The HCl concentration (1 M) used in this study ensured that only sulfur from monosulfides and unbound sulfide was volatilized. Greigite (Fe_3S_4) dissolves only at higher HCl concentrations (3 M) (Cutter and Oatts, 1987). Pyrite (FeS_2) only dissolves in the presence of a stronger reductant like Cr(II) (Zhabina and Volkov, 1978), which releases also other trace metals associated with or bound in pyrite.

2.2.6. Bonding form analysis of metals

The bonding forms in the sludge were determined with a modified Tessier sequential extraction procedure as described by Osuna et al. (2004) (Table 2.4). The sequential extraction yields four bonding form fractions, which are referred to as: (1) adsorbed, (2) acid-soluble, (3) oxidizable and (4) residual. The extractions were performed in triplicate on subsamples of about 1 g wet sludge. The concentrations are given based on the total solid content determined in triplicate on subsamples. This scheme was chosen for its relatively good precision (van Hullebusch et al., 2005b). Reducible fractions, *e.g.* metals bound to Fe oxides, are not extracted because their extraction tends to oxidize the anoxic samples and thus overestimates metals bound to oxidized compared to reduced compounds (Ngiam and Lim, 2001). Above that, Fe and Mn oxides are also relatively scarce in anaerobic granular sludge (van Hullebusch et al., 2005b).

The transition from step 3 to 4 of the scheme was slightly modified. The extract and the residue of the third step were separated via filtration (589[1] Black ribbon ashless). The centrifuge vessels were rinsed with UPW. The filter together with the residual sludge is transferred to a microwave vessel for digestion. No water was added to the residue of the sludge prior to digestion. Regarding the anaerobic nature of the sludge, the method was further modified by maintaining anoxic conditions during the first two extraction steps. To prevent unwanted oxidation, the extraction solutions for these steps were purged with Ar for approximately 30 min. to remove dissolved oxygen (Butler et al., 1994) and manipulations took place in an anaerobic glove box.

Table 2.4. Modified Tessier sequential extraction procedure.

Fraction	Extracting agent	Extraction conditions Shaking time[a]	Temperature (°C)
1.Exchangeable	10 mL NH_4CH_3COO (1M, pH = 7)	1 h	20
2. Carbonates	10 mL CH_3COOH (1M, pH = 5.5)	1 h	20
3. Organic matter/sulfides	5 mL H_2O_2 (30%, pH = 2)	3 h	35
4. Residual	10 mL *Aqua regia* (HCl/HNO_3, 3:1)	26 min	Microwave-oven[b]

[a] Shaking was applied at 100 rpm
[b] Extraction of the residual fraction in the microwave was equal to the total extraction method.

As a control, the retrieval of each element was checked ($Element_{tot}$; Eq. 2.4). Regarding the potential errors, a deviation of ± 20 % was regarded as plausible. The possible causes for

deviations between the sum of the fractions and the total element content are the same as for the sulfur fractionation. Additionally, a loss of material during the successive extraction steps can occur.

$$[\text{Element}_{\text{sum}}] = \sum_{i=1}^{4} [\text{Element}_{\text{fract.i}}] \approx [\text{Element}_{\text{tot}}] \pm 20\,\% \qquad (2.4)$$

2.2.7. Correlation Analysis

Statistical data analysis has been performed with the statistical software package SPSS for Windows (Rel. 12.0.1. 2003; SPSS Inc., Chicago, IL, USA). The Spearman rank coefficient (r_s) and its statistical significance (α) was calculated for the element content of each reactor separately.

2.3. RESULTS

2.3.1. Total element content

The total element concentrations of the sludge from both reactors are plotted together with the bonding form distribution in Fig. 2.2-2.4. R1 is characterized by significantly higher concentrations of most of the elements than R2. In both sludges, no clear trend in enrichment or loss can be seen for most of the elements investigated (Fig. 2.2-2.4). Fe_{tot} increases strongly after startup of R1 (day 0: 27 ± 0 mg·g^{-1} TS compared to day 22: 30 ± 0 mg·g^{-1} TS). No clear trend of iron loss or enrichment can be seen afterwards. In R2, Fe_{tot} seems to decrease since startup, although the trend is not very strong (Fig. 2.4). Mg_{tot} is generally enriched in both R1 and R2 (Fig. 2.2). In contrast, Mn_{tot} of R1 shows a strong decrease in the first 22 days of operation (day 0: 405 ± 7 µg·g^{-1} TS; day 22: 210 ± 3 µg·g^{-1} TS). The loss continues, but it is not as high as in the first period (day 43: 187 µg·g^{-1} TS). In R2, Mn_{tot} is relatively stable (29 ± 5 µg·g^{-1} TS).

The difference in the influent composition of the two reactors is clearly visible in the enrichment of either Co (R1) or Ni (R2) in the sludge (Fig. 2.4). However, omission of either Co (R2) or Ni (R1) from the feed leads to only a very limited decrease in their concentration. In fact, Co_{tot} of R2 sludge can be regarded as stable (10 ± 1 µg·g^{-1} TS). In R1, Ni_{tot} remained stable over the first 35 days (Ni: 44 ± 2 µg·g^{-1} TS) and decreased significantly only after that time (day 43: 33 ± 2 µg·g^{-1} TS).

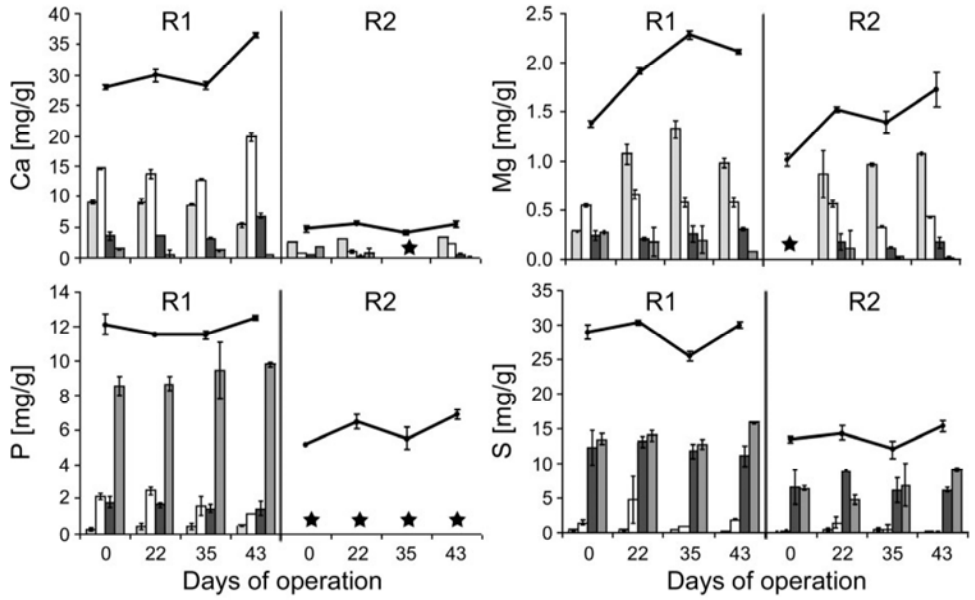

Figure 2.2. Bonding form distribution and total contents of Ca, Mg, P and S in anaerobic granular sludge from methanol-fed UASB reactors (R1, R2). ▫ Adsorbed; □ Acid-soluble; ■ Oxidizable; ▪ Residual ; ─ Total (★: data eliminated due to retrieval above/below criterium, cf. Eq. 2.4).

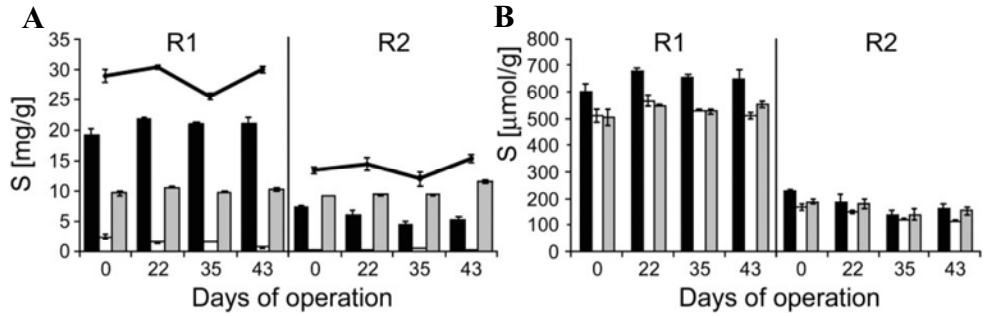

Figure 2.3. Development of S and Fe over a reactor operation time of 43 days in anaerobic granular sludge from methanol-fed UASB reactors (R1, R2). A: ─ Total S and S fractions (■ AVS: acid-volatile S; □ ASS: acid-soluble S; ▪ S_{res}: residual S). B: Molar concentrations of ■ AVS, □ simultaneously extracted Fe (Fe_{SEM}) and ▪ total Fe (Fe_{tot}).

2.3.2. Sulfur fractionation and simultaneously extracted metals

Fig. 2.3 shows the development of the S fractions and S_{tot} in the sludge during reactor operation. Although the sludges originate from different wastewater treatment plants and differ strongly in S_{tot} (R1: 28.6 ± 2.2 mg·g^{-1} TS; R2: 13.8 ± 1.4 mg·g^{-1} TS), both reactors contain about

the same amount of S_{res} over the investigated period (R1: 10.0 ± 0.4 mg·g^{-1} TS; R2: 9.8 ± 1.1 mg·g^{-1} TS). AVS instead shows a greater difference between both sludge types. In R1, AVS prevails with an average of 64 % of S_{sum}, while in R2 this fraction corresponds to 36 %. ASS is a minor component in both sludges (R1: 5 %; R2: 2 %). In R1, this fraction declines over time, while it is relatively stable in R2.

In the investigated sludges, the Fe concentration also dominates compared to the other metals (Table 2.5). The concentration of other sulfide-forming metals is very low compared to Fe (*e.g.* R1: Fe: 530 µmol·g^{-1} TS, sum of Co, Mn and Ni: 4 µmol·g^{-1} TS). In both reactors, the molar AVS concentration is clearly higher than the simultaneously extracted Fe (Fe_{SEM}) and Fe_{tot} (Fig. 2.3), which implies the presence of sufficient sulfide in the sludges to form other metal sulfides besides FeS. Examples for other metal sulfides, which might be present in the sludge, are FeS_2 and also trace metal sulfides like CoS or NiS. The probability of the formation of pure phases of trace metal sulfides is relatively low compared to the high content of Fe, therefore mixed metal sulfides will predominate.

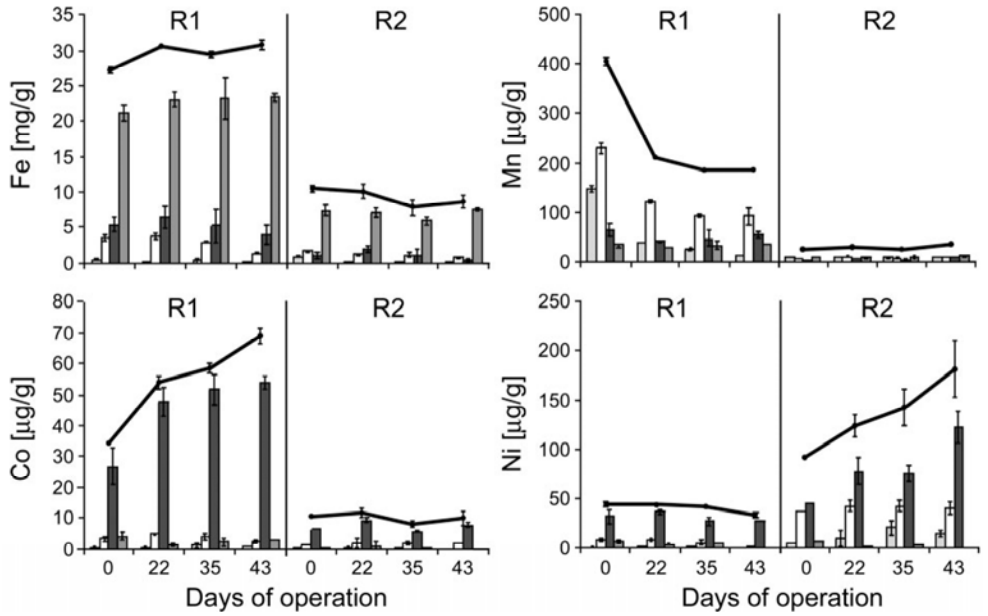

Figure 2.4. Bonding form distribution and total contents of Fe, Mn, Co and Ni in anaerobic granular sludge from methanol-fed UASB reactors (R1, R2). ■ Adsorbed; □ Acid-soluble; ■ Oxidizable; ■ Residual ; ─ Total.

Table 2.5. Sequential extractions performed under oxic (ox.) and anoxic (anox.) conditions (n = 4; averaged percentage of summed concentration of all four fractions ± standard deviation).

Fraction	Fe		Mn		Co		Ni		S		Zn	
	ox.	anox.	ox.	anox.	ox.	anox.	ox.	anox.	ox.	anox.	ox.	anox.
Adsorbed	2±0	1±1	33±4	31±1	2±0	2±1	2±1	2±1	0±0	1±0	2±1	3±2
Acid-soluble	11±8	11±2	40±7	50±4	8±5	10±3	9±7	18±6	1±1	5±2	6±2	6±1
Oxidizable	16±5	18±3	22±9	12±3	76±10	77±4	76±8	67±6	38±4	43±5	66±6	78±3
Residual	71±3	70±1	5±3	8±0	14±6	11±1	13±3	14±1	60±4	51±4	26±7	12±1

2.3.3. Bonding form distribution

2.3.3.1. Oxic vs. anoxic extraction.

A comparison of oxic vs. anoxic conditions during the sequential extraction shows that not all elements are significantly affected (Table 2.5). The bonding form distribution of Fe, Co and Cu (Table 2.5; Cu: data not shown) exhibits almost no change between the two extraction modes. In contrast, Mn, Ni and Zn are affected the most. For Mn and Ni, the changes occur in the acid-soluble and the oxidizable fraction. The acid-soluble fraction is lower under oxic than under anoxic conditions, while the oxidizable fraction behaves contrary. Therefore, the acid-soluble fraction must also contain metals from very reactive sulfides, which readily oxidize and will be removed from the sludge during sample preparation under oxic conditions. Zn shows a transition between the oxidizable and the residual fraction, while the potentially more mobile fractions (adsorbed and acid-soluble) are not affected. The oxidizable fraction is significantly higher under anoxic than under oxic conditions. The oxidized Zn is extracted as residual Zn. Therefore, oxic extraction of Zn leads to an underestimation of the potential mobility under changing redox conditions. Overall, it should be mentioned that the standard deviation for the oxic extraction mode is higher than that for the anoxic mode, making the latter extraction method more reliable.

2.3.3.2. Bonding form fractionation during reactor operation

In both reactors, Ca is mostly present in adsorbed and acid-soluble forms (Fig. 2.2). During operation of R1, Ca is lost from the adsorbed fraction (9.1–5.3 $mg \cdot g^{-1}$ TS). The amount of Ca in the acid-soluble fraction seems to follow Ca_{tot}. In R2, Ca increased in the acid-soluble fraction during reactor operation (0.9–2.4 $mg \cdot g^{-1}$ TS).

The bonding form distribution of Mg is similar in both reactors, which is dominated by the adsorbed fraction during reactor operation (50 % of Mg_{sum}). The increase of Mg_{tot} corresponds to an increase of the adsorbed fraction in both reactors during reactor operation. The residual fraction decreases over time in R1 and R2. In R2, a strong change of Mg in the acid-soluble fraction occurred.

Although the sequential extraction scheme is mostly focused on metal cations, simultaneously released P and S were also measured in the extracts because of the fixation of metals as *e.g.* phosphates or sulfides. For P only data for R1 is available due to the poor recovery of P in the extraction of granules from R2. P bound in the residual fraction predominates the bonding form distribution of R1 (65–76 % of P_{sum}) and increases over time (day 0: 8.5 $mg \cdot g^{-1}$ TS; day 43: 9.7 $mg \cdot g^{-1}$ TS). During reactor operation, there is a loss of P from the acid-soluble (-47 %) and the oxidizable (-20 %) fractions. Adsorbed P increases over time (day 0: 2 % of P_{sum}; day 43: 4 % of P_{sum}; Fig. 2.2).

In both reactors, S is mostly extracted in oxidizable (average: R1: 40–48 % of S_{sum}; R2: 46– 67 % of S_{sum}) and residual (average: R1: 46–58 % of S_{sum}; R2: 35–68 % of S_{sum}) forms. Adsorbed and acid-soluble S plays a minor role (*e.g.* adsorbed S: R1: 1 % of S_{sum}; R2: 3 % of S_{sum}). In R1, the difference between oxidizable and residual S is not large, except for day 43, when residual S exceeds oxidizable sulfur. In R2, the proportions between the two forms changes without a trend (Fig. 2.2).

Fig. 2.4 clearly shows that the residual fraction predominates the bonding form distribution of Fe in both reactors (70–90 % of Fe_{sum}). Over time there is a slight loss of Fe from the acid-soluble and the oxidizable fraction. The adsorbed fraction is almost negligible compared to the others (R1: 0.3–1.2 % of Fe_{sum}; R2: 1.5–8.2 % of Fe_{sum}).

In both reactors, Mn is present in relatively high amounts in all four bonding form fractions (Fig. 2.4). While Mn is almost evenly distributed over all fractions in R2, the acid-soluble fraction predominates in R1 (47–53 % of Mn_{sum}). As the Mn concentration of R2 changes little over time (29 ± 5 $\mu g \cdot g^{-1}$ TS), there is also little change in the bonding form distribution. The strong decrease of Mn in R1 (day 0: 410 ± 7 $\mu g \cdot g^{-1}$ TS; day 43: 187 ± 2 $\mu g \cdot g^{-1}$ TS) results from a decline of the adsorbed and the acid-soluble fraction, which is the strongest in the adsorbed phase. Here not only the absolute but also the relative concentration of Mn in the adsorbed phase declines steadily (day 0: 31 % of Mn_{sum}; day 43: 7 % of Mn_{sum}).

Feeding Co to R1 led to a significant increase in its concentration in the sludge of R1. This enrichment is reflected by the increase of the oxidizable bonding form, which prevails with more than 80 % of Co_{sum} (Fig. 2.4). The other fractions change very slightly over time. The sludge loses acid-soluble Co between day 22 and day 43. The bonding form distribution of Co in the sludge of R2 is predominated by the oxidizable fraction as well, although the relative content is slightly lower than in R1 (max. 80 % of Co_{sum}). The omission of Co from the influent of R2 did not lead to a significant decrease of Co_{tot} in the sludge (day 0: 11 ± 0 $\mu g \cdot g^{-1}$ TS, day 43: 10 ± 2 $\mu g \cdot g^{-1}$ TS). The fluctuation of Co_{tot} is reflected by the fluctuation of the oxidizable fraction. The disappearance of the adsorbed and the residual phase in R2 on day 43 is due to the low Co concentration, which fell below the detection limit.

The bonding form distribution of Ni, like Co, is predominated by the oxidizable fraction (Fig. 2.4), although at the higher concentrations of Ni in R2 other bonding forms become important as well (oxidizable: R1: 70–95 % of Ni_{sum}; R2: 50–70 % of Ni_{sum}). The enrichment of Ni_{tot} in R2 is accompanied by a strong increase of the oxidizable (day 0: 45 $\mu g \cdot g^{-1}$ TS; day 43: 123 ± 16 $\mu g \cdot g^{-1}$ TS) and a slight increase of the acid-soluble (day 0: 5 $\mu g \cdot g^{-1}$ TS; day 43: 15 ± 3 $\mu g \cdot g^{-1}$ TS) fraction. The omission of Ni from the influent to R1 led to its loss from the acid-soluble (-72 %) and the oxidizable fraction (-18 %).

2.4. DISCUSSION

2.4.1. Metal sulfides

The predominating role of sulfides in metal fixation in anaerobic systems is supported by the high AVS content (Fig. 2.3) and the high metal content in the oxidizable (containing both sulfidic and organic bonding forms) fraction (Fig. 2.4). Therefore, the bioavailability or mobility of essential trace metals in the UASB reactors are mainly controlled by the sulfide chemistry. As metal sulfides have a very low solubility product, it would be expected that these metals are non-bioavailable to the methanogenic consortia. Jansen et al. (2007) nevertheless showed that in most cases the dissolution rates of Co and Ni sulfides are not limiting to methanogenic activity in anaerobic wastewater treatment. It should be noted that this was based on batch tests with methanogenic enrichments and freshly precipitated metal sulfides. Ageing of sulfidic precipitates, which takes place in the sludge during reactor operation, will lower the dissolution rates and might therefore lower the metal bioavailability (Gonzalez-Gil et al., 2003).

The concentration of adsorbed metals is low in the anaerobic granular sludges of both reactors (Fig. 2.4), although they are continuously dosed via the influent. The reaction of metals with sulfide, which is present in sufficient concentration (Table 2.6), is fast enough to remove metals from the adsorbed fraction. The metals are removed relatively quickly from the solution via metal sulfide precipitation or replacement of Fe from FeS (Arakaki and Morse, 1993). The excess of AVS compared to metals of the SEM fraction leads to several possible explanations: the molar concentration difference could be explained either by the presence of free or dissociated sulfide in the granules or the metal sulfides could contain an excess of S (FeS_{1+x}). Fig. 2.5 demonstrates the excess of S (AVS) compared to Fe_{SEM} and Fe_{tot}.

In R1, AVS correlates positively with Co_{SEM} ($r_s = 0.830$, $\alpha = 0.01$, $n = 13$). Within the oxidizable fraction, some of the Fe (R1, R2), Co (R2) and Ni (R1) correlate positively with S (R1, Fe–S: $r_s = 0.708$, $\alpha = 0.01$, $n = 17$; R1, Ni–S: $r_s = 0.873$, $\alpha = 0.01$, $n = 17$; R2: Fe–S: $r_s = 0.793$, $\alpha = 0.01$, $n = 15$; R2, Co–S: $r_s = 0.743$, $\alpha = 0.01$, $n = 15$). It has to be noted that Co and Ni only correlate with S in the reactors, where they were not dosed during reactor operation. A possible cause might be, besides the operational character of the fraction, that different types of metal scavenging by sulfide contribute to the metal enrichment. Metals can form metal

sulfides not only via direct precipitation ($Me^{2+} + S^{2-} \rightarrow MeS$), but also *e.g.* via adsorption on metal sulfides (Morse, 1994; Morse and Luther Iii, 1999). X-ray analysis of sludge from the same full-scale reactors did not reveal any crystalline sulfides, *e.g.* pyrite (van Hullebusch et al., 2005b), in the sludge. This reported lack of detection might be explained by a multiplicity of metal sulfides involved and a detection limit of approximately 1 wt.-% of a mineral in a material depending on sample composition and preparation.

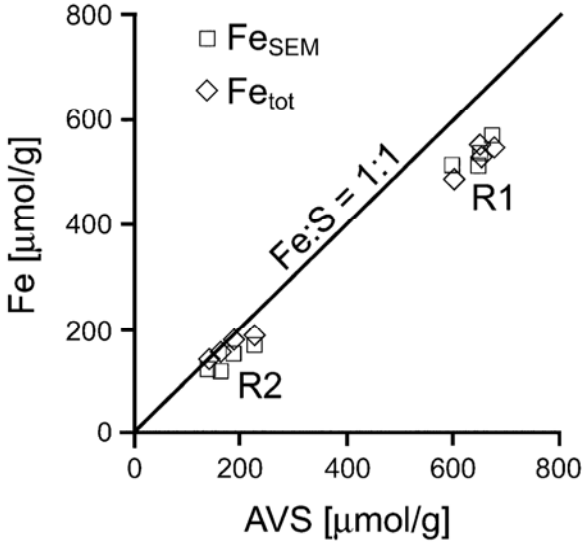

Figure 2.5. Scatterplot of simultaneously extracted Fe (Fe_{SEM}) and total Fe (Fe_{tot}) against acid volatile sulfide (AVS) in anaerobic granular sludge from R1 and R2.

Further characterization of residual S (S_{res}), *e.g.* by XANES, might be helpful in assessing metal mobility, *e.g.* the presence of stable metal sulfides (FeS_2, pyrite). The application of more elaborate S extraction schemes, yielding more specific fractions including elemental S and disulfides (Canheld et al., 1998; Van der Veen, 2004), which have been successfully applied to freshwater and marine sediments, can also contribute to a better characterization of the sulfur pool in anaerobic granular sludge.

Table 2.6. Simultaneously extracted metals (SEM) and acid volatile sulfide (AVS) compared to metals and sulfur of the oxidizable fraction (ox.) and the molar ratio of SEM/AVS in the anaerobic granular sludge from the methanol-fed UASB reactors (R1, R2).

Reactor	Days	Fe		Mn		Co		Ni		S		SEM/AVS
		[mg·g^{-1}TS]		[µg·g^{-1}TS]		[µg·g^{-1}TS]		[µg·g^{-1}TS]		[mg·g^{-1}TS]		Molar
		SEM	ox.	SEM	ox.	SEM	ox.	SEM	ox.	AVS	ox.	ratio
R1	0	28.5±1.3	5.4±1.0	381±28	64±12	26±1	27±6	31±3	32±7	19.3±1.0	12.2±2.5	0.87±0.01
	15	31.7±1.2	6.4±1.5	191±5	40±0	33±1	48±5	33±1	36±2	21.7±0.4	13.1±0.7	0.85±0.02
	35	29.7±0.1	5.2±2.3	161±1	44±15	32±1	51±5	31±1	29±2	20.9±0.4	11.7±1.0	0.83±0.02
	43	28.5±0.7	4.0±1.4	141±4	53±4	33±1	55±1	28±2	26±2	20.9±1.1	11.1±1.5	0.80±0.02
R2	0	9.3±0.7	1.0±0.5	14±1	4	7±1	7±0	80±6	45±0	7.3±0.3	6.6±2.5	0.77±0.03
	15	8.5±0.3	1.9±0.5	19±3	6±1	7±0	9±1	106±3	84±16	6.0±0.9	9.0±0.1	0.86±0.10
	35	6.8±0.2	1.0±1.0	18±0	4±1	6±0	6±0	110±1	78±8	4.5±0.4	6.1±1.8	0.94±0.07
	43	6.5±0.2	0.4±0.2	23±1	10±1	9±0	7±0	118±2	123±16	5.2±0.6	6.2±0.3	0.78±0.07

2.4.2. Sulfur chemistry

The extracted S fractions of the two applied extraction schemes and total element content (AVS, ASS, S_{res}; adsorbed S, acid-soluble S, oxidizable S, residual S; S_{tot}) do not correspond to each other with just one exception: the only correlation in between the methods (bonding form analysis, S fractionation, total content) was detected between S_{tot} and acid-soluble S of R1 (Spearman correlation coefficient: $r_s = 1.000$, $\alpha = 0.01$, $n = 4$). A weakness of the statistical comparison of the results between different methods is the small number of results due to the necessary computation of average values ($n \leq 4$) compared to the total number of analyses for each method ($n \leq 12$).

The low correspondence of the extraction results can also be assigned to the operational character of the S fractions. For example the oxidizable S in the bonding form analysis contains S from other sources besides monosulfides (*i.e.* AVS), which include disulfides and organically bound sulfur. The related fraction of the sulfur fractionation scheme is AVS, which, in contrast to the oxidizable fraction, is only composed of S from dissolved sulfide, sulfide minerals (mostly mackinawite, FeS), sulfide clusters and nanoparticles (Morse and Rickard, 2004). Comparing the methods, AVS is the more specific S fraction of the two extraction schemes.

The correlation analysis for the S fractionation with the variables AVS, ASS and S_{res} shows that most of the S fractions seem to be independent from each other. R1 shows a significant positive correlation between AVS and S_{res} (R1: $r_s = 0.714$, $\alpha = 0.01$, $n = 13$; Fig. 2.6). A negative correlation between ASS and AVS, as a follow-up to the reduction of sulfate (ASS) and the subsequent formation of sulfide (AVS), could not be detected. This might be explained by a wash-out of part of the sulfate from the sludge via the effluent, which would eventually disturb the relationship of sulfate reduction and sulfide formation.

2.4.3. Bonding form distribution and bioavailability of selected elements

The relative bonding form distribution of the inoculum is similar to those reported by Zandvoort et al. (2003), although some differences can be seen especially in sludge from Eerbeek (R1). Co, Ni and Fe in the sludge from Nedalco (R2) show only minor deviations compared to Zandvoort et al. (2003). The bonding form distribution of Mn in the sludge from Nedalco was predominated by the acid-soluble fraction (Zandvoort et al., 2003), while Mn is now almost evenly distributed over all four fractions. These differences might be explained by differences in operation conditions of the full-scale reactor between both studies, ageing of the sludge after sampling, or by differences in the sample handling and the extraction protocol (oxic vs. anoxic). The operating conditions and the wastewater composition of the full-scale reactor might have the strongest influence, since Mn_{tot} is a factor two lower in the present study.

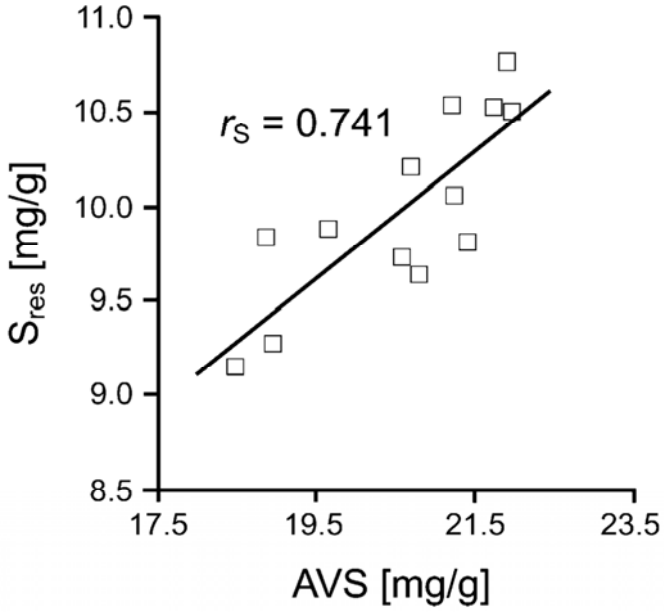

Figure 2.6. Scatterplot and trendline of acid volatile sulfide (AVS) and residual S (S_{res}) in granular anaerobic sludge from R1.

The Eerbeek sludge shows more prominent differences. In the present study, Co is clearly dominated by oxidizable bonding forms, while there was almost no dominance of one of the fractions before (Zandvoort et al., 2003). The oxidizable fraction of Ni predominates in the material for this study, before Ni was retrieved mostly in the residual fraction. The predominance of the adsorbed and the acid-soluble fraction of Mn is now even stronger than in Zandvoort et al. (2003). In the present study, Fe of R1 is clearly dominated by the residual fraction, while Fe_{res} is only slightly higher than the other fractions in inoculum sludge of previous studies (Zandvoort et al., 2003). The divergence could have been caused by an oxidation of the sample followed by release of Fe from oxidized FeS and thus a higher acid-soluble content in the study of Zandvoort et al. (2003). The high Fe_{res} might also be attributed to a readsorption of Fe to the oxidized sample matrix at the third step (Jong and Parry, 2004b). The high concentrations of Fe_{res} are opposed to the high concentrations of Fe_{SEM} that are almost equal to Fe_{tot} in R1 (Fig. 2.3).

The potential bioavailability of metals can be assessed from the bonding form distribution although the fractions are operationally-defined and not phase-specific. The fractions possess a decreasing solubility/reactivity from the first to the last step. This decreasing solubility can be used as a measure of potential bioavailability (Jong and Parry, 2004a), regarding the first fraction, *i.e.* adsorbed, the most bioavailable and the last fraction, *i.e.* residual,

the least bioavailable. Since studies have shown that even sulfides are bioavailable for methanogenic bacteria under anoxic conditions (Gonzalez-Gil et al., 2003), the first three fractions can be added up for a ranking in potential bioavailability. At startup Mn has the highest bioavailability in R1 (Mn > Co > Ni >> Fe), while in R2 Co and Ni have similar potential bioavailability (Co = Ni > Mn >> Fe). During reactor operation, the ranking only changes in R1 (Ni > Co > Mn >> Fe). When taking only the most soluble fractions into account (adsorbed and acid-soluble), Mn has a much higher potential bioavailability compared to the other metals in R1. This is caused by the predominance of the oxidizable fraction in Co and Ni compared to Mn. The effect on Fe is smaller since the bonding form distribution is dominated by the residual fraction (Fig. 2.4). The poor bioavailability of Fe is almost equal to Co or Ni (R1: startup: Mn >> Ni > Fe > Co, day 43: Mn >> Ni > Co > Fe; R2: startup: Mn > Ni > Fe > Co, day 43: Mn > Ni > Co > Fe).

Although the sequential extraction scheme is not explicitly designed for P fractionation, the P content of the extracts was measured as well, since P compounds can fix metals, *e.g.* Fe phosphate (vivianite: $Fe_3(PO_4)_2 \cdot 8H_2O$). This can be used as an assessment of the potential mobility of phosphorus. According to the applied extraction scheme, P is relatively immobile (R1), since 65–76 % was found in the residual fraction. The investigation of P bonding forms with a special P fractionation scheme (Ruban et al., 2001) in combination with the measurement of simultaneously released metals in the P extracts, similar to SEM of the S extraction scheme, could give a better insight into the influence of P on metal bonding forms.

Mn_{tot} of R1 strongly decreased during reactor operation. The trend might be partly assigned to biomass growth, but the dominating effect is most probably the high potential mobility of Mn in reducing environments (Berner, 1970). This can be seen by the high portion of the mobile fractions (adsorbed and acid-soluble Mn) in the sludge. The high potential mobility of Mn can also be derived from the high Mn_{SEM} content, which corresponds to 75–93 % of Mn_{tot} in R1. The bonding form distribution shows that the decrease takes place via a loss from the more mobile phases, which supports the wash-out of Mn via the effluent. These findings confirm the rather poor capacity of UASB reactors to remove Mn from wastewater (Ginter and Grobicki, 1997).

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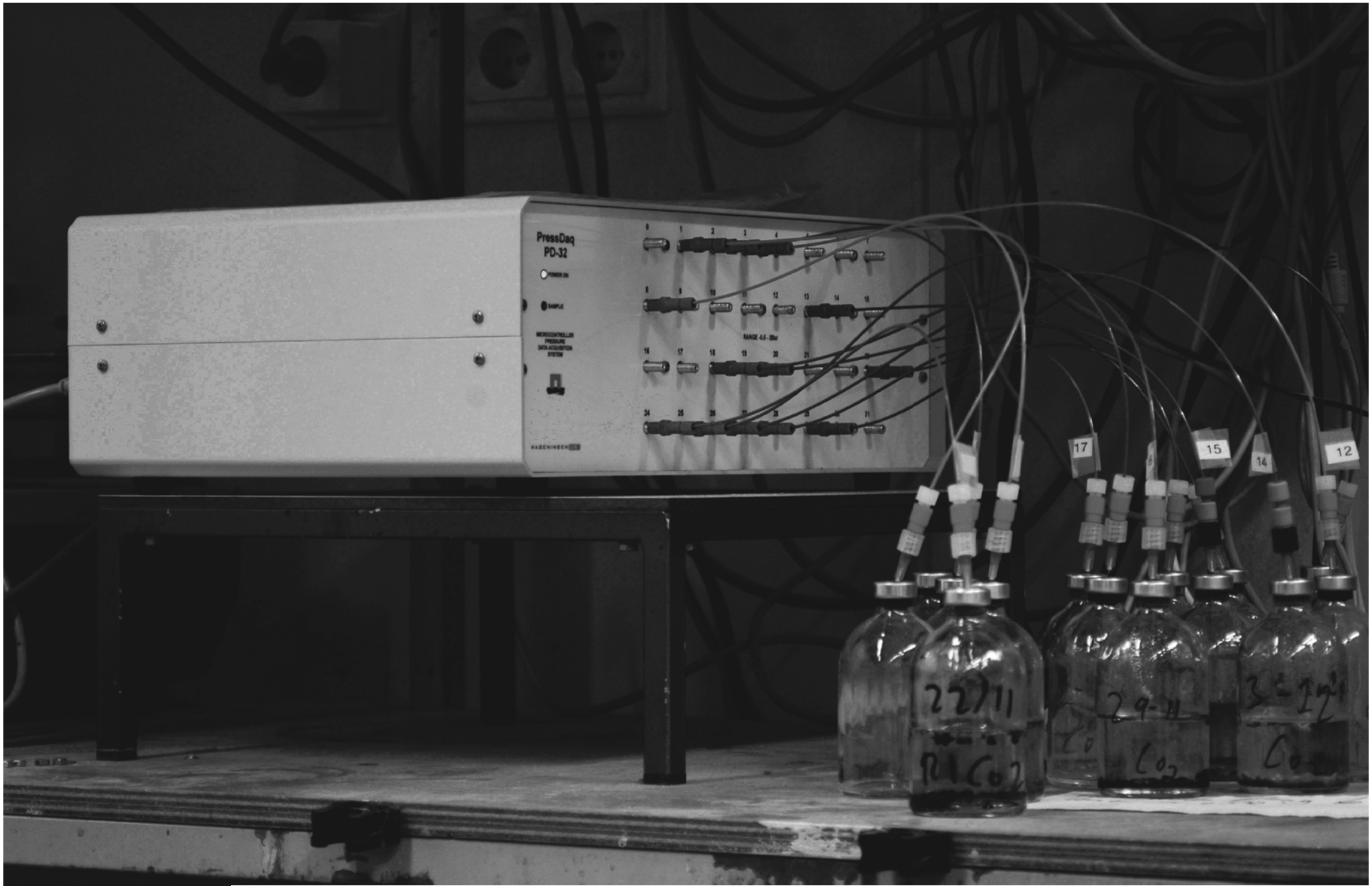
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ACIDIFICATION OF METHANOL-FED ANAEROBIC GRANULAR SLUDGE BIOREACTORS BY COBALT DEPRIVATION: INDUCTION AND MICROBIAL COMMUNITY DYNAMICS

ABSTRACT

The acidification of mesophilic (30°C) methanol-fed Upflow Anaerobic Sludge Bed (UASB) reactors induced by cobalt deprivation from the influent was investigated by coupling the reactor performance (pH 7.0; organic loading rate 4.5 g $COD \cdot L^{-1} \cdot d^{-1}$) to the microbial ecology of the bioreactor sludge. The latter was investigated by specific methanogenic activity (SMA) measurements and fluorescence in situ hybridization (FISH) to quantify the abundance of key organisms over time. This study hypothesised that under cobalt limiting conditions, the SMA on methanol of the sludge gradually decreases, which ultimately results in methanol accumulation in the reactor effluent. Once the methanol accumulation surpasses a threshold value (about 8.5 mM for the sludge investigated), reactor acidification occurs because acetogens outcompete methylotrophic methanogens at these elevated methanol concentrations. Methanogens present in granular sludge at the time of the acidification do not use methanol as the direct substrate and are unable to degrade acetate. Methylotrophic/acetoclastic methanogenic activity was found to be lost within 10 days of reactor operation, coinciding with the disappearance of the *Methanosarcina* population. The loss of SMA on methanol can thus be used as an accurate parameter to predict reactor acidification of methanol-fed UASB reactors operating under cobalt limiting conditions.

3

3.1. INTRODUCTION

Several types of high-rate methanogenic reactors are applied to treat wastewaters predominantly containing methanol (Weijma and Stams, 2001). In these reactors, methanol is converted to methane via several pathways, i.e. direct conversion to methane by methylothrophic methanogens (Nishio et al., 1992), indirect conversion to acetate by acetogens (Van der Meijden et al., 1984) coupled to acetoclastic methanogenesis (Huser et al., 1982) or indirect conversion to H_2 and CO_2 (Cord-Ruwisch et al., 1988) coupled to hydrogenotrophic methanogenesis (Whitman et al., 1982). Cobalt plays an important role in the direct conversion of methanol to methane, as it forms the metallocenter of corrinoid compounds that catalyze the methyl transfer from methanol to methyl-coenzyme M, the common precursor of methane for all methanogenic substrates (Thauer, 1998).

Cobalt also regulates the methanol degradation pathway under anaerobic conditions by affecting the different trophic groups (acetogens or methanogens) involved in methanogenic methanol conversion (Florencio et al., 1994; Florencio et al., 1993). Acetogens have a lower substrate affinity for methanol (K_s = 16 mM) compared to methanogens (K_s = 0.25 mM) (Florencio et al., 1995). Thus, acetogens can only outcompete methanogens at high methanol concentrations. Acidification of upflow anaerobic sludge bed (UASB) bioreactors is the main concern for stable operation of methanol-fed anaerobic wastewater treatment bioreactors. It is due to an imbalance between acetate formation from methanol by acetogenic bacteria and acetate consumption by methanogenic bacteria. Previous research has suggested that UASB acidification is related to elevated methanol concentrations in the bioreactor, which can be induced by the activity decrease due to depletion of the sludge by cobalt (Zandvoort et al., 2002a), but also nickel (Zandvoort et al., 2002b) and even iron (Zandvoort et al., 2003).

Reactor acidification of methanol-fed UASB reactors might proceed via the algorithm presented in Fig. 3.1. According to this hypothesis, acidification is triggered by elevated methanol concentrations in the reactor mixed liquor, which can occur in the case of bioreactor overloading or when the specific methanogenic activity (SMA) of anaerobic granular sludge on methanol gradually decreases, e.g. under metal limiting conditions. Once the methanol accumulation surpasses a threshold value, reactor acidification, i.e. acetate accumulation, will occur (Fig. 3.1). This is due to the development of the homoacetogenic population, induced by their high K_s (Florencio et al., 1995), coupled to a methanogenic population with a sufficiently low acetoclastic activity to restrict conversion of the formed acetate to methane. The latter may be due to the development of a methanogenic community capable only of methylotrophic methanogenesis. For example, methanol-grown cells of *Methanosarcina* sp. strain TM-1 produce methane from acetate alone at a rate about 4% of that from methanol (Zinder, 1985).

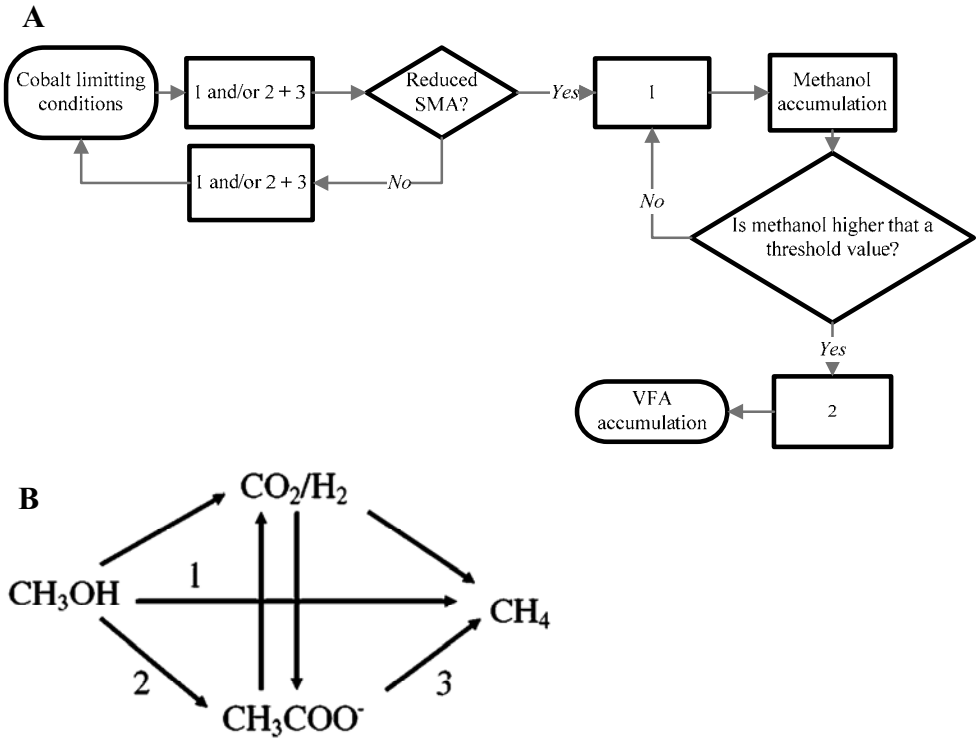

Figure 3.1. A. Proposed outline of the mechanism of induction of cobalt limitation in methanol-fed UASB reactors. B. Main pathways in methanol removal. (1) methylotrophic methanogenesis. (2) acetogenesis. (3) acetotrophic methanogenesis. SMA: specific methanogenic activity with methanol as the substrate.

The aim of this study was to validate the outline of reactor acidification due to cobalt depletion, as presented in Figure 3.1, by coupling bioreactor performance with biomass SMA measurements and the abundance of key organisms. Cobalt deprivation was compared to total metal deprivation by running a second reactor under the same conditions, but from which all metals were omitted from the influent wastewater.

3.2. MATERIALS AND METHODS

3.2.1. Source of biomass

Methanogenic granular sludge was obtained from a full-scale UASB reactor treating alcohol distillery wastewater at Nedalco (Bergen op Zoom, The Netherlands). The total suspended solids (TSS) and volatile suspended solids (VSS) concentration of the wet sludge were 5.23 (± 0.02)% and 4.93 (± 0.03)% (w/w), respectively. The initial cobalt concentration of the sludge was relatively low (11 μg ·g TSS^{-1}).

3.2.2. UASB reactor operation

Two Plexiglas cylindrical UASB reactors, R1 and R2, which were of the same design as described by Zandvoort et al. (2002a), were employed. Each reactor was inoculated with 2.2 l wet sludge. A hydraulic retention time (HRT) of 8 h, a superficial liquid upflow velocity of 0.1 $m \cdot h^{-1}$ and an organic loading rate (OLR) of 4.5 g CH_3OH-$COD \cdot L^{-1} \cdot d^{-1}$ were applied for both reactors.

To avoid precipitation in the storage vessels, R1 and R2 influents were composed of three separate streams: (1) macronutrients (NH_4Cl, $MgSO_4 \cdot 7\,H_2O$, $CaCl_2 \cdot 2\,H_2O$); (2) methanol with $NaHCO_3$ and K_2HPO_4; and (3) dilution water. Tap water (Co < 1 $\mu g \cdot L^{-1}$) was used to prepare the influent and as dilution water. The pH of the reactor liquid was set to pH 7.0 (± 0.2) by the addition of 2.52 g $NaHCO_3 \cdot L_{influent}^{-1}$. In addition, a trace metal solution, from which cobalt was omitted (Table 3.1), was added to the R2 feed (as part of stream 1).

Table 3.1. Composition of trace metal solution added to R2 influent and activity tests media.

Compound	Metal	Conc. [µM]
$FeCl_2 \cdot 4H_2O$	Fe (II)	5
$CuCl_2 \cdot 2H_2O$	Cu (II)	0.5
$ZnCl_2$	Zn (II)	0.5
$MnCl_2 \cdot 4H_2O$	Mn (II)	0.5
$NiCl_2 \cdot 6H_2O$	Ni (II)	0.5
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	Mo (VI)	0.5
$Na_2SeO_2 \cdot 5H_2O$	Se (VI)	0.5
$Na_2WoO_4 \cdot 2H_2O$	W (VI)	0.5
$CoCl_2 \cdot 6H_2O$[a]	Co (II)	0.5

a. Not added in R2 influent, added at operation day 103 in R1 influent.

3.2.3. Specific methanogenic activity tests

The SMA of the inoculum and temporal samples of the reactor biomass was determined in duplicate at 30 (± 2)ºC using on-line gas production measurements as described by Zandvoort et al. (2002a). The SMA of the sludge present in R1 and R2, with methanol (2 g $COD \cdot L^{-1}$) as the substrate, was determined at day 2, 42, 63 (before volatile fatty acids (VFA) accumulation in R2), 73 (VFA accumulation in R2), 91 (before VFA accumulation in R1 and conclusion of R2) and 103 (R1 only - before cobalt addition). The SMA with acetate (2 g $COD \cdot L^{-1}$) as the substrate was determined at day 42 and day 63 in both reactors and at day 91 in R2.

Sludge samples were taken from the middle of the sludge bed on day 63 and 73 in R1 and R2, respectively, as well as on day 91 and 103 in R1. To study potential segregation of the SMA

in the sludge bed, samples were taken from the top and the bottom of the sludge bed on day 42 in R1 and R2, and on day 91 in R2.

3.2.4. Fluorescent in situ hybridization

Anaerobic granules were fixed in 4% (w/v) paraformaldehyde in phosphate buffered saline (PBS; 130 mmol·L^{-1} sodium chloride and 10 mmol·L^{-1} sodium phosphate [pH 7.2]) and cross-sections (thickness, 16 µm; diameter, 0.5-1.5 mm) were prepared as described previously by Sekiguchi et al. (1999). Fluorescent in situ hybridization (FISH) was performed as described by Schramm et al. (1998) using Cy3-labeled 16S rRNA-targeted oligonucleotide probes (Biomers.net, Germany): *Eury498* (Burggraf et al., 1994) and *Sarci551* (Sorensen et al., 1997), specific for the Kingdom *Euryarchaeota* and *Methanosarcina* genus, respectively. Specimens were viewed using a Nikon E600 epifluorescent microscope equipped with a 100 W mercury bulb and a Cy3 filter set. The abundance of *Euryarchaeota* or *Methanosarcina* was determined as a fraction of the total area of respective granule sections.

3.2.5. Chemical analyses

Methanol and VFA concentrations were determined using gas liquid chromatography as described by Weijma et al. (2000). The total suspended solids (TSS) and volatile suspended solids (VSS) concentrations were determined according to Standard Methods (APHA/AWWA 1998). All chemicals were of analytical or biological grade and were purchased from E. Merck AG (Darmstadt, Germany).

3.3. RESULTS

3.3.1. Effect of trace metals and cobalt deprivation on reactor performance

Methanol removal was complete in both reactors during the first 33 days of operation (Fig. 3.2). The increase in influent methanol concentration to 1800 mg COD·L^{-1} on day 33 resulted in methanol accumulation in R1 and R2 effluent. Following this, the R1 and R2 influent methanol concentration were reduced to 1500 mg COD·L^{-1} on day 40, which facilitated complete methanol removal again until day 60.

Methanol accumulation was detected in R2 (solely cobalt deprivation) effluent on day 60, reaching a value of 544 mg COD·L^{-1} on day 69, when VFA were also observed in the effluent. From day 69, methanol and VFA accumulation in R2 effluent increased to a maximum of 870 mg COD·L^{-1} (on day 76) and 676 mg COD·L^{-1} (on day 81), respectively. VFA accumulation remained constant and methanol accumulation decreased to 440 mg COD·L^{-1} at the end of the R2 trial (day 91). Concurrently (day 60 to 90), only methanol accumulation was observed in the R1 (deprivation of all trace metals) effluent (maximum, 520 mg COD·L^{-1}). No VFA

accumulation was observed in R1 during this period. Only on day 90, when the methanol concentration in the effluent had increased to 460 mg COD·L^{-1}, VFA were detected in the effluent. From day 90 until day 105, both methanol and VFA accumulated in the R1 effluent at maximum levels of 1550 mg COD·L^{-1} and 260 mg COD·L^{-1}, respectively (Fig. 3.2). The biogas production of both reactors decreased during VFA accumulation (from day 90 and day 60 onwards in R1 and R2, respectively; Fig. 3.3).

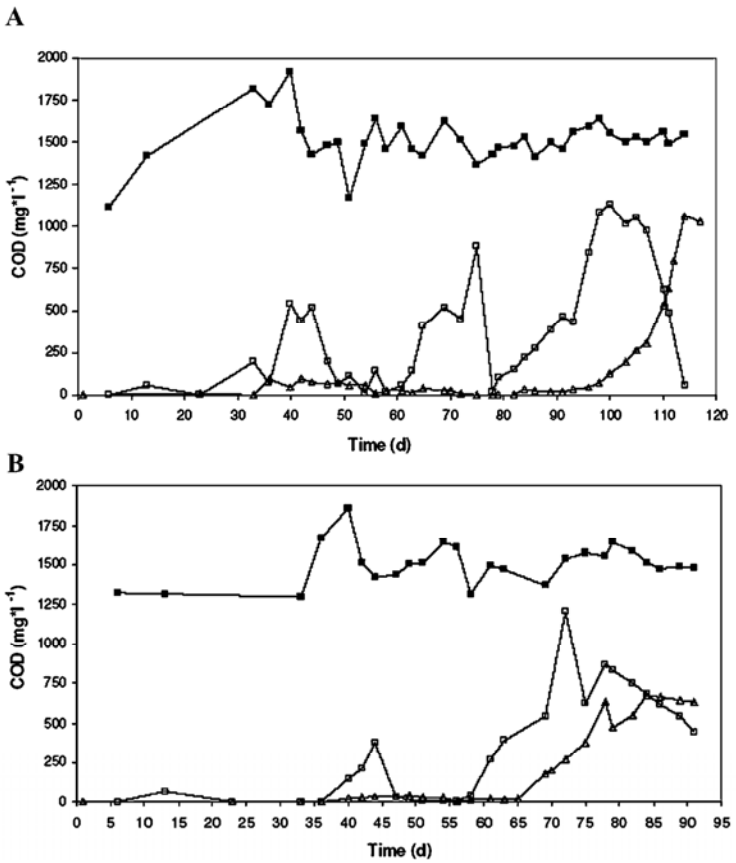

Figure 3.2. Evolution of reactor performance of (A) R1 and (B) R2 with time. Influent methanol (■), effluent methanol (□) and the effluent VFA (Δ) concentration.

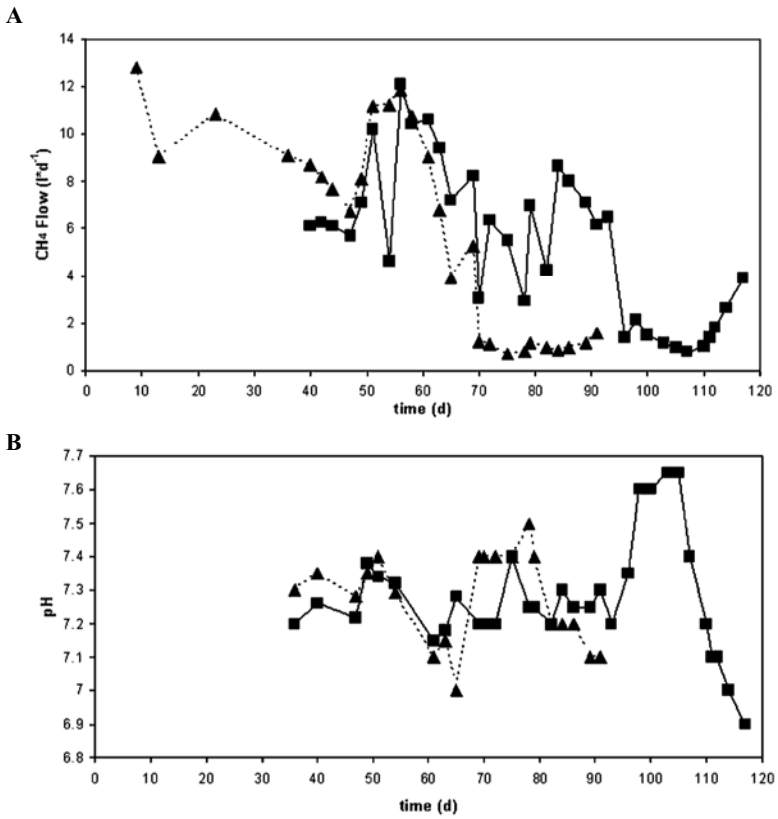

Figure 3.3. Evolution of (A) CH_4 production and (B) pH in the effluent of R1 (-■-) and R2 (-▲-) with time.

The highest SMA of the R1 sludge with methanol as the substrate on day 103 was observed during an assay performed using a trace metal solution containing only cobalt (Table 3.2). In order to test whether methanol conversion could be stimulated, cobalt was dosed (0.5 μM) to the R1 influent from day 105 until the end of the experiment (day 117). A reduced R1 effluent methanol concentration (55 mg $COD \cdot L^{-1}$) was observed following cobalt addition. However, the VFA production was stimulated (up to 1060 mg $COD \cdot L^{-1}$) of which acetate represented up to 96%, leading to reactor acidification (pH dropped from 7.6 to 6.9, Fig. 3.3).

Table 3.2. Evolution of the specific methanogenic activity (mg CH_4-COD·g VSS^{-1}·d^{-1}) with methanol as the substrate as a function of time and trace metal content of the medium.

R1	Day of operation		Specific maximum methanogenic activity [mg CH_4-COD·g VSS^{-1}·day^{-1}]				Relative abundance[a]	
			No metals	Complete trace metal solution except cobalt	Complete trace metal solution	Cobalt solely	*Eury498*	*Sarci551*
	2		380±50	345±12	655±62	498	+++	+++
	42	Top	353±7	349±20	1288±90	ND	ND	++
		Bottom	357±19	288±40	1324±122	ND	ND	+
	63		368±20	287	1550	ND	ND	+++
	73		386±33	278±12	1677±36	ND	ND	+++
	91		358	334±3	1695±76	ND	+++	++
	103		121±26	65±18	115±25	140	ND	+
	117		ND	ND	ND	ND	++	+
R2	Day of operation		Specific maximum methanogenic activity [mg CH_4-COD·g VSS^{-1}·day^{-1}]				Relative abundance[a]	
			No metals	Complete trace metal solution except cobalt	Complete trace metal solution	Cobalt solely	*Eury498*	*Sarci551*
	2		380±50	345±12	655±62	498	+++	+++
	42	Top	372±7	366±14	1414±57	ND	ND	++
		Bottom	396±4	446±35	1327±27	ND	ND	+
	63		320±6	196±48	1583±230	ND	ND	++
	73		45±3	23±16	75±3	ND	ND	+
	91	Top	ND	135±11	271±27	465±23	+	-
		Bottom	ND	137±1	643±12	859±59	+	-

ND: not determined; [a]Relative abundance calculated as proportion of hybridized cells with *Eury498* or *Sarci551* as a function of total section area (- = no positive cells detected).

3.3.2. Evolution of the SMA on methanol

Zandvoort et al. (2002a) demonstrated that cobalt addition stimulated the SMA of the inoculum used in this study. On day 2 of reactor operation, the SMA increased from 380 to 498 mg CH_4-COD·g VSS^{-1}·d^{-1} in batch assays as a result of 0.5 μM Co^{2+} addition to a medium without metals. This cobalt concentration increased the SMA of the latter sludge from 345 to 655 mg CH_4-COD·g VSS^{-1}·d^{-1} in a medium with complete trace metal solution except cobalt (Table 3.2).

After 42 days of reactor operation, the SMA in a medium with complete trace metal solution except cobalt was similar to that on day 2 (Table 3.2; Fig. 3.4C,E). However, the time

required to reach the maximum activity was shorter than day 2 (230 hours on day 2 and 50 hours on day 42), which is likely to be attributed to adaptation of the sludge to methanol. The addition of Co^{2+} (0.5 μM) to the medium (containing the complete trace metal solution except cobalt) induced a 100% increase in the SMA compared to that obtained on day 2 (Table 3.2; Fig. 3.4A,D).

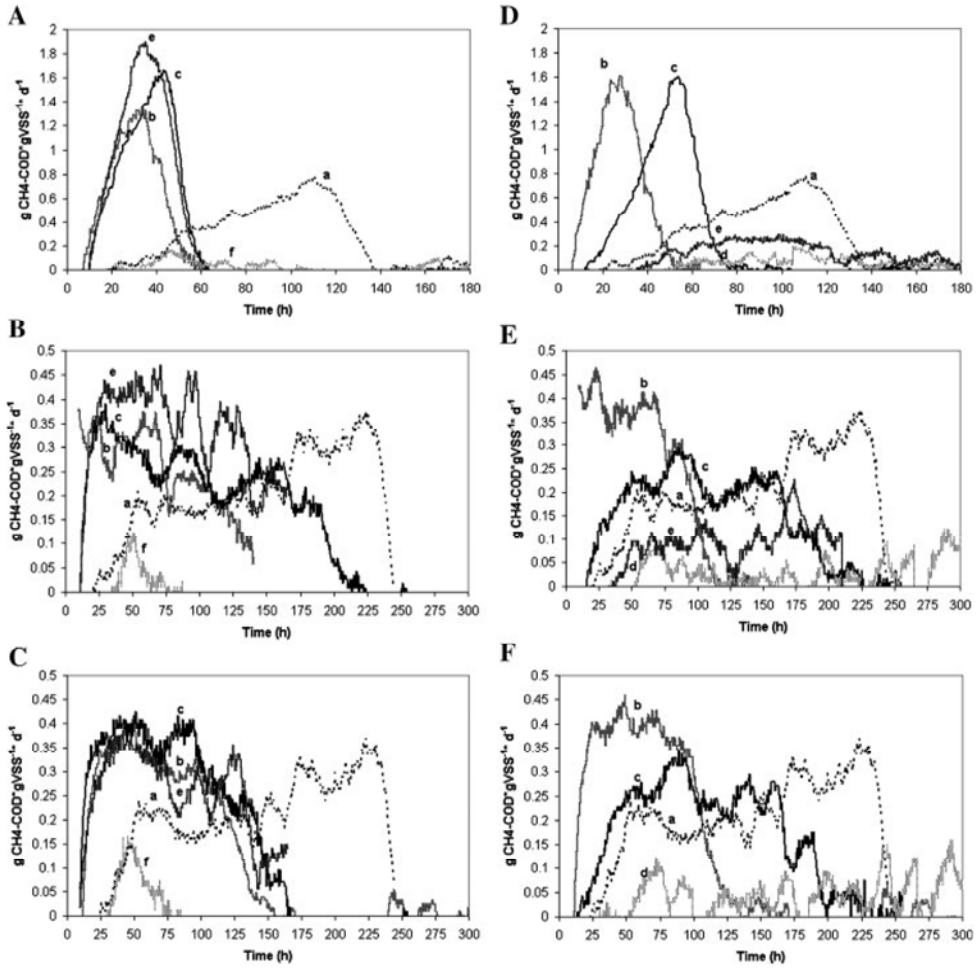

Figure 3.4. Specific methanogenic activity (pH 7; 30°C) with methanol as the substrate. (A, D) Test medium containing the complete trace metal solution, utilizing sludge from R1 and R2, respectively. (B, E) Test medium containing the complete trace metal solution except cobalt, utilizing sludge from R1 and R2, respectively. (C, F) Test medium no containing metals, utilizing sludge from R1 and R2, respectively. a, b, c, d, e and f represent the sludge present in the reactor after 2, 42, 63, 73, 91 and 103 days, respectively.

On day 63, when methanol accumulation was observed in R2, the SMA had decreased to 196 mg CH_4-COD·g VSS^{-1}·d^{-1} in a medium containing the complete trace metal solution except cobalt. The addition of Co^{2+} resulted in a similar SMA to that achieved on day 42 under similar conditions. On day 73, when VFA accumulation (R2) was observed, the SMA had decreased to as low as 23 mg CH_4-COD·g VSS^{-1}·d^{-1} in a medium containing the complete trace metal solution except cobalt. Surprisingly, this time, the addition of Co^{2+} to the medium did not significantly increase the SMA. At the conclusion of the R2 trial (day 91), when the effluent methanol concentration was 440 mg COD·L^{-1}, the SMA was slightly increased (136 mg CH_4-COD·g VSS^{-1}·d^{-1} in a medium with the same metal concentration as in the R2 influent. Addition of Co^{2+} to the medium only marginally increased the SMA of the R2 sludge (Table 3.2).

The SMA of the R1 sludge on days 63, 73 and 91 was similar to the values obtained on day 42 for all the metal media investigated (Table 3.2; Fig. 3.4 A,B,C). However, on day 103, when the VFA accumulated in the R1 effluent, the SMA had decreased to as low as 65 mg CH_4-COD·g VSS^{-1}·d^{-1} in a medium with complete trace metal solution except cobalt. Addition of 0.5 μM Co^{2+} (complete trace metal solution) did not significantly increase the SMA.

3.3.3. Evolution of SMA with acetate as the substrate

Sludge samples from the top and the bottom of both R1 and R2 had a moderate acetoclastic methanogenic activity (525 mg CH_4-COD·g VSS^{-1}·d^{-1}) on day 42. This methanogenic activity on acetate was completely lost in both reactors between days 42 and 63 (Fig. 3.5). This loss of acetoclastic methanogenic activity due to cobalt depletion occurred prior to the VFA accumulation. On day 91, when the VFA concentration in R2 effluent was 600 mg COD·L^{-1}, the SMA of R2 sludge on acetate was determined and no methane production was detected at the conclusion of the assay (91 days of incubation).

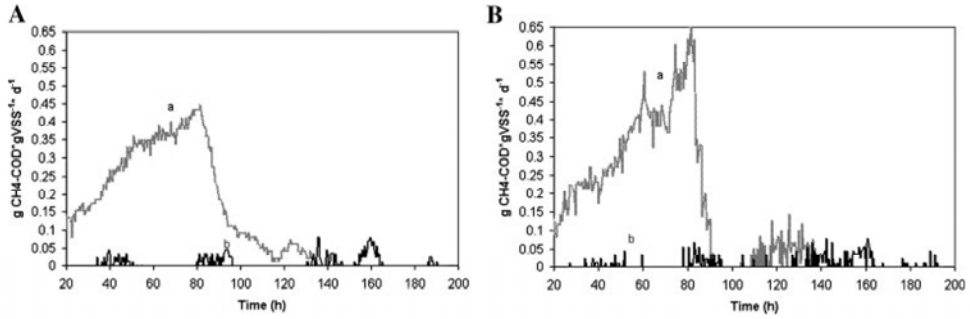

Figure 3.5. Specific methanogenic activity (pH 7; 30°C) with acetate as the substrate. (A, B) Test medium containing the complete trace metal solution except cobalt, utilizing sludge from R1 and R2, respectively. a and b represent the sludge present in the reactor after 42 and 63 days of operation, respectively.

3.3.4. Effect of sample location in the sludge bed on SMA

On day 42, there was no difference in the SMA with methanol as the substrate between sludge samples from the top and the bottom of R1 and R2 (Table 3.2). However, sludge taken from the bottom of R2 on day 91 had a 2.5 times higher SMA compared to biomass from the top (Table 3.2), but this was only observed when cobalt was included in the medium.

3.3.5. Evolution of key microbial populations

The abundance of the *Euryarchaeota* decreased during reactor operation (Fig. 3.6). Cells hybridizing to *Eury498* comprised up to 55% of the area of cross-sections of sludge granules on day 2 (seed sludge). This proportion was typically 35% and 40% for granules from the bottom and top of the R1 sludge bed, respectively, on day 117 (conclusion of the R1 trial), and 20% and 25% for R2 granules from, respectively, the bottom and the top of the R2 sludge bed on day 91 (conclusion of the R2 trial).

Genus-specific hybridizations with the *Sarci551* probe revealed a temporal evolution of the *Methanosarcina* populations in R1 and R2. Firstly, *Sarci551*-positive cells were typically observed in densely packed clusters, which were abundant throughout the sections of the seed sludge (Fig. 3.7a). The evolution of the *Methanosarcina* population in R1 sludge was dynamic, and the relative abundance of hybridized cells in R1 specimens had decreased on day 42. However, large (diameter, 100-200 μm) *Sarci551*-positive clusters were abundant (>50% of granule area) in the R1 biomass by day 63, and again on day 73 (Fig. 3.7b). A reduction was evident by day 91 and on day 103, with fewer cells, which were more dispersed, rather than in clusters, and concentrated around the periphery of the sections (Fig. 3.7c). R1 specimens examined on day 117 indicated very few *Methanosarcina* cells in sludge granular sections obtained from the bottom of the sludge bed. However, some large, defined clusters of hybridized cells appeared in the sludge from the top of the bed.

In R2 sludge, an equally dynamic *Methanosarcina* population was monitored, but a different temporal trend was observed. *Sarci551*-positive clusters were abundant in R2 sections on days 42 and 63, although these were mainly located towards the surface of the granules (Fig. 3.7d,e). By day 73, the relative abundance of the *Methanosarcina* in R2 was reduced, which were present in relatively smaller, infrequent clusters around the edge of the granule sections (Fig. 3.7f). Finally, no *Sarci551*-positive cells could be detected in the R2 specimens on day 91.

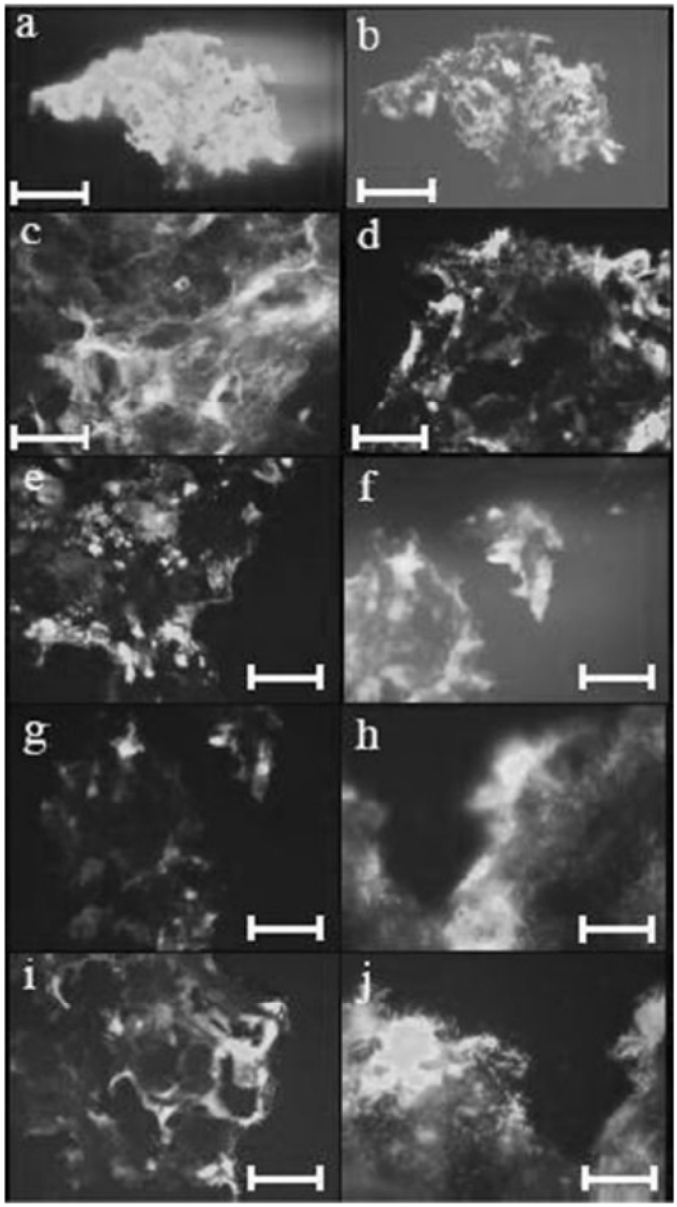

Figure 3.6. In situ hybridizations using cross-sections of anaerobic granules illustrating (a) DAPI staining with seed sludge (inoculum); and *Euryarchaeota* (*Eury498*) in (b) seed sludge (day 0); (c) R1 day 91; (d) R1 [bottom] day 117; (e) R1 [top] day 117; (f) DAPI staining, R2 [bottom] day 91; Eury 498 in (g) R2 [bottom] day 91; (h) R2 [bottom, 100X at periphery (surface) of granule] day 91; (i) R2 [top] day 91; (j) R2 [top, 100X at surface] day 91. Scale bar: a, b, c, d, e, f, g, i = 250µm; Scale bar: h, j = 20 µm.

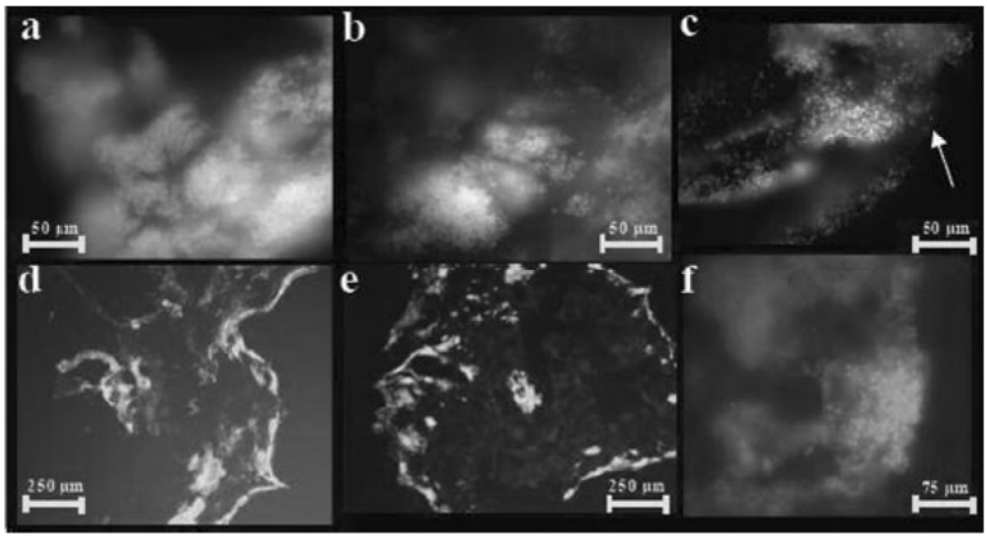

Figure 3.7. In situ hybridizations using the *Sarci551* probe and anaerobic granule sections illustrating *Methanosarcina* populations; (a) in defined clusters of coccoid cells in seed sludge; (b) large clusters in R1 biomass on day 63; (c) dispersed *Methanosarcina* cells toward the granule surface (indicated by arrow) from R1 on day 91; (d) R2 granule section illustrating abundant hybridization, mainly toward the granule surface, on day 42; (e) entire R2 granule section on day 63; (f) hybridized cluster on R2 granule surface on day 73.

3.4. DISCUSSION

3.4.1. Methanogenesis under cobalt limiting conditions

This study shows that omission of cobalt from the feed of methanol-fed anaerobic granular sludge bioreactors lowers the methanol removal efficiency followed by the enhanced formation of acetate as shown in Fig. 3.1. Under non-limiting conditions, methylothrophic methanogens, such as *Methanosarcina* spp. (Florencio et al., 1995), are the predominant methanol degrading population in mesophilic methanol-fed anaerobic granular sludge bioreactors. In the present study, cobalt depletion led to a reduced SMA on methanol and, consequently, to methanol accumulation (Fig. 3.1). Zandvoort et al. (2006), working under cobalt limiting conditions, observed a similar SMA reduction and consequent methanol accumulation. Thus, during methylothrophic methanogenic degradation of methanol, the decrease of SMA on methanol of the sludge is the variable that should be considered when predicting possible methanol accumulation and further acetate accumulation.

Cobalt supplementation is required to maintain a high SMA, and therefore, a stable methanogenic methanol-fed reactor in case of cobalt limitation (Zandvoort et al., 2003). Different cobalt dosing strategies have been studied, such as continuous dosing (Zandvoort et al., 2002a), pulse dosing or pre-loading in the sludge (Zandvoort et al., 2004). For these cobalt

dosing strategies, cobalt losses from the reactor were found to be considerably high. Therefore, other kinds of cobalt dosing strategies that minimize the metal losses by manipulating the cobalt dissolution rate need to be developed in further reserach, e.g. by slow cobalt release capsules or electrochemical release of cobalt (Cosnier et al., 1994).

It should also be noted that the same cobalt dependent enzyme, coenzyme M methyltransferase, is involved in the degradation pathway (Sauer and Thauer, 2000) of other substrates, such as methanethiol (Van der Maarel and Hansen, 1997) or trimethylamine (Zhilina and Zavarzin, 1990). Thus, as discussed in this paper, the effect of cobalt limitation can also be expected to play a key role in reactors treating those substrates. Moreover, not only cobalt but also other metals can play a key role in bioconversions. Speece et al. (1986) showed that the removal capacity of 10 out 30 anaerobic treatment systems could be improved by cobalt, nickel and iron supply. Metal deficiencies of anaerobic and aerobic treatment systems are, therefore, certainly not a rare phenomenon and rational trace metal supply is required in wastewater treatment systems in order to achieve maximal substrate conversion rates.

3.4.2. Methanogenesis versus acetogenesis

Once the methanol accumulation surpasses a threshold value, about 8.5 mM in the present study, acetogens could outcompete methanogens for methanol, due to their higher affinity for methanol at high methanol concentrations. Florencio et al. (1995) concluded that significant acetate formation will occur when the effluent methanol concentration would exceed 20 mM. This is in accordance with the methanol concentration, 18 mM, in the effluent of an acidified nickel limited methanol-fed UASB reactor (Chapter 4). Therefore, maintaining a low methanol concentration in the reactor mixed liquor is the key parameter to maintain methanol-fed UASB reactors methanogenic.

3.4.3. Acetogenesis under cobalt limiting conditions

After day 63, acetogenesis in R2 dominated over methanogenesis (Fig. 3.2B), which was accompanied by a reduction in the relative abundance of *Methanosarcina* in granule sections. By day 91, an overall reduction in the abundance of *Eury498*-positive (methanogenic) cells in R2 sludge was observed and no *Sarci551*-positive cells were detected anymore, indicating the loss of *Methanosarcina spp*. This is in accordance with Bahar and Orhan (2000), who observed a *Methanosarcina* population decrease as VFA increased in an acidifying reactor (pH 6, 35 °C) fed with wastewater from a milk and cream bottling plant.

The lack of acetoclastic activity and the recovery of SMA on methanol on day 91 (136 mg CH_4-COD·g VSS^{-1}·d^{-1} on day 91 versus 23 mg CH_4-COD·g VSS^{-1}·d^{-1} on day 73), may point towards to a partial recovery of methylotrophic methanogenic activity or, albeit more unlikely, to hydrogenotrophic methanogenic activity. Under thermophilic conditions, a substantial

fraction of methane is formed by the oxidation of methanol to H_2/CO_2 and subsequent hydrogenotrophic methanogenesis (Paulo et al., 2004) Interestingly, cobalt dosage was ineffective under these thermophilic conditions as methane formation via H_2 is a cobalt-independent pathway. The syntrophic conversion of acetate to methane via H_2/CO_2 is the major metabolic pathway under thermophilic conditions (Zinder, 1990), but insignificant under mesophilic conditions (Florencio et al., 1994). Therefore, the role of methanol-oxidation to H_2/CO_2 in the present study was expected to be insignificant.

3.4.4. Activity segregation along the sludge bed

Zandvoort et al. (2006) found that newly grown cells, present in the top of the sludge bed, did not granulate, but had a 4 times higher activity compared to sludge from the bottom of the sludge bed. Buyukkamaci and Filibeli (2004) reported a higher methanogenic activity in the upper part and a higher acetogenic activity in the bottom of a hybrid (UASB and anaerobic filter) reactor. In contrast with these findings, the activity was not segregated over the sludge bed of R2 on day 42 (Table 3.2). R2 granules from the top of the sludge bed on day 42 contained approximately 50% more hybridized *Sarci551*-positive cells than the equivalent granules from the bottom (Table 3.2). Thus, although less hybridized cells were detected in the granules from the bottom of the sludge bed, this biomass had a similar methanogenic activity as the biomass at the top of the reactor.

Sludge taken from the bottom of R2 on day 91 had a 2.5 times higher SMA on methanol compared to that of the top (Table 3.2). However, this was only observed when cobalt was included in the medium, and no differences in activity were observed in the absence of cobalt (Table 3.2). FISH data did not provide an ecological basis for this 2.5 higher SMA in the bottom than in the top part of the reactor on day 91. The difference in activity could indicate a different cobalt affinity of the methanogenic population depending on the location within the sludge bed, e.g. a higher cobalt affinity in areas closer to the feeding point.

3.4.5. Reactor recovery by cobalt addition

The addition of Co^{2+} (0.5 μM) to the R1 influent on day 105 led to increased acidification (Fig. 3.2A) and a consequent reduction in the pH of the reactor liquor (Fig. 3.3B). The small increase in methane production in these last days of operation (Fig. 3.3A), in parallel with decreased methanol accumulation due to increased VFA production (Fig. 3.2A), may indicate that methylothrophic methanogens could eventually outcompete acetogens for methanol due to the higher methanol affinity of methylothrophic methanogens (Florencio et al., 1995) at low methanol concentrations (below 8.5 mM). FISH experiments indicated indeed a re-emergence of hybridized *Methanosarcina*-like cells as defined clusters in sludge from the top of the sludge bed at the conclusion of the R1 trial (day 117), although few *Methanosarcina* cells were present

in granules from the bottom of the sludge bed. This suggests that a recovery of the *Methanosarcina* population might be possible by cobalt addition, but further research is required to inequivocally demonstrate this.

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ROLE OF NICKEL IN HIGH RATE METHANOL DEGRADATION IN ANAEROBIC GRANULAR SLUDGE BIOREACTORS

ABSTRACT

The effect of nickel deprivation from the influent of a mesophilic (30°C) methanol fed Upflow Anaerobic Sludge Bed (UASB) reactor was investigated by coupling the reactor performance to the evolution of the *Methanosarcina* population of the bioreactor sludge. The reactor was operated at pH 7.0 and at organic loading rate of 5 to 15 g $COD \cdot L^{-1} \cdot day^{-1}$ for 191 days. A clear limitation of the specific methanogenic activity (SMA) on methanol cause by the absence of nickel was observed after 129 days of bioreactor operation: the SMA of the sludge in medium with the complete trace metal solution except nickel amounted to 1.164 (± 0.167) g CH_4-$COD \cdot g\ VSS^{-1} \cdot day^{-1}$ compared to 2.027 (± 0.111) g CH_4-$COD \cdot g\ VSS^{-1} \cdot day^{-1}$ in a medium with the complete (including nickel) trace metal solution. The methanol removal efficiency during these 129 days was 99 %, no volatile fatty acid (VFA) accumulation was observed and the size of the *Methanosarcina* population increased compared to the seed sludge. Continuation of the UASB reactor operation with the nickel limited sludge led to incomplete methanol removal, and thus methanol accumulation in the reactor effluent from day 142 onwards. This methanol accumulation subsequently induced an increase of the acetogenic activity in the UASB reactor on day 160. On day 165, 77 % of the methanol fed to the system was converted to acetate and the *Methanosarcina* population size had substantially decreased. Inclusion of 0.5 µM Ni (dosed as $NiCl_2$) to the influent from day 165 onwards led to the recovery of the methanol removal efficiency to 99 % without VFA accumulation within two days of bioreactor operation.

4.1. INTRODUCTION

Biological wastewater treatment processes require both macronutrients (Goodwin et al., 1990) and micronutrients for bacterial metabolism, growth and activity (Florencio et al., 1994; Cook and Shiemke, 1996; Mulrooney and Hausinger, 2003). The optimal operation of upflow anaerobic sludge bed (UASB) reactors and similar anaerobic wastewater treatment systems are highly dependent on the presence of metal ions in the influent wastewater (Speece et al., 1983; Oleszkiewicz and Sharma, 1990; Singh et al., 1999). Methanolic wastewater is commonly treated at full-scale in UASB reactors (Weijma and Stams, 2001). Methanol is an important constituent of wastewater generated in the petrochemical industry and in the widely used "kraft" process for wood pulping in the paper industry.

Methanogenic *Archaea* use several pathways to reduce the various carbon substrates involved in methanol degradation (methanol, acetate and CO_2/H_2) (Fig. 4.1A), but each pathway converges to the common intermediate methyl-S-CoM (Thauer, 1998). Methyl-S-CoM and coenzyme B are the substrates of methyl-S-CoM reductase, whereas methane and heterodisulfide are its products (Bobik et al., 1987; Ellermann et al., 1988) (Fig. 4.1B).

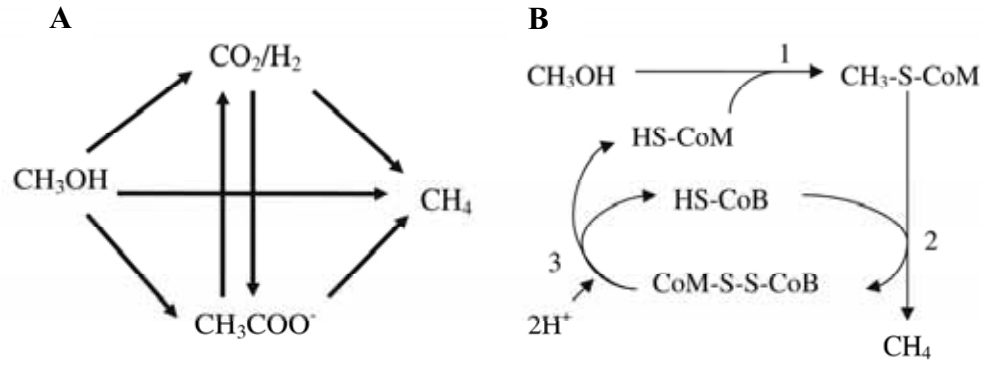

Figure 4.1. (A) Main pathways in methanogenic methanol removal and (B) Enzymes involved in methanogenesis from methanol. (1) Coenzyme M methyltransferase. (2) Methyl-coenzyme M reductase. (3) Heterodisulfide reductase.

Depletion of cobalt (Zandvoort et al., 2006b) has been shown to induce methanol accumulation, and, later, acidification, of methanol-fed UASB reactors (Chapter 3). This is due to the key role of cobalt in the direct conversion of methanol to methane, as it forms the metallocenter of corrinoid compounds that catalyze the methyl transfer from methanol to methyl-S-CoM (Thauer, 1998). Similarly, nickel plays also a key role in the methanogenic conversion of methanol: one mol of methyl-S-CoM reductase contains 2 mol of tightly, but not covalently, bound coenzyme F_{430} (Ellefson et al., 1982). F_{430} is a nickel porphinoid (Fig. 4.2). In addition, many hydrogenase enzymes involved in hydrogen formation possess nickel

(Deppenmeier et al., 1992; Kemner and Zeikus, 1994). For example, carbon monoxide dehydrogenase (CODH), which possesses two nickel-containing metallocentres, is present in both acetoclastic methanogens and acetogenic microorganisms (Hausinger, 1987). In medium rate methanol-fed UASB reactors (organic loading rate (OLR) of 7.5 g $COD \cdot L^{-1}$ day^{-1}), omission of nickel from the influent clearly induced a nutrient (nickel) limitation of the methanol conversion by the granular sludge (Zandvoort et al., 2002b).

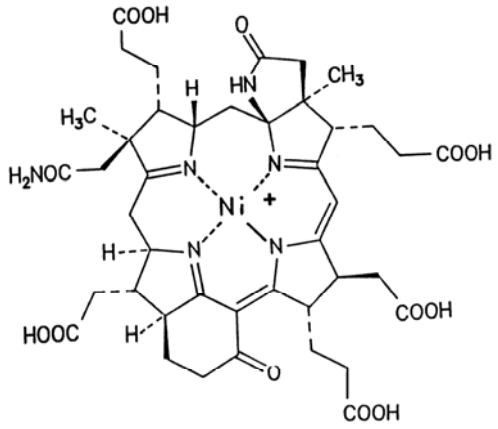

Figure 4.2. Structure of coenzyme F_{430} in the Ni^{2+} oxidation state. From Thauer (1998).

The granular sludge present in UASB reactors consists of dense conglomerates of biomass. The bioavailability of a metal in a sludge granule depends on the chemical speciation of the metal and physical processes within the granular matrix, e.g. adsorption and precipitation. In studies on metal bioavailability in complex biological systems as a UASB granule, it is usually assumed that the metal bioavailability is correlated with the free metal ion concentration in the liquid phase (Worms et al., 2006).

This study aims to evaluate the effect of nickel deprivation on the performance of a high-rate methanol-fed UASB reactor (OLR: 15 g $COD \cdot L^{-1}$ day^{-1}). Specifically, it was investigated whether the mechanism of nickel deprivation during high rate methanol conversion is similar to the outline for cobalt deprivation (Chapter 3) resulting in acidification of the UASB reactor (Fig. 4.3) or if it follows the outline proposed by Zandvoort et al. (2002b), where long-term operation of medium rate UASB reactors without nickel in the influent appeared to induce a nickel independent methanol degradation route. The fate and removal efficiency of methanol, along with the metal content of the UASB sludge were monitored as a function of time. The metabolic properties (specific methanogenic activity, SMA), the abundance of the key organism *Methanosarcina* and possible nickel limitation of the sludge that developed in the UASB reactor

were characterized. The effect of nickel speciation on the uptake and activity of nickel-limited anaerobic granular sludge was studied as well.

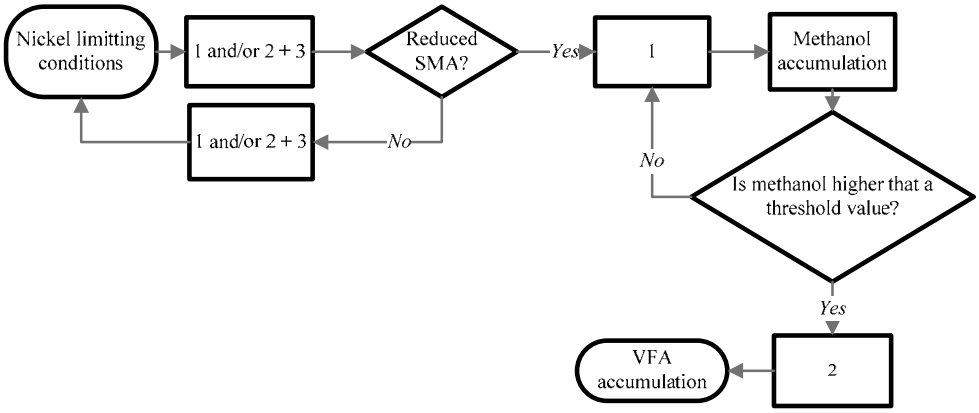

Figure 4.3. Proposed outline of the mechanism of induction of nickel limitation in methanol-fed UASB reactors. Numbers indicate predominant process. (1) methylotropihic methanogenesis. (2) acetogenesis. (3) acetotrophic methanogenesis. (From Chapter 3).

4.2. MATERIALS AND METHODS

4.2.1. Source of biomass

Methanogenic granular sludge was obtained from a full-scale UASB reactor treating paper mill wastewater (pH 7.0, 30°C) at industriewater Eerbeek (Eerbeek, The Netherlands), described in detail by Zandvoort et al. (2006a).

4.2.2. Medium composition

The reactor was fed a basal medium as synthetic watewater, consisting of methanol, macronutrients and a trace metal solution (Table 4.1). The inorganic macronutrients contained ($mg \cdot L^{-1}_{basal\ medium}$): NH_4Cl (280), K_2HPO_4 (250), $MgSO_4 \cdot 7H_2O$ (100) and $CaCl_2 \cdot 2H_2O$ (10). To ensure pH stability, 2.52 g (30 mM) of $NaHCO_3$ was added per litre of basal medium. To avoid precipitation in the storage vessels prior to reactor feeding, the UASB influent was composed of 3 streams: (1) macronutrients and trace metal solution without K_2HPO_4 and $NaHCO_3$, (2) methanol with $NaHCO_3$ and K_2HPO_4 and (3) dilution water. Tap water was used to prepare the influent and as dilution water.

Table 4.1. Composition of the trace metal solution supplied to influent and activity test media.

Compound	Metal	Conc. [μM]
$FeCl_2 \cdot 4H_2O$	Fe (II)	5
$CuCl_2 \cdot 2H_2O$	Cu (II)	0.5
$ZnCl_2$	Zn (II)	0.5
$MnCl_2 \cdot 4H_2O$	Mn (II)	0.5
$NiCl_2 \cdot 6H_2O$ [a]	Ni (II)	0.5
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	Mo(VI)	0.5
$Na_2SeO_4 \cdot 5H_2O$	Se (VI)	0.5
$Na_2WoO_4 \cdot 2H_2O$	Wo (VI)	0.5
$CoCl_2 \cdot 6H_2O$	Co (II)	0.5

a. added from operation day 165 to the influent.

4.2.3. UASB reactor operation

The experiment was performed in a Plexiglas cylindrical UASB reactor with a working volume of 7.25 L and an inner diameter of 0.1 m. The reactor was operated at 30 (± 2)°C in a temperature-controlled room. The UASB reactors were inoculated with 8.02 g $VSS \cdot L^{-1}_{reactor}$ and operated at a hydraulic retention time (HRT) of 8 h. The conical bottom of the reactors was filled with glass marbles (1 cm diameter) to evenly distribute the influent over the sludge bed. Peristaltic pumps (type 505S, Watson and Marlow, Falmouth, U.K.) were employed for influent flow, whereas no effluent recycle was applied. The superficial liquid upflow velocity was 0.1 $m \cdot h^{-1}$.

The produced biogas was led through a water lock filled with a concentrated NaOH (15 %) solution in order to remove CO_2 and H_2S. The volume of the produced methane was measured with a wet gas meter (Schlumberger Industries Dordrecht, The Netherlands).

The reactor run was divided into three periods. Period I (PI, days 0-137) focused on methanogenesis from methanol under nickel limiting conditions. On day 137, 65 % of the sludge bed (calculated based on bed height) was removed from the reactor to perform kinetic studies with the nickel-limited methanogenic sludge. Period II (PII, days 137-165) focused on methylotrophic acetogenesis under nickel-limited conditions. Period III (PIII, days 165-191) focused on the potential recovery of methylotrophic methanogenesis by the continuous addition of nickel (0.5 μM Ni^{2+} as $NiCl_2$) to the bioreactor influent.

4.2.4. Specific methanogenic activity assays

The SMA of the sludge that developed in the reactor was determined in duplicate at 30 (±2)°C using on-line gas production measurements as described by Zandvoort et al. (2002a). Briefly, approximately 1 g (wet weight) of granular sludge was transferred to serum bottles

(0.12 L) containing 0.05 L of basal medium with the same composition as the reactor basal medium, but which was supplemented with methanol (4 g $COD \cdot L^{-1}$) or acetate (2 g $COD \cdot L^{-1}$) as the substrate. The data were plotted in a rate versus time curve, using moving average trend lines with an interval of 10 data points.

The SMA with methanol as the substrate was determined on days 22, 35, 45, 64, 82, 95, 110, 129 and 137, and with acetate on days 129 and 168. The effect of different metal concentrations in the test medium was also studied for the different sludge samples. Sludge samples for SMA were always taken from the same place (the mid-height) in the sludge bed.

4.2.5. Metal uptake kinetics

The apparent affinity constant (K_M) and maximum specific methanogenic activity (SMA_{max}) with methanol for nickel addition, as $NiCl_2$ and $NiEDTA^{2-}$, were determined with nickel-deprived granular sludge harvested from the UASB reactor on day 137. The evolution of the methanol and dissolved nickel concentration as well as pressure increase during methanogenic methanol degradation upon addition of $NiCl_2$ or $NiEDTA^{2-}$ (0.5 µM) were monitored. The K_M and SMA_{max} were calculated using Michaelis-Menten kinetics, following SMA assays with different initial nickel concentrations. The free nickel concentration in the liquid media was calculated by equilibrium speciation modeling (MINTEQA2).

4.2.6. Fluorescent in situ hybridization

The microbial population present in the anaerobic granules was studied by fluorescent in situ hybridization (FISH) as described in Chapter 3. Granules were fixed in 4 % (w/v) paraformaldehyde in phosphate buffered saline (PBS; 130 $mmol \cdot L^{-1}$ sodium chloride and 10 $mmol \cdot L^{-1}$ sodium phosphate [pH 7.2]) at 4°C for 6 h, and cross-sections (thickness, 16 µm; Ø, 0.5-1.5 mm) were prepared as described previously (Sekiguchi et al., 1999). FISH was performed as described by Schramm et al. (1998) using a Cy3-labeled 16S rRNA-targeted oligonucleotide probe (Biomers.net, Germany), *Sarci551* (Sorensen et al., 1997), specific for *Methanosarcina*. The stringency in the hybridization buffer used was achieved by adding formamide to a final concentration (v/v) of 20 %. The *Non338* probe (Wallner et al., 1993) was used as a negative control, whereas pure cultures of *Methanosarcina barkeri* were used to positively control the *Sarci551* probe. Specimens were viewed using a Nikon E600 epifluorescent microscope equipped with a 100 W mercury bulb and a Cy3 filter set. The abundance of *Methanosarcina* was determined as the fraction of the total area of respective granule sections.

4.2.7. Metal composition of the sludge

The total metal content of the sludge per gram of total solids was determined after destruction with *Aqua regia* (mixture of 2.5 mL 65 % HNO_3 and 7.5 mL 37 % HCl) added to approximately 1 g (wet weight) of granular sludge samples. After microwave destruction (Milestone ETHOS E temperate controlled; Milestone INC; Monroe, CT, USA), the samples were paper-filtered (Schleider Schuell 589, Germany) and diluted to 0.1 L with demineralised water. The total metal concentration was determined by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES; Varian, Australia) as described by van Hullebusch et al. (2005)

Ultra-pure water (Milli-Ro System, Millipore, Bedford, MA, USA) was used to prepare standard solutions of all reagents, which were of suprapur quality (Merck, Darmstadt, Germany) and were checked for possible trace metal contamination. All glassware and plastic material used was treated for 12 h with 10 % (v/v) HNO_3 and rinsed vigorously with demineralized water.

4.2.8. Chemical analyses

Methanol and VFA were determined using gas liquid chromatography as described by Weijma et al. (2000). The total suspended solids (TSS) and volatile suspended solids (VSS) concentrations were determined according to Standard Methods (APHA/AWWA, 1998). All chemicals were of analytical or biological grade and purchased from E. Merck AG (Darmstadt, Germany).

4.3. RESULTS

4.3.1. Reactor operation

After a start-up period of 12 days, methanol was fully converted to methane in the UASB reactor (Fig. 4.4). The organic loading rate (OLR) was increased twice during PI, first from 5 to 10 g $COD \cdot L^{-1} \cdot day^{-1}$ on day 64 and then from 10 to 15 g $COD \cdot L^{-1} \cdot day^{-1}$ on day 113, without resulting in methanol or VFA accumulation in the effluent (Fig 4.4). The methanol effluent concentration started to accumulate on day 130, reaching a maximum value of 1380 mg COD-$MeOH \cdot L^{-1}$ on day 133, corresponding to 31 % of the influent methanol concentration (4500 mg COD-$MeOH \cdot L^{-1}$). On day 137, the applied OLR was reduced from 15 to 5 g $COD \cdot L^{-1}$ day^{-1} in order to avoid overloading of the reactor due to the sludge removal to perform kinetic studies on the reactor sludge. Methanol was completely removed without any VFA accumulation from day 137 (PII) until day 142. From day 142 onwards, however, methanol accumulation was observed in the UASB reactor, reaching 819 mg COD-$MeOH \cdot L^{-1}$ by day 147. From day 149, the concentration of accumulated methanol decreased. This was accompanied by an increase in the VFA concentration, mainly acetate (around 86 %). On day 163, VFA effluent concentrations

reached a maximum of 1150 mg COD-VFA·L^{-1}, corresponding to 95 % of the influent methanol being converted to VFA.

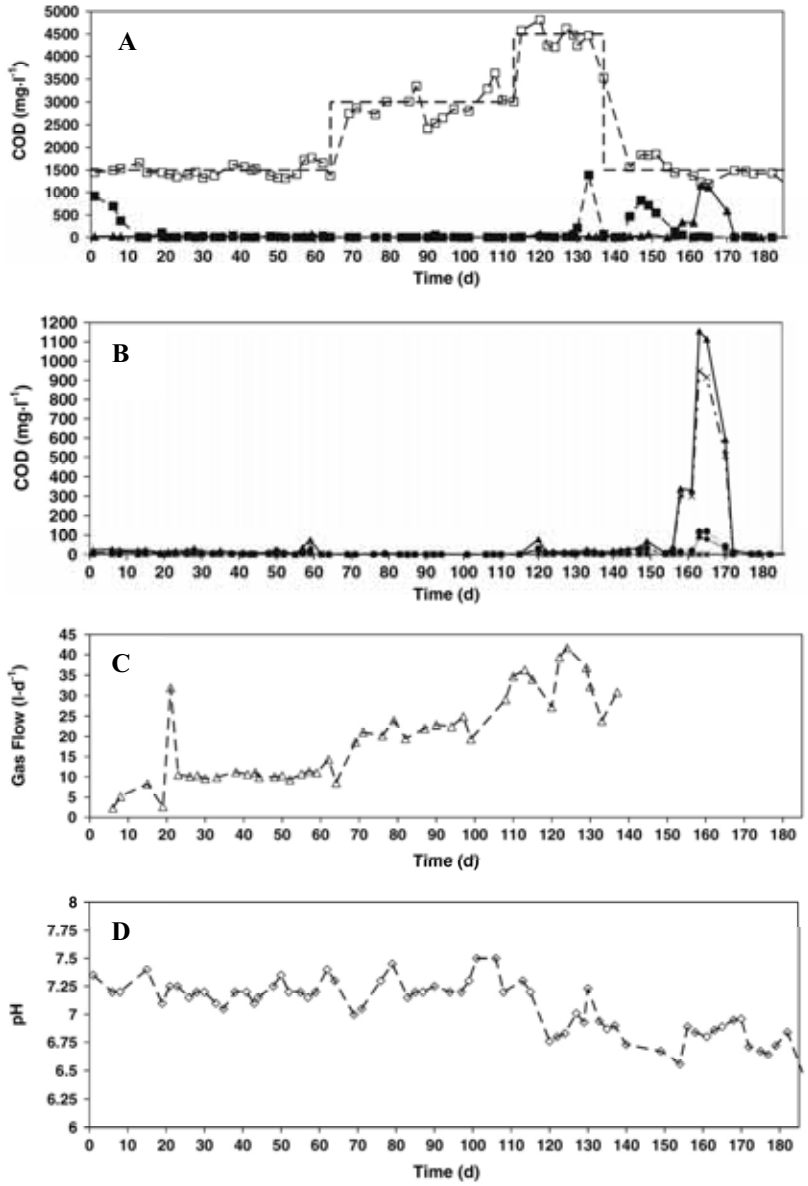

Figure 4.4. Evolution of the reactor performance with time. (A) Calculated influent methanol (---), measured influent methanol (□), effluent methanol (■), and effluent VFA (▲) concentrations. (B) Total VFA (▲), acetate (x), propionate (●), butyrate (♦), and valerate (*) concentration. (C) CH_4 production (Δ), note that gas production was not measured from day 137 onwards due to technical problems. (D) pH (◊) of UASB reactor mixed liquor.

Methanol was fully removed from the wastewater by the UASB reactor from day 155 (prior to the nickel additions) onwards, but mainly converted to VFA (Fig. 4.4A). The continuous addition of 0.5 µM Ni^{2+} as $NiCl_2$ to the influent from day 165 onwards (PIII) resulted in an immediate reduction in the effluent VFA concentration. From day 172 (7 days after the initiation of nickel supplementation) until the end of the experiment (day 191), the methanol was fully removed without any VFA accumulation. In fact, no VFA were detected in the effluent anymore after day 172.

4.3.2. Evolution of SMA over time

A slight nickel limitation of methylotrophic methanogenesis was observed in the beginning of the reactor operation. The SMA on methanol in a medium with the complete metal solution was 6.5 % (day 22) and 17 % (day 35) higher than in a medium with the complete metal solution except nickel (Fig. 4.5). On day 45, the SMA on methanol was double compared to day 35 for all the trace metal solutions tested (Table 4.2), which likely can be attributed to the adaptation of the sludge to methanol. Similar SMA values to those on day 45 were measured on day 64 for all the different trace metal solutions tested (Table 4.2). On day 82, a 37 % higher SMA on methanol was achieved in a medium with complete metal solution compared to a medium with complete metal solution except nickel. Similar SMA values were measured on day 82 and on day 95 for all the trace metal solutions tested (Table 4.2). On day 110, the SMA on methanol increased with around 25 % compared to day 95 for all the different trace metal solutions tested, suggesting a further adaptation of the sludge to methanol (Table 4.2).

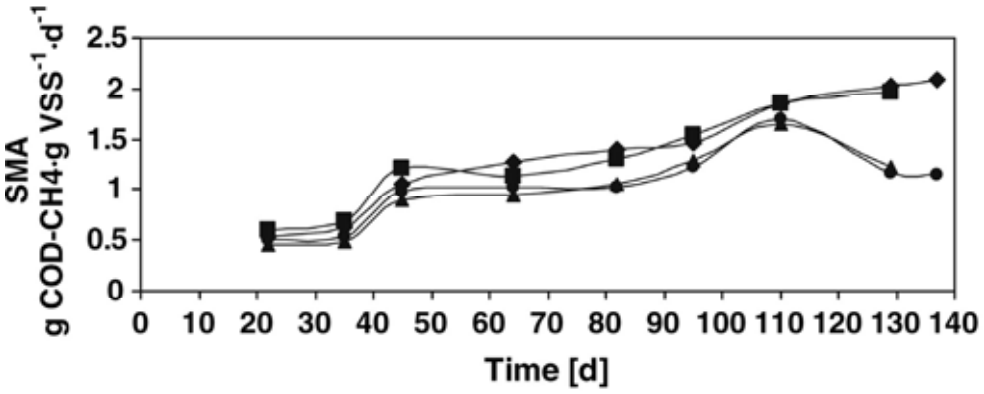

Figure 4.5. Evolution of the specific methanogenic activity (mg CH_4-COD·g VSS^{-1}·d^{-1}) with methanol as the substrate as a function of time and trace metal content of the medium. No metal addition (▲), addition of complete trace metal solution except nickel (●), addition of complete trace metal solution (♦), and addition of nickel alone (■). Note that at operation day 165, the UASB reactor was completely acidified and no activity on methanol was measured.

On day 129, which was 1 day before the methanol accumulation was observed, the SMA on methanol had decreased to 1.164 g CH_4-COD·g VSS^{-1}·d^{-1} in a medium containing the complete trace metal solution except nickel, corresponding to a 31 % reduction in SMA compared to day 110 for the same test medium. However, in a medium containing a complete metal solution, the SMA on methanol on day 129 increased to 2.027 g CH_4-COD·g VSS^{-1}·d^{-1}, corresponding to a 9 % higher SMA than on day 110 for the same test medium. No methanogenic activity on acetate was observed either on day 129 or on day 168.

Table 4.2. Evolution of the specific methanogenic activity (mg CH_4-COD·g VSS^{-1}·d^{-1}) with methanol as the substrate as a function of time and trace metal content of the medium.

Day of operation	Specific maximum methanogenic activity [g CH_4-COD·g VSS^{-1}·day^{-1}]			
	No metals	Complete trace metal solution except nickel	Complete trace metal solution	Nickel solely
22	0.45	0.503±0.03	0.537	0.593
35	0.494	0.527±0.010	0.636±0.006	0.696±0.013
45	0.902±0.035	0.973±0.048	1.059±0.022	1.207±0.008
64	0.951±0.031	1.026±0.081	1.270±0.026	1.125±0.049
82	1.047±0.043	1.021±0.023	1.399±0.135	1.298
95	1.291±0.223	1.222±0.028	1.463±0.093	1.544±0.158
110	1.652±0.149	1.679±0.104	1.852±0.055	1.851
129	1.225±0.121	1.164±0.167	2.027±0.111	1.959±0.158
137	n.d.	1.154±0.094	2.085	n.d.

n.d.: not determined

4.3.3. Metal concentration in the sludge

Distinct differences in the retention of cobalt, zinc and copper were observed during the entire experiment, even when these metals were dosed at the same concentration (Table 4.1). After 60 days of UASB reactor operation at pH 7.2 (Fig. 4.4D), the cobalt and copper concentrations in the sludge had increased by about 2.5 and 4.5 times, respectively. From day 60 to day 110, the cobalt concentration slightly decreased, while the copper concentration increased about 7 times compared to the initial concentration in the seed sludge. The nickel and zinc concentrations in the sludge were stable over time during the first 110 days of operation, whereas, the iron concentration halved over the same period (Fig. 4.6, Table 4.3).

A decrease in concentration of all the measured metals occurred between day 110 and 134, at the same time that methanol accumulated and a small pH drop (from 7.25 on day 110 to 6.90 on day 134) occurred (Fig. 4.4D). The cobalt and nickel concentration on day 134 was

about 3 times lower than the concentration on day 110. In the same period, the zinc and iron concentration decreased by 50 %, while the copper decreased by only 20 % (Fig. 4.6, Table 4.3).

During acetate accumulation (PII, days 134 to 169), the cobalt, copper and zinc content of the sludge increased slightly. In the same period, the nickel and iron concentration slightly decreased. The nickel concentration in the sludge doubled between days 169 (one day after initiation of continuous nickel addition) and 191 (conclusion of the trial).

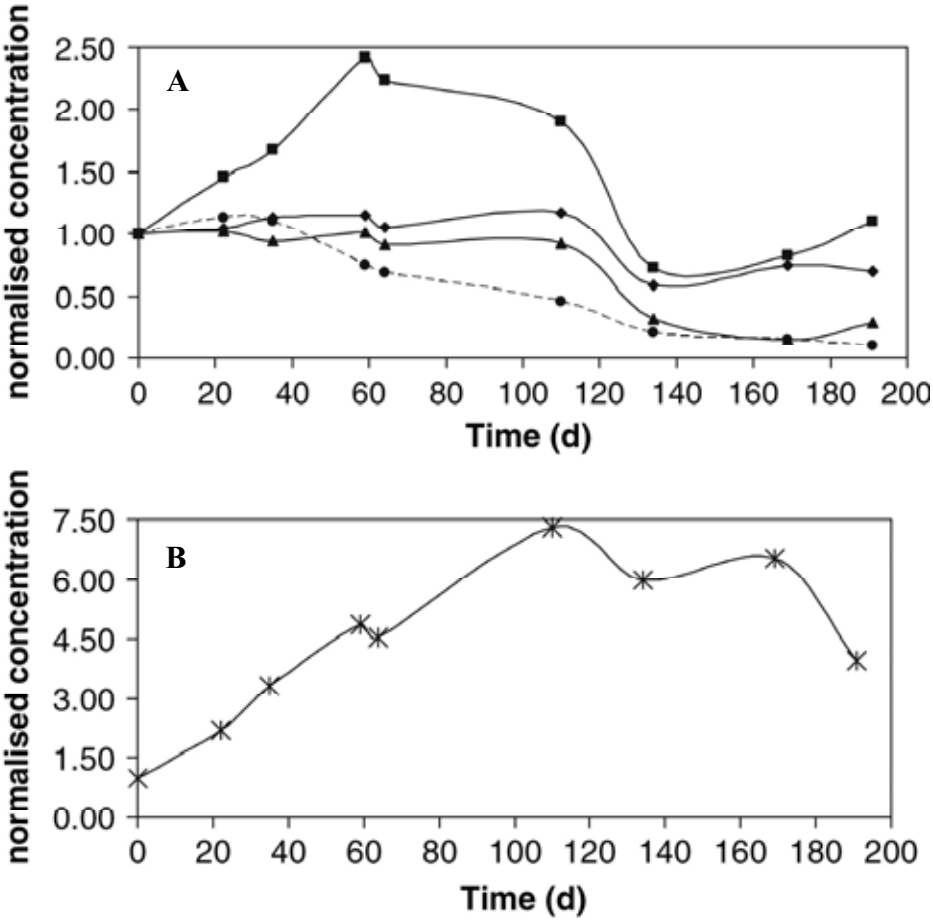

Figure 4.6. Evolution of the normalized by t = 0 metal concentration in the sludge as function of reactor operation. (A) Nickel (▲), cobalt (■), zinc (♦), and iron (●). (B) Copper (X).

Table 4.3. Evolution of the metal concentration (μg ·g TSS^{-1}) in the sludge as a function of time.

Day	Ni	Co	Cu	Zn	Fe
0	43	35	156	219	27119
22	44	51	343	229	30556
35	41	58	516	248	29950
59	43	84	757	253	20185
64	39	78	705	230	18477
110	39	66	1135	257	12262
134	14	25	930	127	5534
169	6	29	1016	163	3914
191	12	38	614	152	2778

4.3.4. Evolution of key microbial population

Genus-specific hybridizations with the *Sarci551* probe revealed a temporal evolution of the *Methanosarcina* populations in the reactor. In the seed sludge, *Sarci551*-positive cells were typically observed in densely packed clusters (Fig. 4.7A). The distribution pattern of *Methanosarcina* cells in the granules was dynamic during the start-up period, and more dispersed cells were observed on day 35 (Fig. 4.7B) than in the seed sludge. However, the relative abundance of hybridized cells appeared constant and was similar on day 35 compared to the seed sludge.

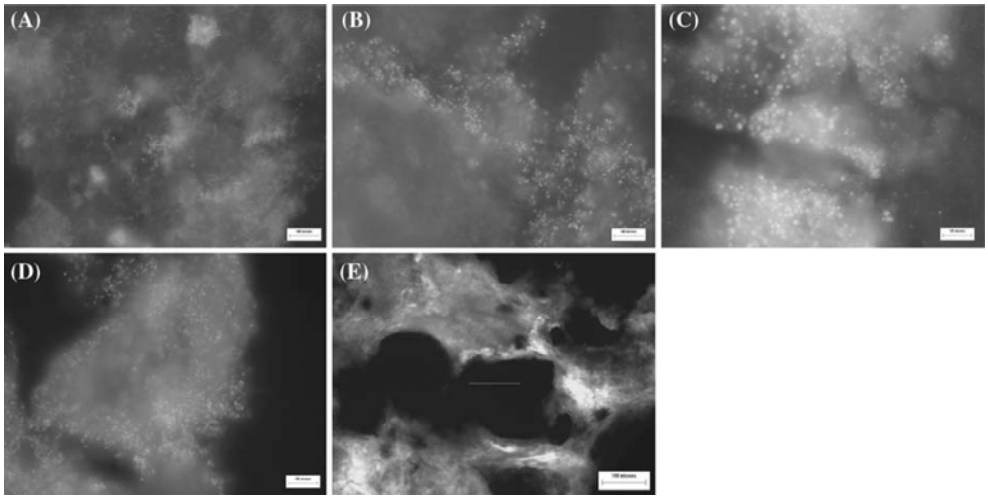

Figure 4.7. In situ hybridizations of anaerobic granule sections with the *Sarci551* probe illustrating the evolution of the *Methanosarcina* populations as a function of time. (A) Seed sludge, (B) day 35, (C) day 110, (D) day 168 and (E) day 191. Scale bar: 100 μm.

An increased abundance of *Sarci551*-positive cells was detected in the biomass by day 110 (Fig. 4.7C). *Sarci551*-positive cells were typically observed in loosely-packed clusters by day 110, but they were less dispersed than on day 35. A reduction in the number of *Sarci551*-positive cells was evident by day 168, with fewer cells, which were more dispersed, rather than in clusters, and which, interestingly, were concentrated around the periphery of the sections (Fig. 4.7D). The relative abundance of hybridized cells on day 191 (Fig. 4.7E) was similar to day 168 (Fig. 4.7D). However, specimens examined on day 191 indicated that the *Sarci551* cells were mainly packed in clusters or micro-colonies of cells, also mainly located in the periphery of the granule cross sections.

4.3.5. Kinetic parameters of the nickel-limited granular sludge

The SMA_{max} of the sludge in the medium containing $NiEDTA^{2-}$ (2.62 g CH_4-COD·g VSS^{-1}·d^{-1}) was 20 % higher than the medium with $NiCl_2$ (3.08 g CH4-COD·g VSS^{-1}·d^{-1}). The K_M values of the UASB granules show that the nickel concentration required to overcome the limitation on nickel is low: 0.16 μM and 0.18 μM of Ni^{2+} for $NiCl_2$ and $NiEDTA^{2-}$, respectively (Fig. 4.8A). This corresponds to a free nickel concentration in the liquid medium of, respectively, $2 \cdot 10^{-3}$ and $1 \cdot 10^{-7}$ μM of Ni^{2+} for $NiCl_2$ and $NiEDTA^{2-}$.

The evolution of the dissolved nickel concentration during a methanogenic activity test was different when nickel was dosed as $NiEDTA^{2-}$ (Fig. 4.8B) or as $NiCl_2$ (Fig. 4.8C). Already 10 hours after of the start of the experiment, the dissolved nickel concentration when nickel was added as $NiCl_2$ was only 20 % of the total added nickel, whereas 105 % of the total added nickel was presented as dissolved nickel when it was added as $NiEDTA^{2-}$. The dissolved nickel in the experiments decreased during the period with methylotrophic methanogenic activity at a rate of about 1.6 nM·h^{-1} and about 5 nM·h^{-1} for, respectively, $NiCl_2$ and $NiEDTA^{2-}$. After the activity had finished and substrate was consumed the rate decreased up to 1.6 nM·h^{-1} in the experiment with $NiEDTA^{2-}$ (Fig. 4.8B). Interestingly, a slow nickel re-dissolution took place at a rate of about 0.3 nM·h^{-1} in the experiment with $NiCl_2$ upon completion of the methane production (Fig. 4.8C).

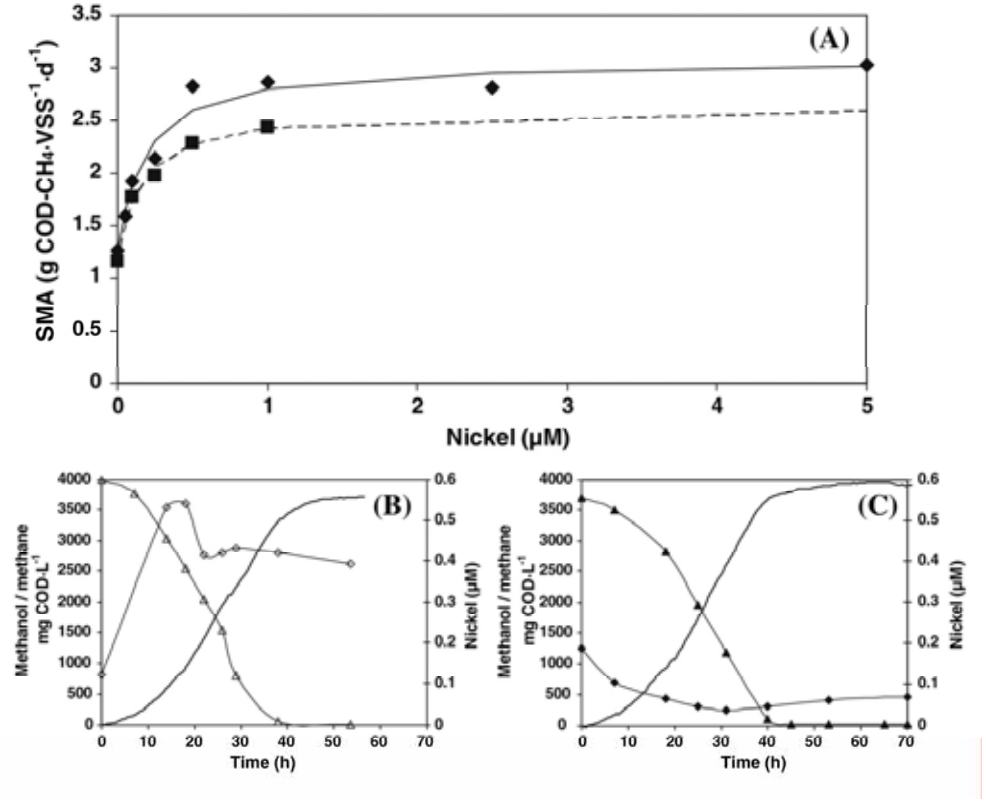

Figure 4.8. (A) Effect of nickel concentration on the specific methanogenic activity with methanol as the substrate of nickel limited sludge (sampled on day 137). Nickel added as $NiCl_2$ (■) and $NiEDTA^{2-}$ (♦). (B) Evolution of dissolved nickel added as $NiEDTA^{2-}$ (◊) during methanol (Δ) conversion to methane (—). (C) Evolution of dissolved nickel added as $NiCl_2$ (♦) during methanol (▲) conversion to methane (—).

4.4. DISCUSSION

4.4.1. Methanogenesis under nickel-limiting conditions

This study shows that lack of nickel in the influent reduces the methanol removal efficiency of methanol-fed anaerobic granular sludge bioreactors, and, ultimately, leads to reactor acidification. Under non-limiting conditions, methylothrophic methanogens, such as *Methanosarcina* (Florencio et al., 1995), are the predominant methanol-degrading population in mesophilic methanol-fed anaerobic granular sludge bioreactors. In the present study, nickel depletion led to a reduced SMA on methanol (Fig. 4.5) and, consequently, to methanol accumulation in the effluent after 140 days of operation (Fig. 4.4). Moreover, the nickel limitation occurred in a similar way as for cobalt shown in Chapter 3, according to the algorithm presented in Fig. 4.3. Apparently, the shorter operation time and the higher OLR applied in this

study did not allow the induction of a nickel independent pathway, as proposed by Zandvoort et al. (2002b). The time period required to achieve nickel limitation, as also observed by Zandvoort et al. (2002b), was much longer compared to that required for cobalt: at an OLR of 5 g COD·L^{-1}·day^{-1}, cobalt limitation is achieved in 15 days (Chapter 3).

In the present study, the SMA on methanol in the absence of nickel on day 110 was similar to the SMA in the presence of nickel and 3 times higher than the SMA on methanol in the absence of nickel on day 35 (Table 4.2). FISH data provided an ecological basis for this 3 times higher SMA: the abundance of *Methanosarcina* cells on day 110 was higher than in the seed sludge and on day 35 (Fig. 4.7b,c). The same increase in *Methanosarcina* cell number over time was also found in a stable methanogenic methanol-fed UASB (pH 7, 30 °C, 5 g COD·L^{-1}·day^{-1}) (Chapter 3). The increased SMA during PI is most likely due to biomass adaptation to the substrate, as evidenced by the increase in activity (Table 4.2) and *Methanosarcina* abundance (Fig. 4.7).

Kida et al. (2001) observed that the methanogenic activity with acetate as the substrate (pH 7, 37 °C), as well as F_{430} and corrinoid concentrations in methanogenic mesophilic biomass from a sewage treatment plant decreased with decreasing amounts of Ni^{2+} and Co^{2+} added. The omission of nickel might thus result in a reduced amount of coenzyme F_{430}, thereby decreasing the SMA of the sludge. In the present study, the reduced SMA in the absence of nickel was not observed until day 129. The initial concentration of nickel in the seed sludge (43 μg·g TSS^{-1}; Table 4.3) was probably enough to support the enzymatic activity and synthesis of coenzyme F_{430} for a considerably long time, even if the *Methanosarcina* population increased. Moreover, many organisms respond to lower metal bioavailability by an increased synthesis of metals transporters (Sunda and Huntsman, 1998). This type of adaptation to low nickel concentrations might also have contributed to the longer time-period before a decrease in methylotrophic activity is observed.

4.4.2. Methanogenesis with different nickel species

Nickel bound to $EDTA^{4-}$ induced a similar K_M, but higher SMA_{max} of the nickel limited sludge with methanol as the substrate as compared to nickel chloride (Fig. 4.8). This disagrees with the assumption that the free metal species is the bioavailable fraction in a biofilm system (Worms et al., 2006) as the initial free nickel concentration in $NiEDTA^{2-}$ amended medium is much lower (< 0.1 %) compared to $NiCl_2$ (c.a. 28 %) amended medium. This discrepancy might be due to the formation of different nickel species in the batch test medium or granular matrix that alter the metal bioavailability. Indeed, a lower total dissolved nickel concentration was measured in the nickel chloride activity test medium (Fig. 4.8C). For example, sulfide ions, which are always present in the granular sludge (van Hullebusch et al., 2003), may react with the free nickel ions to form metal sulfides. As the free nickel concentration is higher with nickel

chloride than with nickel bound to $EDTA^{4-}$, more nickel sulfide will be formed in the nickel chloride medium. Osuna et al. (2004), using a sequential extraction protocol with the same seed sludge as used in the present study, showed that nickel is mainly present in the sulfide fraction. The latter is a strongly bound fraction, which could make nickel less available for the methanogens (Jansen et al., 2005). In this way, the nickel bound to $EDTA^{4-}$ is less available to nickel sulfide formation and can thus penetrate the granular matrix without precipitation. Local and homogeneous dissociation of nickel bound to $EDTA^{4-}$ within the granule will lead to higher methanogenic activities. Therefore, the addition of nickel bound to $EDTA^{4-}$ or other strong ligands to UASB reactors will facilitate homogeneous distribution of nickel in the sludge present in the reactor.

The bioavailability of metal species depends on the species formed. In a medium with sulfide, carbonates, phosphates and $EDTA^{4-}$ as the main metal ligands, it is more likely that the $EDTA^{4-}$ determines the nickel bioavailability, and thus nickel remains mainly dissolved (Fig. 4.8B). However, in a medium with only sulfide, carbonates and phosphates, precipitation determines the nickel bioavailability (Fig. 4.8C). Further research is required to confirm this, measuring nickel bioavailability with specific techniques, as e.g. by the donnan membrane technique (DMT) or diffusive gradient in thin films (DGT) techniques (Kalis et al., 2006).

The retention and bioavailability of the different nickel species in anaerobic reactors should also be taken into account when the hydraulic retention time in anaerobic reactor operation is shorter than the time to reach physical and chemical equilibrium. This can be done using techniques that give a better understanding of the different metal retention mechanisms, e.g. spectrophotometric measurement of compounds that become fluorescent upon metal complexation, as e.g. Newport green with nickel (Wuertz et al., 2000). The latter compound becomes a fluorescent molecule when bound to nickel. Using this technique, nickel retention in a biofilm was shown to be not only due to cellular sorption but also to extracellular binding by extracellular polymeric substance.

4.4.3. Acetogenesis under nickel-limiting conditions

Once the methanol accumulation surpasses a threshold value (about 18 mM in the present study; Fig. 4.4), acetogens outcompeted methanogens for methanol. This is due to the higher affinity of acetogens for methanol at high methanol concentrations (Florencio et al., 1995). Methanol accumulation was followed by acetate accumulation, similar to the algorithm of cobalt limitation of methanol-fed UASB reactors (Chapter 3), even when nickel and cobalt are components of different enzymes in the anaerobic methanol degradation (Fig. 4.1B). This suggests that even other metals present in the enzyme system involved in methanol degradation could follow the same algorithm as well, e.g. iron which is present in heterodisulfide reductase

(Deppenmeier et al., 1999) or zinc which is present in coenzyme M methyltransferase (Sauer and Thauer, 2000) .

Florencio et al. (1995), working with a methanol fed UASB reactor (pH 7.0 and 30 °C), concluded that even if enough bicarbonate and cobalt are supplied to the media, significant acetate formation will occur when the effluent methanol concentration would exceed 20 mM in case of e.g. overloading of the reactor or other metal deficiency. Therefore, maintenance of the SMA high enough to keep the methanol effluent concentration low is required to maintain methanol-fed UASB reactors methanogenic.

4.4.4. Reactor recovery by nickel addition

The addition of nickel (0.5 μM) to the influent almost instantaneously decreased the acidification and recovered the methanol removal efficiency (Fig. 4.4). The decreased VFA accumulation and complete methanol removal in these last days of operation (Fig. 4.4), coupled to the lack of acetoclastic methanogenic activity, indicates that methylothrophic methanogens recovered their activity upon the nickel addition. The nickel addition also influenced the *Methanosarcina* population dynamics inside the granule: the relative abundance of *Methanosarcina* cells did not change, but after 25 days of nickel addition (day 191) the *Methanosarcina* cells were much more packed in cluster than after 3 days (day 168) of nickel addition. The aggregation in clusters of methanogens after nickel addition is similar to that observed after 110 days of reactor operation. The growth of methanogens in clusters seems to be correlated with the development of the methanogenic population. Similarly, in Chapter 3 was observed abundant clusters of *Methanosarcina* cells in a cobalt limited methanogenic methanol-fed UASB reactor during stable operation. Also Sekiguchi et al (1999) showed that methanogenic archaea are mainly present in clusters in sucrose and VFA fed mesophilic granular sludge.

In contrast with the methanogenic reactor recovery upon nickel addition, cobalt addition in a cobalt-deprived reactor enhanced the acetogenic activity of the biomass (Chapter 3). This different behaviour is probably due to the high cobalt dependence (Bainotti and Nishio, 2000), but slightly nickel dependence of the enzymatic system of acetogenesis (Diekert et al., 1981). Thus, in the case of nickel limitation, dosing nickel can enhance methylotrophic methanogenic activity, without boosting acetogenesis.

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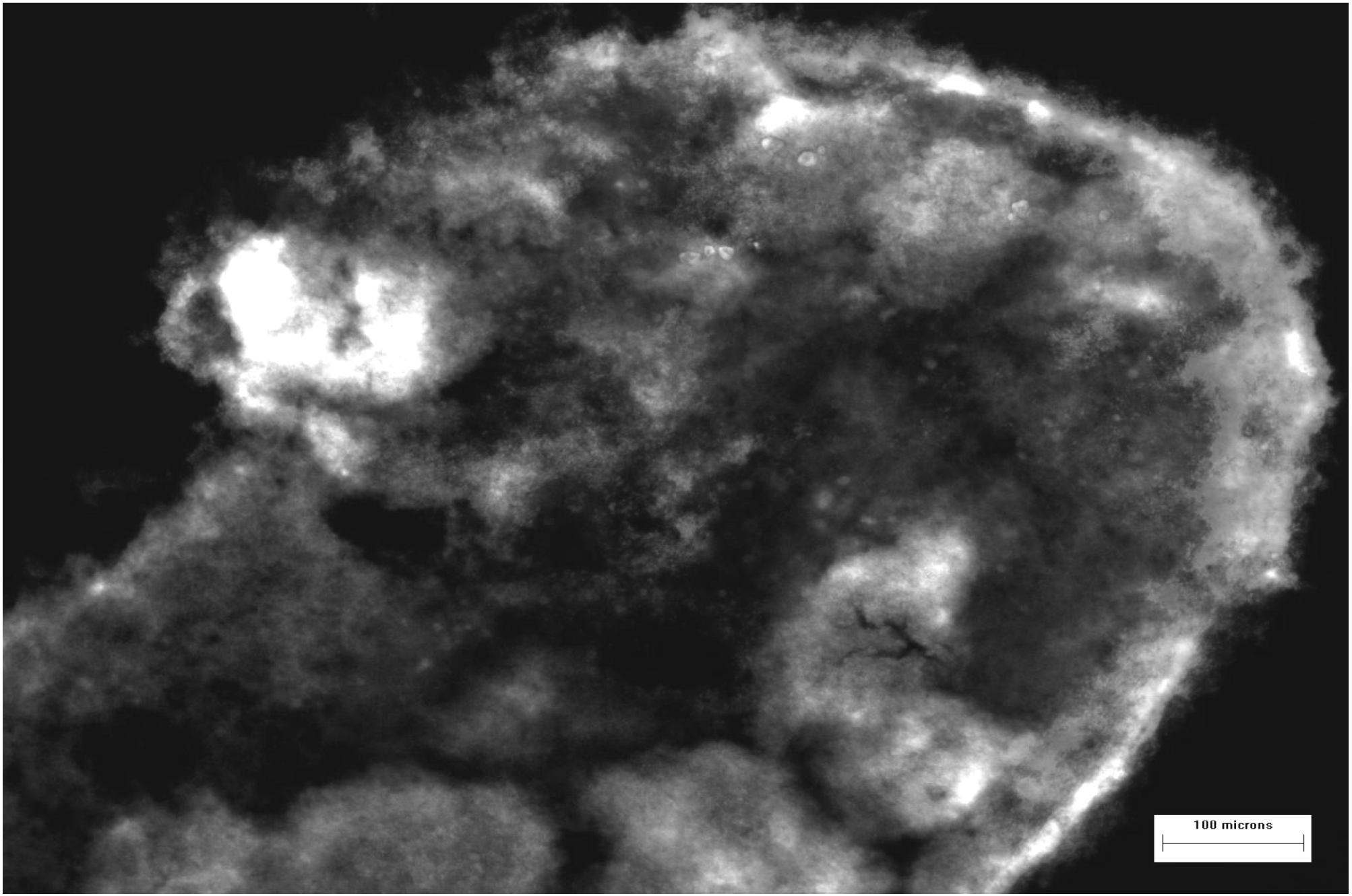
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ZINC DEPRIVATION OF METHANOL FED ANAEROBIC GRANULAR SLUDGE BIOREACTORS

ABSTRACT

The effect of omitting zinc from the influent of mesophilic (30 °C) methanol fed upflow anaerobic sludge bed (UASB) reactors, and latter zinc supplementation to the influent to counteract the deprivation, was investigated by coupling the UASB reactor performance to the microbial ecology of the bioreactor sludge. Limitation of the specific methanogenic activity (SMA) on methanol due to the absence of zinc from the influent developed after 137 days of operation. At that day, the SMA in medium with a complete trace metal solution except Zn was 3.4 g CH_4-COD·g VSS^{-1}·day^{-1}, compared to 4.2 g CH_4-COD·g VSS^{-1}·day^{-1} in a medium with a complete (including zinc) trace metal solution. The methanol removal capacity during these 137 days was 99 % and no volatile fatty acids (VFA) accumulated. Two UASB reactors, inoculated with the zinc deprived sludge, were operated to study restoration of the zinc limitation by zinc supplementation to the bioreactor influent. In a first reactor, no changes to the operational conditions were made. This resulted in methanol accumulation in the reactor effluent after 12 days of operation, which subsequently induced acetogenic activity 5 days after the methanol accumulation started. Methanogenesis could not be recovered by the continuous addition of 0.5 µM $ZnCl_2$ to the reactor for 13 days. In the second reactor, 0.5 µM $ZnCl_2$ was added from its start-up. Although the reactor stayed 10 days longer methanogenically active than the reactor operated without zinc, methanol accumulation was observed in this reactor (up to 1.1 g COD-MeOH·L^{-1}) as well. This study shows that zinc limitation can induce failure of methanol fed UASB reactors due to acidification, which can not be restored by resuming the continuous supply of the deprived metal.

5.1. INTRODUCTION

Balanced operation of anaerobic biological treatment processes, like the upflow anaerobic sludge bed (UASB) reactor, highly depends on the presence of sufficient trace metals to support the growth of the anaerobic microorganisms (Oleszkiewicz and Sharma, 1990; Singh et al., 1999; Jefferson et al., 2001). Trace metal bioavailability affects the overall functioning of anaerobic treatment systems due to their role in enzymatic activity, membrane stability, nutrient transport and energy conservation in methanogenic biomass (Whitman et al., 1982; Gavel et al., 1998; Sauer and Thauer, 2000; Mulrooney and Hausinger, 2003).

Fig. 5.1 depicts the algorithm how depletion of granular sludge by cobalt (Zandvoort et al., 2006; Chapter 3) or nickel (Zandvoort et al., 2002b; Chapter 4) induces methanol accumulation and latter acidification in methanol-fed UASB reactors.

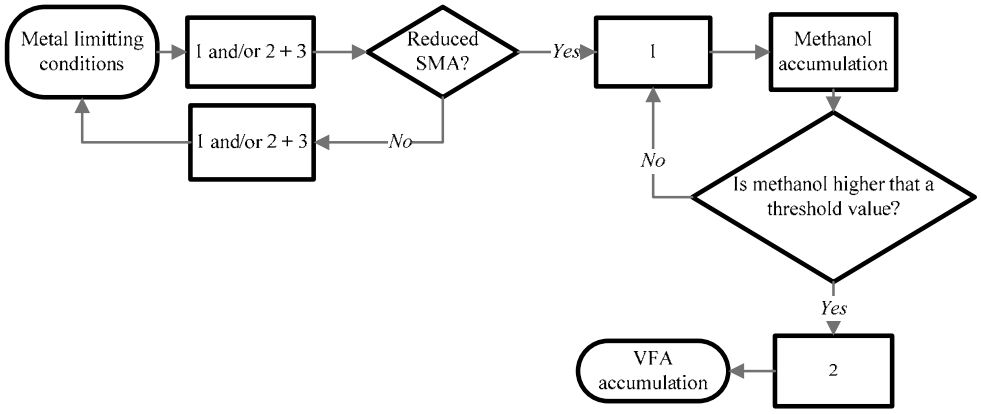

Figure 5.1. Proposed outline of the mechanism of induction of metal limitation in methanol-fed UASB reactors. Numbers indicate predominant process. (1) methylotropihic methanogenesis. (2) acetogenesis. (3) acetotrophic methanogenesis. (From Chapter 3).

Zinc is another key element in methanogenesis (Fig. 5.2) and its potential deprivation from anaerobic granular sludge has thus far not been investigated. Zinc is involved in coenzyme M activation during the formation of methyl-coenzyme M (Sauer and Thauer, 2000) (Fig. 5.2). This enzyme system from *Methanosarcina barkeri* is composed of two methyltransferases, designated MT1 (methanol: 5- hydroxybenzimidazolylcobamide (B12-HBI) methyltransferase) and MT2 (Co-methyl-5-hydroxybenzimidazolylcobamide (CH_3-B12-HBI): HS-CoM methyltransferase), which catalyze the formation of methyl-coenzyme M from coenzyme M and methanol (van der Meijden et al., 1983). MT1 in the cob(I)amide-reduced form catalyzes methylation with methanol (Fig. 5.2) (Daas et al., 1996). MT2 is a zinc containing monomeric protein which catalyzes methyl transfer from methylated MT1 to coenzyme M (Fig. 5.2) (Harms

and Thauer, 1996). The methylation of coenzyme M to methyl-coenzyme M is a reaction in which a thiol group is alkylated, and all enzymes catalyzing alkyl transfers to thiols are zinc containing proteins (Matthews and Goulding, 1997; Penner-Hahn, 2007). The postulated role of zinc in these enzymes is that of a Lewis acid that activates the thiol groups to be alkylated (Goulding and Matthews, 1997). In the methanogenic degradation of other substrates, such as dimethylsulfoniopropionate (van der Maarel and Hansen, 1997) or methylamines (Zhilina and Zavarzin, 1990), enzymatic alkyl transfer reactions are involved as well, which could thus also be affected upon zinc deprivation.

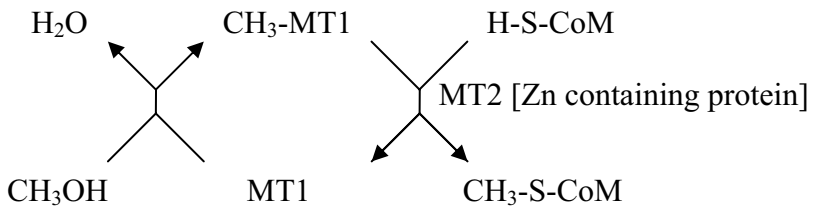

Figure 5.2. Methyl-coenzyme M formation from methanol and coenzyme M (From Sauer and Thauer, 2000).

The aim of this study is to evaluate the effect of long-term zinc deprivation on the performance of methanol fed UASB reactors and if the outline found for long-term cobalt and nickel deprivation (Fig. 5.1) also applies to zinc. Moreover, the possible restoration of the activity of the zinc deprived sludge by continuous zinc addition to the influent was investigated as well. Therefore, the UASB reactor performance was monitored as a function of time. The metabolic properties and possible zinc deficiencies of the sludge that developed in the UASB reactor were characterized by specific methanogenic activity tests. The microbial population dynamics in the sludge granules were observed by Fluorescent In Situ Hybridization (FISH).

5.2. MATERIALS AND METHODS

5.2.1. Source of biomass

Methanogenic granular sludge was obtained from a full-scale UASB reactor treating alcohol distillery wastewater at Nedalco (Bergen op Zoom, The Netherlands). The total suspended solids (TSS) and volatile suspended solids (VSS) concentration of the wet sludge were 5.23 (± 0.02) % and 4.93 (± 0.03) %, respectively.

5.2.2. Feed composition

The reactors were fed a basal medium consisting of methanol, macronutrients and a trace metal solution (Table 5.1) dissolved in demineralised water. The inorganic macronutrients contained (in milligrams per litre of basal medium): NH_4Cl (280), K_2HPO_4 (250), $MgSO_4 \cdot 7H_2O$ (100) and $CaCl_2 \cdot 2H_2O$ (10). To ensure pH stability around 7.0, 2.52 g (30 mM) of $NaHCO_3$ was added per litre of basal medium. To avoid precipitation in the storage vessels, the influent was composed of 3 streams: (1) macronutrients and trace metal solution without K_2HPO_4 and $NaHCO_3$, (2) methanol with $NaHCO_3$ and K_2HPO_4 and (3) dilution water. Demineralised water was used to prepare the influent and as dilution water.

Table 5.1. Composition trace metal solution added to influent and activity tests media.

Compound	Metal	Conc. [μM]
$FeCl_2 \cdot 4H_2O$	Fe (II)	5
$CuCl_2 \cdot 2H_2O$	Cu (II)	0.5
$ZnCl_2$	Zn (II)	0.5 [a] / 5 [b]
$MnCl_2 \cdot 4H_2O$	Mn (II)	0.5
$NiCl_2 \cdot 6H_2O$	Ni (II)	0.5
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	Mo (VI)	0.5
$Na_2SeO_4 \cdot 5H_2O$	Se (VI)	0.5
$Na_2WO_4 \cdot 2H_2O$	W (VI)	0.5
$CoCl_2 \cdot 6H_2O$	Co (II)	0.5

a. 0.5 μM added in R1 after 31 days of operation and from the beginning of R2.
b. 5μM added in R2 after 31 days.

5.2.3. UASB reactor operation

The experiments were performed using 3.25 L glass cylindrical UASB reactors as described in Chapter 3. The reactors were operated in a temperature-controlled room at 30 (±2)°C. The reactors were operated at a hydraulic retention time (HRT) of 12 h and a superficial liquid upflow velocity of 0.35 $m \cdot h^{-1}$. For the influent and recycle flow, peristaltic pumps (type 505S, Watson and Marlow, Falmouth, U.K.) were used. The produced biogas was led through a water lock filled with a concentrated NaOH (15%) solution in order to remove CO_2 and H_2S. The volume of produced methane was measured with a wet gas meter (Schlumberger Industries Dordrecht, The Netherlands).

5.2.4. Experimental design

A UASB reactor (R0) was run during 141 days without zinc in the influent. R0 was inoculated with 1 liter of wet sludge. R0 contained an initial biomass concentration of 6.38 g $VSS \cdot L_{Reactor}^{-1}$ and was operated at an organic loading rate (OLR) of 8 g $COD \cdot L^{-1} \cdot day^{-1}$, corresponding to a specific loading rate (SLR) of 1.25 g $COD \cdot VSS^{-1} \cdot day^{-1}$ based on the initial

VSS concentration. On day 22, the OLR was increased from 8 to 13 g $COD \cdot L^{-1} \cdot day^{-1}$. Sludge growth was excessive and the sludge bed height increased by 80 % during the 141 days of operation.

The biomass present in R0 after the 141 days of operation was split in two UASB reactors, both inoculated with 0.9 L wet sludge from R0, corresponding to an initial biomass concentration of 6.89 g $VSS \cdot L_{Reactor}^{-1}$. Both reactors were operated at an OLR of 6.5 g $COD \cdot L^{-1} \cdot day^{-1}$ (SLR of 0.94 g $COD \cdot gVSS^{-1} \cdot day^{-1}$ based on initial VSS concentration). One UASB reactor (R1) was fed without zinc during 31 days and with 0.5 μM zinc in the influent from day 31 till the end of the experiment (day 44). The second UASB reactor (R2) was fed with zinc in the influent from the start: 0.5 μM zinc during 31 days and 5 μM zinc from day 31 till the end of the experiment (day 44).

5.2.5. Specific methanogenic activity test

The effect of different metal concentrations in test medium and the possible zinc limitation of the sludge that developed in the reactor were investigated by specific methanogenic activity (SMA) tests. The SMA of the sludge was determined in duplicate at 30 (±2) ◦C using on-line gas production measurements as described by Zandvoort et al. (2002a). Approximately 1 g (wet weight) of granular sludge was transferred to 120 mL serum bottles containing 50 mL of basal medium with the same composition as the reactor basal medium, supplemented with methanol (4 g $COD \cdot L^{-1}$), acetate (2 g $COD \cdot L^{-1}$) or hydrogen (1 g $COD \cdot L^{-1}$) as the substrate. Data were plotted in a rate versus time curve, using moving average trend lines with an interval of 15 data points. The SMA with methanol as the substrate was determined on days 50, 69, 91, 111, and 137 for R0, and days 10, 18 and 44 for R1 and R2. The SMA with acetate as the substrate was determined on days 94 and 113 for R0, day 10 for R1 and day 18 for R2. The SMA with hydrogen was determined on day 113 for R0. The samples were always taken from the same place (the mid-height) in the sludge bed.

5.2.6. Fluorescent in situ hybridization

Anaerobic granules from operation days 22, 50, 91, 115, 130 and 135 of R0; 10, 18, 30 and 44 of R1 and 10, 18, 30 and 44 of R2 were fixed in 4% (w/v) paraformaldehyde in phosphate buffered saline (PBS; 130 $mmol \cdot L^{-1}$ sodium chloride and 10 $mmol \cdot L^{-1}$ sodium phosphate [pH 7.2]). Cross-sections (thickness, 16 μm; Ø, 0.5-1.5 mm) were prepared as described previously by Sekiguchi et al. (1999). Fluorescent in situ hybridization (FISH) was performed as described by Schramm et al. (1998) using Cy5 and Cy3-labeled 16S rRNA-targeted oligonucleotide probes (Biomers.net, Germany): Eub338 (Stahl and Amann, 1991), *Eury498* (Burggraf et al., 1994) and *Sarci551* (Sorensen et al., 1997), specific for the kingdom *Eubacteria* and *Euryarchaeota* and *Methanosarcina* genus, respectively. Hybridization

stringencies in the hybridization buffer used were achieved by adding formamide. The *Non338* probe (Wallner et al., 1993) was used as a negative control. Specimens were viewed using a Nikon E600 epifluorescent microscope equipped with a 100 W mercury bulb and a Cy3 and Cy5 filter set. The abundance of *Eubacteria, Euryarchaeota* and *Methanosarcina* was determined as a fraction of the total area of respective granule sections by image analysis with ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2007.).

5.2.7. Metal composition of the sludge

The metal composition of the sludge was measured on days 22, 50, 81, 107 and 141 for R0; days 10, 18, 31 and 40 for R1 and 10, 18 and 40 for R2. The metal composition of the sludge was determined after destruction with Aqua regia (mixture of 2.5 mL 65% HNO_3 and 7.5 mL 37% HCl) as described in Chapter 4.

5.2.8. Chemical analyses

Methanol and VFA were determined using gas liquid chromatography as described by Weijma et al. (2000). Total dissolved metal concentrations in the influent and effluent were determined by ICP-OES (Varian, Australia) in samples acidified with 0.1 M HNO_3. The samples were centrifuged at 10000 rpm to remove particles from the liquid. The total suspended solids (TSS) and volatile suspended solids (VSS) concentrations were determined according to Standard Methods (APHA/AWWA, 1998). All chemicals were of analytical or biological grade and purchased from E. Merck AG (Darmstadt, Germany).

5.3. RESULTS

5.3.1. Reactor operation

After a start-up period of 10 days, methanol was almost fully converted to methane during the 141 days of R0 operation, despite some incidental VFA accumulation in the effluent on day 45, 76 and 135. VFA accumulated maximally to 0.9 g COD-VFA·L^{-1} and remained at this level not longer than a couple of days (Fig. 5.3). On day 22, the OLR was increased from 8 to 13 g COD·L^{-1}·day^{-1} without accumulation of methanol or VFA in the influent. The pH was constant (6.86 ± 0.09) during the entire run (Fig. 5.3). The sludge bed height increased by 80 % during the 141 days of operation.

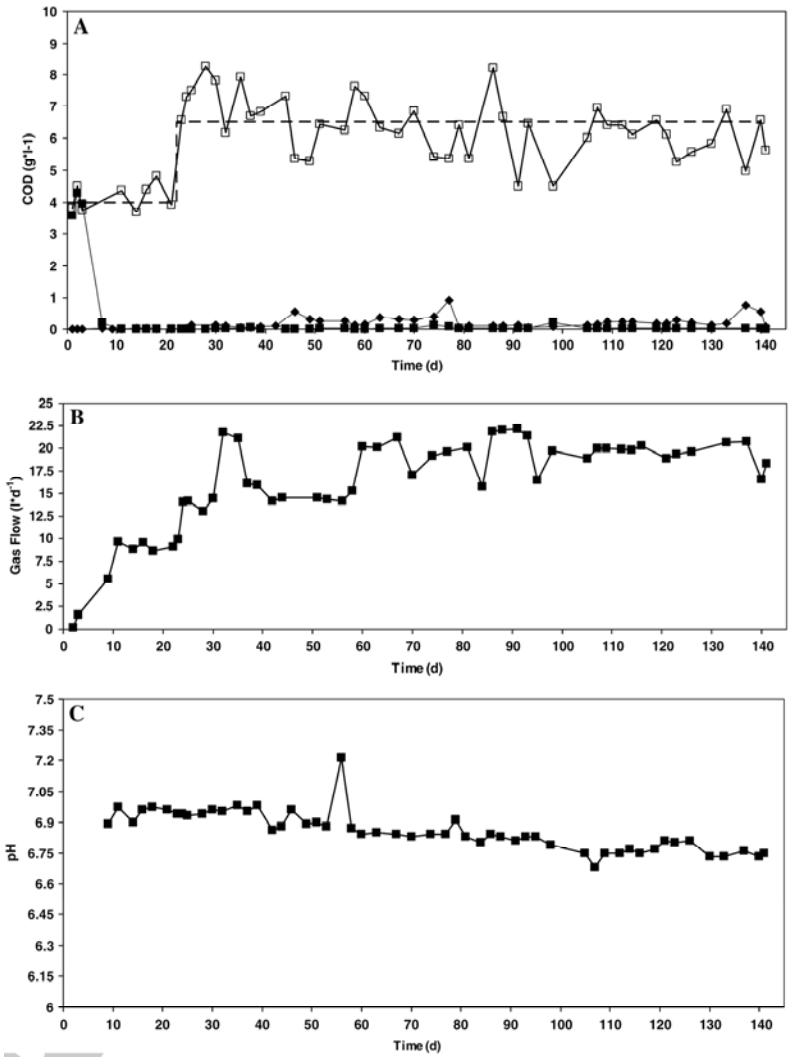

Figure 5.3. Evolution of R0 performance with time. (A) Calculated influent methanol (---), measured influent methanol (□), effluent methanol (■) and effluent VFA (♦) concentrations, (B) CH_4 production and (C) pH in reactor.

The methanol effluent concentration of R1 started to accumulate on day 10, reaching a maximum value of 3.9 g COD-MeOH·L^{-1} on day 19, corresponding to 65 % of the influent methanol concentration (Fig. 5.4). On day 16, VFA started to accumulate and the pH of the R1 effluent dropped from 6.8 to 6.0. The VFA concentration reached 1.8 g COD-VFA·L^{-1} on day 19 and no methane was produced anymore. The OLR was decreased to 2.5 g COD·L^{-1}·day^{-1} on day 19, resulting in a decrease in the methanol effluent concentration of R1 up to 0.5 g COD-

MeOH·L^{-1}. However, the R1 effluent VFA concentration was stable at 1.8 g COD-VFA·L^{-1}. On day 31, 0.5 µM of zinc was included in the R1 influent. The methanol accumulation gradually decreased up to 0.14 g COD-MeOH·L^{-1}. However, the methane production did not recover and all supplied methanol was converted to VFA (mainly acetate) at the end of the experiment (day 44).

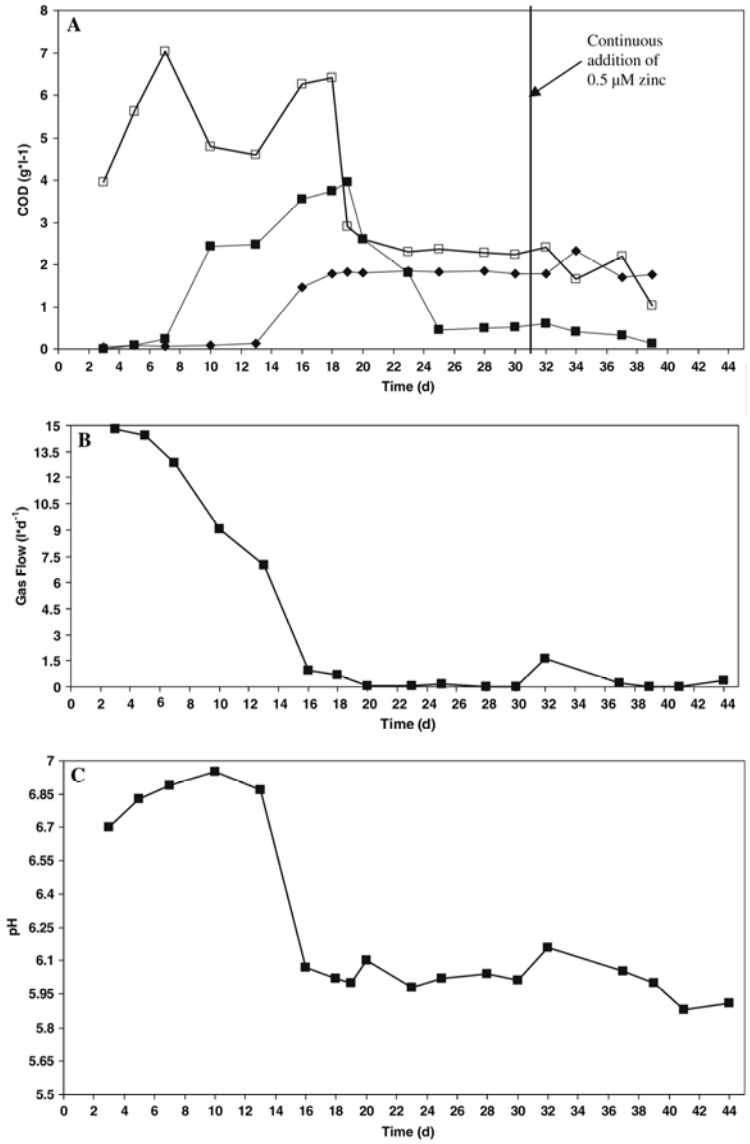

Figure 5.4. Evolution of R1 performance with time. (A) Influent methanol (□), effluent methanol (■) and effluent VFA (♦) concentrations, (B) CH_4 production and (C) pH in reactor.

Complete methanol removal was observed during 16 days of R2 operation (Fig. 5.5). On day 16, the methanol effluent concentration started to accumulate to 0.3 g COD-MeOH·L^{-1} and on day 18, VFA started to accumulate as well. On day 19, R2 effluent methanol and VFA concentrations reached 1.1 g COD-MeOH·L^{-1} and 1.1 g COD-VFA·L^{-1}, respectively, and only 62 % of the supplied methanol was converted to methane.

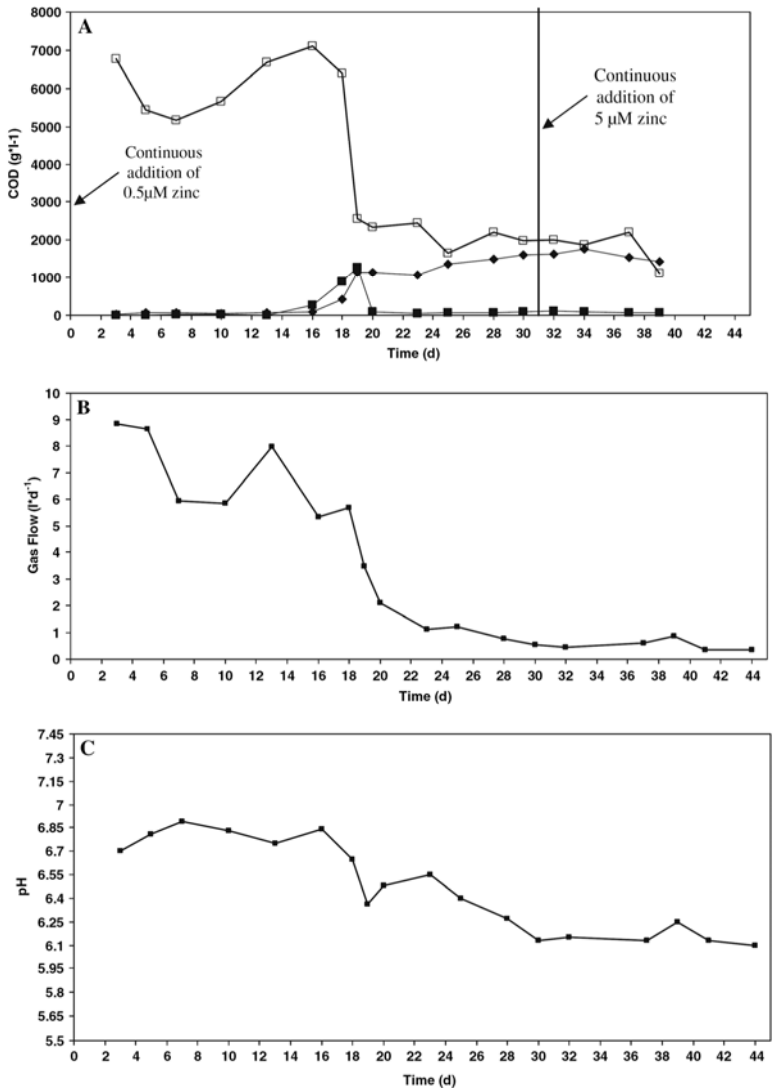

Figure 5.5. Evolution of R2 performance with time. (A) Influent methanol (□), effluent methanol (■) and effluent VFA (♦) concentrations, (B) CH_4 production and (C) pH in reactor.

The pH of the R2 effluent dropped from 6.8 (day 16) to 6.2 (day 19). The OLR was therefore decreased to 2.5 g $COD \cdot L^{-1} \cdot day^{-1}$ on day 19, resulting in a gradual decrease in methanol accumulation to 0.09 g $COD\text{-}MeOH \cdot L^{-1}$ on day 23. However, the R2 effluent VFA concentration increased up to 1.6 g $COD\text{-}VFA \cdot L^{-1}$ on day 30 and only 15 % of the fed methanol was converted to methane on that day. On day 31, the zinc concentration in the R2 influent was increased to 5 μM. This did not induce significant changes in the R2 effluent methanol or VFA concentration within the time span investigated (day 31-44).

5.3.2. Evolution of SMA over time

Induction of zinc limitation was observed in R0 sludge after 50 to 70 days of operation (Fig. 5.6A; Table 5.2). On day 50, the SMA with methanol as the substrate in a medium with no metals was 14 % lower than in a medium with zinc solely. Similarly, the SMA with methanol as the substrate in a medium with no metals on day 69 was 18 % and 21 % lower than in a medium with zinc solely and in a medium with the complete metal solution except zinc, respectively. It should be noted that on day 69, the SMA on methanol had increased for all the different trace metal solutions tested compared to day 50.

On day 90, a further increase in SMA of the R0 sludge with methanol as the substrate was observed (Table 5.2), but no metal limitation of the SMA on methanol was observed on day 90 as the tests with zinc and without zinc had a similar SMA (Table 5.2). On day 111, the SMA on methanol increased again for all the trace metal solutions tested and metal limitation was observed again. The SMA on methanol with the complete metal solution was 7 %, 13 % and 24 % higher than in, respectively, a medium with no metal, a medium with the complete metal solution except zinc and a medium with zinc solely. The SMA with acetate and hydrogen as the substrates, measured on day 111, were 0.51 g $CH_4\text{-}COD \cdot g\ VSS^{-1} \cdot d^{-1}$ and 0.82 g $CH_4\text{-}COD \cdot g\ VSS^{-1} \cdot d^{-1}$, respectively. The latter values indicate that maximally 38 % of the total amount of methane formed could be produced via acetate and/or hydrogen as the intermediates if they are compared with the SMA on methanol on the same day, 3.49 g $CH_4\text{-}COD \cdot g\ VSS^{-1} \cdot d^{-1}$ (Table 5.2).

On day 137, a clear zinc limitation was observed (Table 5.2; Fig. 5.6A): the SMA on methanol with the complete metal solution except zinc or in a medium with no metals at all was 24 % and 28 % lower than the SMA on methanol in the medium with the complete metal solution. The SMA on methanol with the complete metal solution was slightly higher (3 %) than in a medium with zinc solely.

After 10 days of operation of R1, at the time that methanol accumulation was observed, the SMA on methanol had decreased to 2.26 g $CH_4\text{-}COD \cdot g\ VSS^{-1} \cdot d^{-1}$ in a medium containing the complete metal solution except zinc, corresponding to a 52 % lower SMA than the R0 sludge had on day 137 for the same medium (Table 5.2; Fig. 5.6B). The SMA of the R1 sludge with acetate as the substrate on day 10 (0.31 g $CH_4\text{-}COD \cdot g\ VSS^{-1} \cdot d^{-1}$) indicates that maximally 13 %

of the methane formed could be produced via acetate as an intermediate (Table 5.2). On day 18, when VFA accumulation was observed, the SMA with methanol as the substrate had decreased to 0.96 g CH_4-COD·g VSS^{-1}·d^{-1} in a medium containing the complete trace metal solution except zinc. On day 44, 13 days after zinc addition (0.5 μM) to the R1 influent, the SMA with methanol as the substrate had decreased to as low as 0.17 g CH_4-COD·g VSS^{-1}·d^{-1} in a medium containing the complete trace metal solution except zinc (Table 5.2).

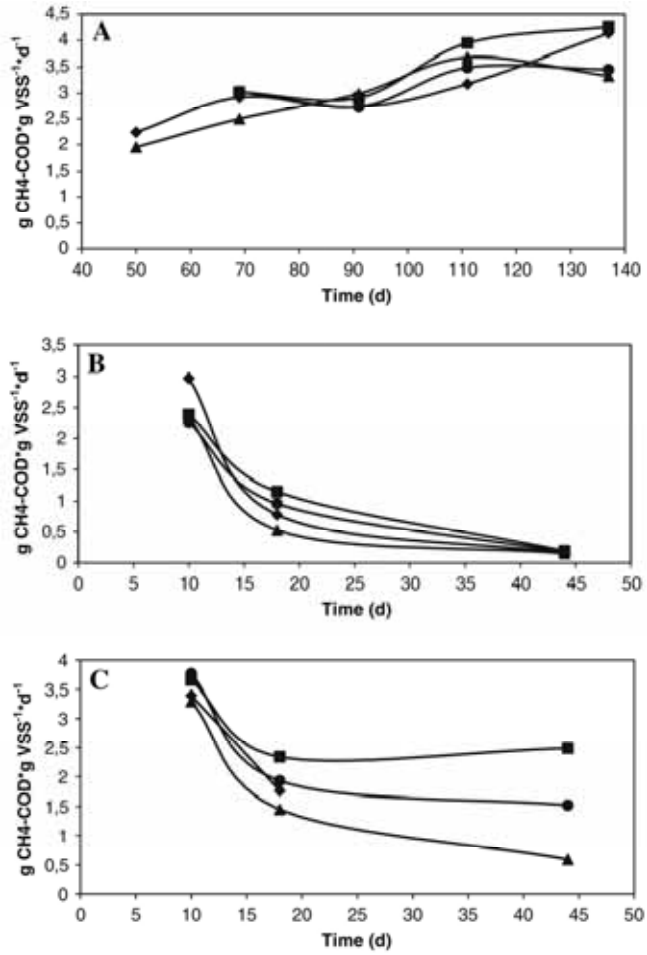

Figure 5.6. Evolution of SMA on methanol with time. (A) R0. (B) R1. (C) R2. No metal addition (▲), addition of complete trace metal solution except zinc (●), addition of complete trace metal solution (■) and addition of zinc alone (♦).

Table 5.2. Evolution of the specific methanogenic activity (g CH_4-COD·g VSS^{-1}·d^{-1}) as a function of time, organic source and trace metal content of the medium.

	Specific maximum methanogenic activity [g CH_4-COD g VSS^{-1} day^{-1}]											
	Methanol				Acetate				Hydrogen/CO_2			
Day of operation	No metals	Complete trace metal solution except zinc	Complete trace metal solution	Zinc solely	No metals	Complete trace metal solution except zinc	Complete trace metal solution	Zinc solely	No metals	Complete trace metal solution except zinc	Complete trace metal solution	Zinc solely
R0												
50	1.95±0.12	ND	ND	2.24±0.03	ND	ND	ND	ND	ND	ND	ND	ND
69	2.50	3.03±0.08	3.02±0.05	2.92±0.08	ND	ND	ND	ND	ND	ND	ND	ND
91	2.99±0.02	2.74±0.07	2.91±0.12	2.73±0.08	ND	ND	ND	ND	ND	ND	ND	ND
111	3.68±0.23	3.49	3.95±0.55	3.18±0.09	0.42±0.01	0.51±0.01	0.45	0.46±0.02	0.75±0.01	0.82±0.02	0.87±0.06	0.73±0.13
137	3.33±0.15	3.45±0.21	4.26	4.14	ND	ND	ND	ND	ND	ND	ND	ND
R1												
10	2.34±0.08	2.26±0.20	2.39±0.18	2.97±0.20	0.30	0.31	0.33	0.31	ND	ND	ND	ND
18	0.53±0.11	0.96±0.02	1.15±0.01	0.78±0.07	ND	ND	ND	ND	ND	ND	ND	ND
44	0.15	0.17	0.19±0.01	0.15±0.03	ND	ND	ND	ND	ND	ND	ND	ND
R2												
10	3.30±0.30	3.7 ±0.3	3.66 ±0.30	3.40 ±0.1	ND	ND	ND	ND	ND	ND	ND	ND
18	1.45±0.03	1.93±0.03	2.34±0.04	1.76±0.11	0.35	0.36±0.01	0.35±0.01	0.32±0.03	ND	ND	ND	ND
44	0.59±0.23	1.52±0.08	2.49	ND	ND	ND	ND	ND	ND	ND	ND	ND

ND: Not determined

After 10 days of operation of R2, the SMA of the R2 sludge on methanol was similar to the SMA observed of R0 sludge on day 137. Interestingly, no differences in SMA with methanol as the substrate were observed with the different metal solutions used (Table 5.2; Fig 5.6C). On day 18, when methanol and VFA accumulated, the SMA had decreased to 1.93 g CH_4-COD·g VSS^{-1}·d^{-1} in a medium containing the complete trace metal solution except zinc. A low SMA of the R2 sludge with acetate as the substrate was observed at this day (0.35 g CH_4-COD·g VSS^{-1}·d^{-1}). On day 44, 13 days after the zinc increase in the R2 influent to 5 μM, the SMA of the R2 sludge had decreased to 1.52 g CH_4-COD·g VSS^{-1}·d^{-1} in a medium containing the complete trace metal solution except zinc (Table 5.2).

5.3.3. Metal dynamics

Zinc was lost from the granular sludge at a rate of 10 μg·g TSS^{-1}·d^{-1} during the first 80 days of R0 operation (Fig. 5.7A). In the same period, zinc was washed out from the reactor via the effluent at a rate of 0.48 μg·L^{-1}·d^{-1}. Nickel and iron concentrations in the granular sludge decreased at a rate of 1.3 and 106 μg ·g TSS^{-1}·d^{-1}, respectively (Fig. 5.7C,E). The cobalt concentration in the R0 sludge was, however, constant during the first 80 days (0.032 ± 0.005 μg·g TSS^{-1}) (Fig. 5.7C). Cobalt and nickel showed a distinct difference in retention, even when they were dosed at the same concentration (Table 5.1) during the same period. From day 80 to the end of R0 operation (day 141), the zinc, cobalt, nickel and iron concentration in the granular sludge were constant: 0.18 (± 0.04), 0.040 (± 0.07), 0.06 (± 0.01) and 1.7 (± 0.5) μg·g TSS^{-1}, respectively (Fig. 5.7A,C,E).

The zinc concentration of the R1 sludge on day 19 was about half compared to day 13: 0.030 and 0.058 μg·g TSS^{-1}, respectively (Fig. 5.7B). The decrease in the zinc concentration of the R1 sludge coincides with the pH drop from 6.8 to 6.0 of the R1 mixed liquor (Fig. 5.4C). Zinc addition (5 μM) to R1 influent from day 32 onwards did not increase the zinc concentration of the granular sludge (Fig. 5.7B). In the same period, the cobalt and nickel concentration almost doubled compared to the concentration in the R1 sludge prior to the addition (Fig. 5.7D).

After 31 days of zinc addition to R2, the zinc concentration in the R2 sludge had almost doubled compared to the seed sludge used to inoculate R2 (Fig. 5.7B). The zinc concentration of the R2 sludge had not increased on day 40, 9 days after the increase of the zinc concentration in the influent from 0.5 μM to 5 μM. During the 44 days of R2 operation, the cobalt and iron concentration in the R2 sludge were stable at 0.056 (± 0.003) μg ·g TSS^{-1} and 1.15 (± 0.1) μg ·g TSS^{-1}, respectively (Fig. 5.7D, F). In the same period, the nickel concentration in the R2 sludge increased from 63 μg·g TSS^{-1} on day 10 to 158 μg·g TSS^{-1} on day 40 (Fig. 5.7D).

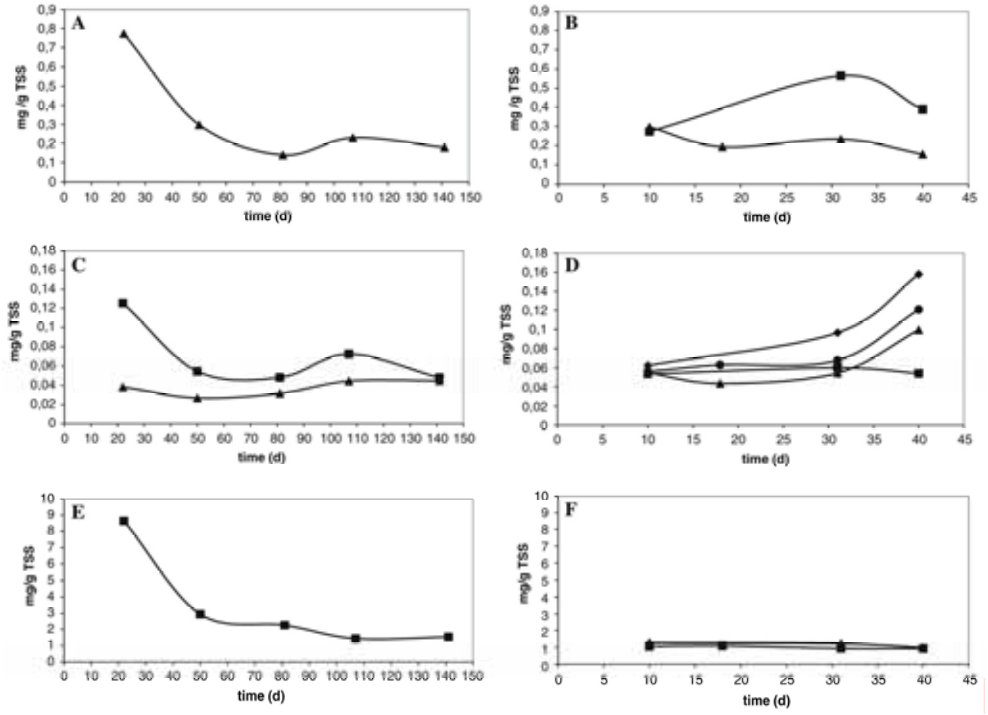

Figure 5.7. Evolution of trace metal and sulfur concentration in the sludge over time. (A) Zinc concentration in the sludge in R0. (B) Zinc concentration in the sludge in R1 (▲) and R2 (■). (C) Cobalt (▲) and nickel (■) sludge concentration in R0. (D) Cobalt (▲) and nickel (●) sludge concentration in R1 and cobalt (■) and nickel (♦) sludge concentration in R2. (E) Iron sludge concentration in R0. (F) Iron sludge concentration in R1 (■) and R2 (▲).

5.3.4. Evolution of key microbial population

The abundance of the *Euryarchaeota* and *Methanosarcina* in the R0 granules was stable during the 141 operation days (Fig. 5.8A, C, D, F). The abundance of the *Eubacteria* followed a slight decreasing trend. On day 130, the abundance of cells hybridizing to *Eub338* had decreased compared to day 50 (Fig. 5.8B, E). On day 135, an increase of cells hybridizing to *Eub338* was observed, which coincides with a short VFA accumulation in the effluent (Fig. 5.3A versus Fig. 5.8B, E).

Genus-specific hybridizations with the *Eury498* and *Eub338* probe revealed a temporal evolution of the *Euryarchaeota* and *Eubacteria* populations in the R1 run. Firstly, *Eury498*-positive cells were abundant around the periphery of respective sections on day 10 (Fig. 5.9A). *Eub338*-positive cells indicated very few *Eubacteria* cells, which were mainly present in infrequent clusters around the edge of the granule sections on day 10 (Fig. 5.9B). On day 18, the *Euryarchaeota* and *Eubacteria* populations were located throughout the sections of the sludge.

An increase in *Eubacteria* population was observed on day 18. By day 30, the relative abundance of the *Euryarchaeota* was reduced in R1 sludge, which were present in relatively smaller, infrequent clusters around the edge of the granule sections (Fig. 5.9A). However, the *Eubacteria* population on day 30 (Fig. 5.9E) was as abundant as on day 18. On day 44, after zinc addition in R1, a further reduction in the *Euryarchaeota* population was observed (Fig. 5.9D). The *Eub338*-positive cells were typically observed in densely packed clusters on day 44, but they were less dense than on day 30 (Fig. 5.9E). The abundance of the *Methanosarcina* followed a slight decreasing trend. On day 30, the abundance of cells hybridizing to *Sarci551* had decreased compared to day 10 (Fig. 5.9C). A further reduction was evident by day 44 (Fig. 5.9F).

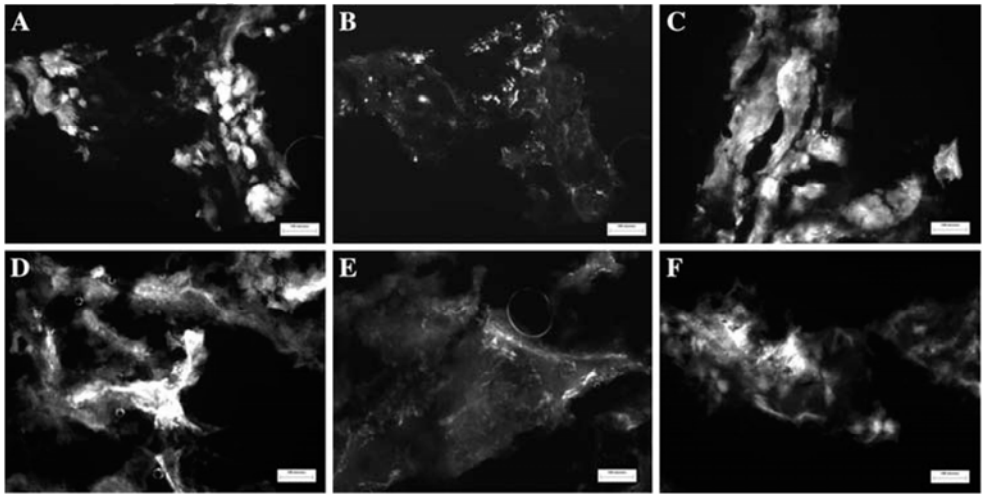

Figure 5.8. In situ hybridizations using cross-sections of anaerobic granules in R0 illustrating (a) Eury498, (b) Eub338 and (c) Sarci551 in day 50; (d) Eury498, (e) Eub338 and (f) Sarci551 in day 135. Scale bar = 100μm.

The *Euryarchaeota* and *Methanosarcina* populations were dynamic in the R2 granules. Firstly, *Eury498*-positive cells were observed in densely packed clusters in the centre of the granule section on day 10 (Fig. 5.10A). *Sarci551*-positive cells were present in clusters around the edge of the granule sections on day 10 (Fig. 5.10C). By day 18, an increased abundance of *Eury498*-positive clusters was detected. The *Methanosarcina* population was observed in less dense clusters than on day 10. On day 30, a slight reduction in the number of *Euryarchaeota* was observed. A slight increase of *Sarci551*-positive cells was evident by day 30, which were more dispersed than on day 18. On day 44, after the zinc concentration was increased in R2 influent, a reduction in the *Methanosarcina* population size was observed (Fig. 5.10F). More *Eury498*-positive dispersed cells were observed on day 44 (Fig. 5.10D) than on day 30. *Eub338*-

positive cells in R2 were abundant around the periphery of respective sections on day 10 (Fig. 5.10B). On day 18, a decrease in abundance of cells hybridizing to *Eub338* compared to day 10 was observed (Fig. 5.10B). No clear further reduction was observed by days 30 and 44 (Fig. 5.10E).

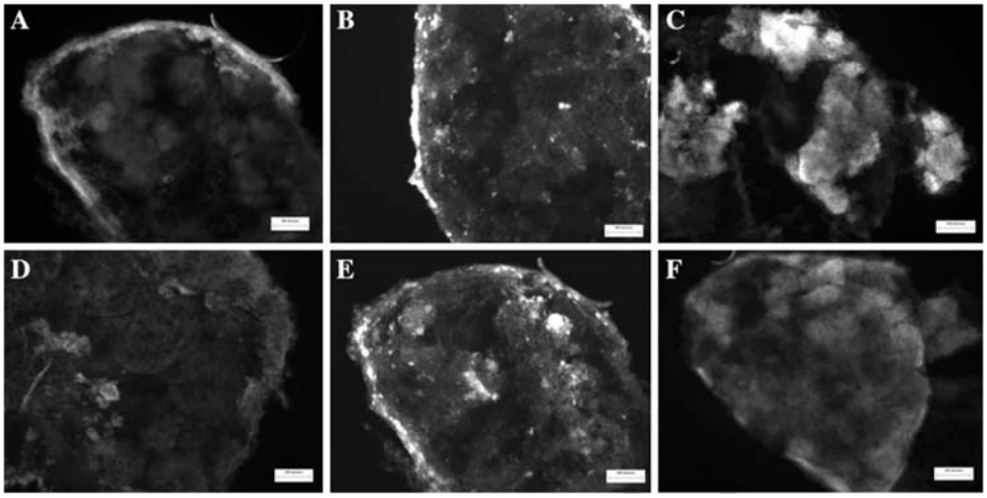

Figure 5.9. In situ hybridizations using cross-sections of anaerobic granules in R1 illustrating (a) Eury498 in day 30; (b) Eub338 in day 10; (c) Sarci551 in day 10; (d) Eury498 in day 44; (e) Eub338 in day 30 and (f) Sarci551 in day 44. Scale bar = 100 µm.

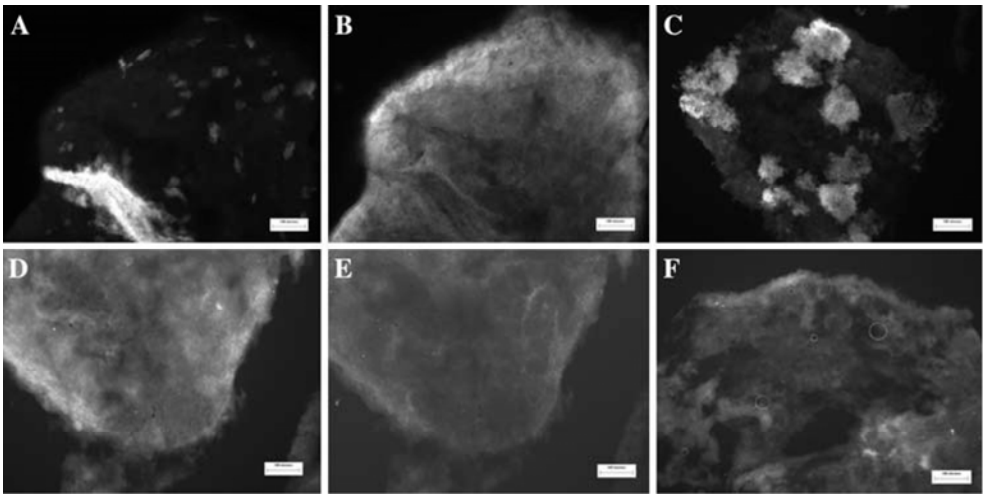

Figure 5.10. In situ hybridizations using cross-sections of anaerobic granules in R2 illustrating (a) Eury498, (b) Eub338 and (c) Sarci551 in day 10; (d) Eury498, (e) Eub338 and (f) Sarci551 in day 44. Scale bar = 100 µm.

5.4. DISCUSSION

5.4.1. Methanogenesis under zinc limiting conditions

This study shows that omission of zinc from the feed of methanol-fed anaerobic granular sludge bioreactors leads to zinc limitation of the granular sludge (Fig. 5.6A; Table 5.2), which reduces the methanol removal efficiency (Fig. 5.4A), and ultimately leads to acidification of the UASB reactor (Fig. 5.4A,C). Zinc depletion thus leads to a drop in the SMA with methanol as the substrate according to the algorithm present in Fig. 5.1, similar to the way cobalt (Zandvoort et al., 2002a; Chapter 3) and nickel (Chapter 4) deprivation of UASB reactors occurs.

Table 5.3 shows that the zinc limitation is less pronounced compared to cobalt or nickel limitation of methanol fed UASB reactors under similar conditions. The rather long time period needed to achieve zinc limitation (>130 days) was also required to achieve nickel limitation (Chapter 4) (Table 5.3). This is, in contrast, much longer compared to the time required for cobalt limitation, which can already develop after 2 days of operation at an OLR of 4.5 g $COD \cdot L^{-1} \cdot day^{-1}$ (Chapter 3; Table 5.3). This clearly reflects the biochemical role of cobalt in the direct methanogenesis with methanol as the substrate, e.g. the central ion of cobalamine is a cobalt ion (Florencio et al., 1994). In contrast, both zinc and nickel play only an indirect role in the functioning of enzymes of methanogenesis, such as methyl-coenzyme M reductase for nickel (Ellefson et al., 1982) or coenzyme M methyltransferase for zinc (Sauer and Thauer, 2000).

The omission of zinc from the UASB reactor influent had some unexpected effects. The maximal SMA achieved in the present study was much higher (3.49 g CH_4-$COD \cdot g\ VSS^{-1} \cdot d^{-1}$) than the maximal SMA achieved in UASB reactors from which nickel or cobalt is omitted from the influent: 1.67 g CH_4-$COD \cdot g\ VSS^{-1} \cdot d^{-1}$ (Chapter 4) and 0.45 g CH_4-$COD \cdot g\ VSS^{-1} \cdot d^{-1}$ (Chapter 3), respectively (Table 5.3). Inhibition by zinc could be one of the reasons for the lower activity of sludge developing under cobalt or nickel limitation (but in the presence of zinc). Tan et al. (2004) showed that zinc could replace nickel in Acetyl Coenzyme A synthase and subsequently inactivate this enzyme, involved in acetogenesis from methanol. Moreover, the sludge bed growth was much higher compared to that in cobalt or nickel deprived reactors (Table 5.3). This might be due to the higher methanogenic activity of the sludge present in the zinc limited reactor or that the presence of zinc in the cobalt and nickel limited reactors had a growth retarding effect (Salminen et al., 2001).

Only a few papers have so far reported on zinc deprivation during methanogenesis. Osuna et al. (2002) studied metal deprivation in VFA fed UASB reactors. The latter authors found that when adding solely zinc to the medium, the SMA with acetate as the substrate of metal deprived sludge increased by 36 %. Sauer and Thauer (1997) found in enzymatic studies that the transfer of the methyl group from CH_3-MT1 to coenzyme M by MT2 depends on the Zn^{2+} concentration. The K_M value of this transfer reaction amounted to 0.25 nM free Zn^{2+}. This K_M value is around 240 times lower than the dissolved zinc concentration in the medium of the

reactor (60 nM) at the moment the limitation was developed in the present study. This difference between the K_M value found by the latter authors (with enzymes) and the dissolved zinc concentration at the time of limitation in the present study (with sludge granules) can be explained by several interactions between the metal cation and the heterogeneous and complex environment inside a UASB reactor. Complexation by bicarbonates, phosphates, soluble sulfides or soluble extrapolymeric substance produced by the biomass reduce the free metal concentration (van Hullebusch et al., 2003).

The dissolved metal concentrations can be only 0.5-4 % of the total metal concentration in anaerobic environments (Osuna et al., 2002), since metal speciation under these conditions depends on the concentration of the metal, its redox state and competition with other metals for adsorption and complexation sites. Assuming the K_M value for the zinc requirement of *Methanosarcina* cells is in the range reported by Sauer and Thauer (1997) and the maximum dissolved zinc concentration is 4 % of the total zinc concentration (Osuna et al., 2002), the required free zinc fraction would be only around 0.016 % of the total zinc concentration present. As the reactor contains already a substantial zinc pool via the inoculum sludge (Fig. 5.7A), dosing zinc to bioreactors will not always be necessary to support the zinc requirement of the methanogens. A slight change in the speciation of zinc, e.g. by a change in redox or by exposure to an electrokinetic field (Virkutyte et al., 2005), can release the zinc present into a bioavailable pool, and thus support the zinc dependent enzymatic processes.

5.4.2. Acetogenesis under zinc limiting conditions

Due to the SMA drop with methanol as the substrate, methanol started to accumulate in R1 (Fig. 5.4) and in R2 (Fig. 5.5). Methanol accumulation was followed by acetate accumulation, similar to the outline shown in Fig. 5.1. Once the methanol accumulation surpasses a threshold value, about 50 mM in R1 and 20 mM in R2, acetogens outcompeted methanogens for methanol, due to their higher affinity for methanol at high methanol concentrations (Florencio et al., 1995). The threshold methanol concentration of cobalt and nickel deprived reactors to get acidification were in the same range as observed in this study with zinc deprived UASB reactors (Table 5.3). The similar threshold value for acidification found for methanol fed UASB reactors limited by different metal (Table 5.3) indicates that enhancement of acetogens upon exceeding the methanol threshold value is metal independant.

Table 5.3. Comparison of kinetic parameters of sludge from methanol fed UASB reactors deprived by different metals.

Deprived metal	Inoculum sludge	Days of operation before limitation is achieved (percentage of limitation)[a]	Maximum limitation reported [%] (operation day when it is achieved)	SMA with the metal under study when maximun limitation is reported [mg CH_4- COD g VSS^{-1} day^{-1}]	OLR [g COD L^{-1} day^{-1}]	Maximum SMA achieved in the reactor run [mg CH_4- COD g VSS^{-1} day^{-1}]	Km [nM]	Methanol threshold for acidification [mM]	Sludge bed growth [%]	Recovery by metal dosing	Reference
Cobalt	Nedalco	0 (52%)	90% (28)	1094	2.6	115	364	No acidification	15%	Yes (before acidification is present)	Zandvoort et al., 2002a
Cobalt	Eerbeek	55 (33%)	33% (55)	2515	17.6	1687	Not reported	15	Not determined	Not tested	Zandvoort et al., 2006
Cobalt	Nedalco	2 (47%)	88% (63)	1583	4.5	446	Not reported	8.5	0%	Not	Chapter 3
Nickel	Nedalco	89 (46%)	46% (89)	546	7.5	292	678	30	Not determined	Not tested	Zandvoort et al., 2002b
Nickel	Eerbeek	82 (27%)	44% (137)	2085	15	1679	160	18	10%	Yes	Chapter 4
Zinc	Nedalco	137 (20%)	20% (137)	4260	13	3490	Not reported	50[b]/20[c]	80%	No	Present study

a: limitation is considered when the SMA with methanol as the substrate in the presence of the metal under study is 20 % higher than the SMA with methanol in medium without the concerned metal.
b: R1
c: R2

5.4.3. Effect of continuous zinc addition

The continuous zinc addition to R2, prior to methanol or acetate accumulation (Fig. 5.5A), supported methanogenesis during a longer period than in R1 to which initially no zinc was added (Fig 4A), but could not prevent its acidification. This suggests that the amount of zinc added to the R2 influent was not enough to support stable high rate methylotrophic and acetoclastic methanogenesis. This contrasts to the almost double zinc concentration of the R2 sludge (Fig. 5.7B). It is expected that the most important process for zinc accumulation in the sludge is zinc sulfide precipitation. The low solubility product of zinc sulfide results in extremely low free zinc ion concentrations (Luther et al., 1996), which might be insufficient to support the trace metal requirement of the methanogens. Therefore, further investigations about dosing methods need to take into account the speciation that involves zinc upon its addition in anaerobic environments (Webb et al., 2000) and the high wash out rates in UASB reactors (Zeng et al., 2005).

The *Methanosarcina* and *Euryarchaeota* populations in R2 were observed in dense clusters in the centre of the granule but in infrequent clusters in the periphery of the granule in R1 after 10 days of operation, when 0.5 µM zinc was continuously added to the influent of the R2 reactor but no zinc to the R1 influent. This suggests that the zinc addition stimulated the growth of methanogens in cluster. Indeed, as shown by other authors (Sekiguchi et al., 1999; Chapter 3, Chapter 4), the association of methanogens in clusters seems to be correlated with the development of the methanogenic population.

Addition of zinc to the reactor influent after methanol and acetate accumulation in the reactors sustained or stimulated acetate accumulation in both reactors (Fig. 5.4A and 5.5A). Methylotrophic or acetoclastic methanogenesis did not recover by the continuous zinc addition to R1 (Table 5.2). In the case of R2 sludge, a five times higher SMA on methanol with zinc in the medium compared to the SMA on methanol in the absence of metals on day 44 (Fig. 5.6C) indicates that the recovery of the methanogenesis by means of zinc addition should be possible. However, the SMA increase on methanol achieved in R2 upon continuous zinc dosing was not high enough to avoid methanol accumulation in the effluent (Fig. 5.5A), which induces reactor acidification (Fig. 5.5C). Both observations for R1 and R2 are in agreement with the FISH pictures, that indicated a further decrease of the *Euryarcheaota* and *Methanosarcina* population after the zinc addition to R1 (Fig. 5.9F) and R2 (Fig. 5.10F) after 31 days of operation.

The same enhancement of acetogenesis and a similar decrease of the *Methanosacina* cell number was observed in Chapter 3 upon cobalt dosing to an acidified cobalt limited methanol fed UASB reactor at pH 7.0. In contrast, in a nickel limited methanol fed UASB reactor with strong acetate accumulation at pH 6.9, the continuous nickel addition enhanced methylotrophic methanogenesis and acidification disappeared within 2 days (Chapter 4). The different behavior upon nickel addition might be due to the lower zinc dependence of the enzymatic system of

methanogenesis (Fig. 5.2) compared to nickel (Sauer and Thauer, 2000). In the case of cobalt, the high cobalt dependence (Bainotti and Nishio, 2000), but slight nickel dependence of the enzymatic system of acetogenesis (Diekert et al., 1981), most probably hampered the restoration of fully methanogenic reactor.

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EFFECT OF TUNGSTEN, MOLYBDENUM AND SELENIUM LIMITATION ON PROPIONATE DEGRADATION IN ANAEROBIC METHANOGENIC GRANULAR SLUDGE

ABSTRACT

Propionate accumulation in anaerobic reactors occurs in case of process failure (e.g. overloading, temperature decrease), and can further increase the negative effects in the process (pH drop, toxicity). Oxidation of propionate to acetate, carbon dioxide, H_2 and formate, can only proceed if the products H_2 and formate are removed by methanogens or other H_2 and formate utilizing bacteria. Molybdenum, tungsten and selenium are essential trace metals for growth of a variety of anaerobic microorganisms and are often essential for enzymes catalyzing reactions, such as formate dehydrogenase (FDH) which catalyzes formate production by propionate oxidizers. The presence of these metals in the anaerobic reactor is essential to keep an optimal enzyme activity and, thus, stable anaerobic reactor operation. In order to supply these essential metals to full scale wastewater treatments in case of limitation, early recognition of the trace metal limitation is essential. The transcription levels of FDH-1 and FDH-2 of *Syntrophobacter* spp. coupled with specific methanogenic activity assays were found to be promising indicators for molybdenum, tungsten and selenium limitation in propionate fed Upflow Anaerobic Sludge Bed (UASB) reactors. According to our results, Q-PCR is a suitable technique to follow FDH-1 and FDH-2 gene expression levels. Therefore, Q-PCR is a promising technique for recognition of trace metal limitation in wastewater treatments.

6.1. INTRODUCTION

Propionate is a key intermediate in the conversion of complex organic matter under methanogenic conditions and it may account for a large fraction of the methane produced (De Bok et al., 2004). Oxidation of propionate to acetate, carbon dioxide, H_2 and formate, can only proceed if the products H_2, formate and to a lesser extent acetate are removed by methanogens or other H_2 and formate utilizing bacteria (Stams, 1994; Schink, 1997). The diffusion of these electron carriers is affected by interspecies distances, therefore, high conversion rates in methanogenic upflow anaerobic sludge bed (UASB) reactors are only observed when propionate-oxidizing bacteria and their methanogenic partners form micro-colonies within densely packed granules (De Bok et al., 2004). *Syntrophobacter* spp. is one of the common propionate degrading bacteria in UASB biofilms (Harmsen et al., 1998; Ariesyady et al., 2007; Fernandez et al., 2007) and is believed to produce not only H_2 but also formate as interspecies electron carrier (Dong et al., 1994).

Molybdenum, tungsten and selenium are essential trace elements for growth of a variety of anaerobic microorganisms and are often essential for enzymes catalyzing reactions, such as formate dehydrogenase (FDH), which catalyzes formate production by propionate oxidizers (Dong et al., 1994). Molybdenum, tungsten and selenium availability is expected to be crucial for formate dehydrogenase activity and subsequent for propionate oxidation (Jiang, 2006) and thus, for UASB reactor performance. In a methanol fed UASB reactor, cobalt limitation decreased SMA (with methanol as substrate) and, thus reactor efficiency (Chapter 3).

The aim of this work was to study the effect of molybdenum, tungsten and selenium limitation in a propionate fed UASB reactor, by coupling the reactor performance with biomass specific methanogenic activity (SMA) measurements and the FDH expression levels in the anaerobic granular sludge. To study the effect of molybdenum, tungsten and selenium limitation on the propionate degradation community in a UASB reactor, we have developed a Quantitative Polymerase Chain Reaction (Q-PCR) method to quantify FDH expression levels. The FDH primers were derived from *Syntrophobacter* spp.

6.2. MATERIALS AND METHODS

6.2.1. Source of biomass

Methanogenic granular sludge was obtained from a full-scale UASB reactor treating alcohol distillery wastewater at Nedalco (Bergen op Zoom, The Netherlands). The total suspended solids (TSS) and volatile suspended solids (VSS) concentration of the wet sludge were 5.23 (± 0.02) % and 4.93 (± 0.03) %, respectively.

6.2.2. Feed composition

The reactor was fed with a basal medium consisting of propionate, macronutrients and a trace element solution (Table 6.1) dissolved in double distilled water. The inorganic macronutrients contained (in milligrams per litre of basal medium): NH_4Cl (280), K_2HPO_4 (250), $MgSO_4 \cdot 7H_2O$ (100) and $CaCl_2 \cdot 2H_2O$ (10). To ensure pH stability around pH 7.0, 2.52 g (30 mM) of $NaHCO_3$ was added per litre of basal medium. To avoid precipitation in the storage vessels, the influent was composed of 3 streams: (1) macronutrients and trace element solution without K_2HPO_4 and $NaHCO_3$, (2) propionate with $NaHCO_3$ and K_2HPO_4 and (3) dilution water. Double distilled water was used to prepare the influent and as dilution water.

Table 6.1. Composition trace element solution added to influent and activity tests media.

Compound	Element	Conc. [μM]
$FeCl_2 \cdot 4H_2O$	Fe (II)	5
$CuCl_2 \cdot 2H_2O$	Cu (II)	0.5
$ZnCl_2$	Zn (II)	0.5
$MnCl_2 \cdot 4H_2O$	Mn (II)	0.5
$NiCl_2 \cdot 6H_2O$	Ni (II)	0.5
$CoCl_2 \cdot 6H_2O$	Co (II)	0.5
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$[a]	Mo (VI)	0.5
$Na_2SeO_4 \cdot 5H_2O$[a]	Se (VI)	0.5
$Na_2WO_4 \cdot 2H_2O$[a]	W (VI)	0.5

a. Added to the reactor influent composition from operation day 249.

6.2.3. UASB reactor operation

The experiments were performed using a 3.25 L glass cylindrical UASB reactor as described in chapter 4. The reactor was operated in a temperature-controlled room at 30 (±2)°C. The reactor was operated at a hydraulic retention time (HRT) of 12 h and a superficial liquid upflow velocity of 0.35 $m \cdot h^{-1}$. For the influent and recycle flow, peristaltic pumps (type 505S, Watson and Marlow, Falmouth, U.K.) were used. The produced biogas was led through a water lock filled with a concentrated NaOH (15%) solution in order to remove CO_2 and H_2S. The volume of produced methane was measured with a wet gas meter (Schlumberger Industries Dordrecht, The Netherlands).

The UASB reactor was operated during 249 days without molybdenum, tungsten or selenium in the influent. The reactor was inoculated with 1 litre of wet sludge. The reactor contained an initial biomass concentration of 6.38 g $VSS \cdot L_{Reactor}^{-1}$ and was operated at an organic loading rate (OLR) of 3.3 (± 1.4) g $COD \cdot L^{-1} \cdot day^{-1}$ between day 0 and day 44 of operation. From day 44 till the end of the experiment (day 276) the OLR applied was 11.8 (± 2.9) g $COD \cdot L^{-1} \cdot day^{-1}$. From day 249 till the end of the experiment, the UASB reactor was supplied with 5 μM of molybdenum, tungsten and selenium.

6.2.4. Specific methanogenic activity test

The activity on different substrates involved in propionate degradation (propionate, acetate, H_2 and formate) and possible metal limitation of the sludge that developed in the reactor were investigated by specific methanogenic activity (SMA) tests. The SMA of the sludge was determined in duplicate at 30 (±2) °C using on-line gas production measurements as described by Zandvoort et al. (2002). Approximately 1 g (wet weight) of granular sludge was transferred to 120 mL serum bottles containing 50 mL of basal medium with the same composition as the reactor basal medium, supplemented with propionate (2 g COD·L^{-1}), acetate (2 g COD·L^{-1}), H_2 (1 g COD·L^{-1}) or formate (2 g COD·L^{-1}) as the substrate. Data were plotted in a rate versus time curve, using moving average trend lines with an interval of 10 data points. The SMA with each of the substrates was determined on days 0, 92, 167, 182, 235 and 249. The samples were always taken from the same place (the mid-height) in the sludge bed.

6.2.5. Metal composition of the sludge

The metal content of the sludge was measured on days 0, 92, 126, 170, 180, 190 and 240, and determined by ICP-OES (Varian, Australia) after destruction with Aqua regia (mixture of 2.5 mL 65% HNO_3 and 7.5 mL 37% HCl) as described in Chapter 4.

6.2.6. RNA Extraction and reverse transcription

In order to perform reverse transcription followed by Q-PCR, RNA was extracted from UASB reactor samples taken at day 40, 81, 123, 180, 231, 246 and 276. Granules were separated from the reactor medium, incubated overnight at 4 °C in RNA stabilization solution "RNA*later*" from Ambion, and stored at –20 °C. To isolate RNA, 50 mg (wet weight) granules were resuspended in 125 µL RNase free water and 375 µL TRIzol Reagent (Invitrogen). This was transferred to a 0.1 mm silica beads containing tube and homogenized 4.5 m·s^{-1} for 30 s in a FastPrep-24 (MP Biomedicals). Tubes were incubated at 20 °C for 5 min and 0.1 mL chloroform was added followed by 15 s manual shaking. After incubation at 20 °C for 5 min, the suspension was centrifuged 9500 × g at 4°C for 15 min. The aqueous phase with an equal amount of 70% ethanol was transferred to an RNeasy mini spin column (Qiagen) and on-column DNaseI digestion with RNase-free DNase I (Qiagen) was performed according to manufacturers instructions. RNA was eluted in 60 µL RNase free water and integrity was confirmed by an Experion RNA StdSens Chip (Bio-Rad) and the Bio-Rad Experion system. Copy DNA was synthesized by SuperScript III reverse transcriptase (Invitrogen) according to manufacturers instructions and random decamers (Ambion).

6.2.7. Q-PCR method development

A Q-PCR method was developed to follow expression levels of four FDH coding genes in *Syntrophobacter* spp. (FDH-1, FDH-2, FDH-3 and FDH-4). The genomes of only two propionate oxidizers have been sequenced: *Syntrophobacter fumaroxidans* and *Pelotomaculum thermopropionicum* strain SI (Doe-Joint-Genome-Institute, 2007). Q-PCR primers specific for FDH and 16S RNA genes (Table 6.2) were designed based on the sequenced genome of *Syntrophobacter fumaroxidans* (GenBank CP000478) (Doe-Joint-Genome-Institute, 2007), and synthesized by BioLegio or Eurogentec. The genome of *P. thermopropionicum* contains genes homologous to FDH-1 and FDH-2 of *S. fumaroxidans*. However, nucleotide sequences differed in such a way that amplification of *P. thermopropionicum* genes with *Syntrophobacter fumaroxidans* FDH primers is highly unlikely.

Q-PCR was performed with the Bio-Rad MyiQ Single color Real-Time PCR detection system. Each 25 μL reaction mixture contained 5 μL 20x diluted cDNA, 12.5 μL of 2x QuantiTect SYBR Green PCR mix (Qiagen), 0.3 μM of each primer. Thermocycling conditions were as follows: 95°C for 10 min, 40 cycles of 95 °C for 15 s, 58 °C for 30 s and 72 °C for 30 s with fluorescence detection at the end of each extension step. The optimal baseline was calculated by Bio-Rad MyiQ system software version 1.0 and Threshold cycle (C_t) values were generated. A second arbitrary baseline was manually defined and the amplification efficiency for each individual reaction was calculated from the kinetic curve (Liu and Saint, 2002). Gene expression values were normalized to 16S rRNA of *Syntrophobacter fumaroxidans*. The 16S rRNA gene – total RNA ratio's remains constant in *Syntrophobacter fumaroxidans* pure cultures (data not shown) indicating that 16S rRNA is suitable as reference gene for *Syntrophobacter fumaroxidans*.

6.2.8. DNA extraction, amplification and DGGE

Total genomic DNA was extracted from 50 mg granules (wet weight) from sample day 40, 81, 123, 180, 231, 246 and 276 with the bead-beat and phenol-chloroform based DNA extraction method (van Doesburg et al., 2005). For each sample day, three replicate RNA extractions were performed. The 16S rRNA genes were amplified by PCR using *Taq* DNA polymerase and thermocycling conditions as described by Sousa et al. (2007). Bacterial and archaeal 16S rRNA genes were selectively amplified for cloning using the forward primers Bact-27-for and 4F respectively and the universal primer 1492-rev. For DGGE analysis, PCR was performed as described by Sousa et al. (2007). Amplicons were separated by DGGE as described by Zoetendal et al. (2001).

Table 6.2. gene specific Q-PCR primers used in this study.

Primer	Sequence (5'-3')	Gene specific code	Reference
FDH-1-fw	CGG CGT CCC GTG AGT T	Sfum_2706 and Sfum_2705	Doe Joint Genome Institute, 2007
FDH-1-rv	GGC AGG GTG CTC TAC CAG TAT	Sfum_2706 and Sfum_2705	Doe Joint Genome Institute, 2007
FDH-2-fw	TGG TGC CAG CAT TCG GTG	Sfum_1274 and Sfum_1273	Doe Joint Genome Institute, 2007
FDH-2-rv	ATG TTC CCC AGG AGC AGC	Sfum_1274 and Sfum_1273	Doe Joint Genome Institute, 2007
FDH-3-fw	GGA CAT CTC GCG TTT GGA C	Sfum_3509	Doe Joint Genome Institute, 2007
FDH-3-rv	CTC GCC TTG ACT TTC ACC TCT	Sfum_3509	Doe Joint Genome Institute, 2007
FDH-4-fw	CTA CCA TAC GCG GAC CCA G	Sfum_0031 and Sfum_0030	Doe Joint Genome Institute, 2007
FDH-4-rv	CGA TTT CAC CCG GAC TTT G	Sfum_0031 and Sfum_0030	Doe Joint Genome Institute, 2007
16SrRNA-fw	ACG CTG TAA ACG ATG AGC ACT A	Sfum_R0013 and Sfum_R0015	Doe Joint Genome Institute, 2007
16SrRNA-rv	GAT GTC AAG CCC AGG TAA GGT	Sfum_R0013 and Sfum_R0015	Doe Joint Genome Institute, 2007
Primer	Sequence (5'-3')	Specificity	Reference
Bact-27-f	GTT TGA TCC TGG CTC AG	Bacterial 16S	Lane, 1991
Uni1492-r	CGG CTA CCT TGT TAC GAC	Universal 16S	Großkopf et al., 1998
4F	TCCGGTTGATCCTGCCRG	Archaea	Kendall et al., 2007
T7	TAA TAC GAC TCA CTA TAG GG	pGEM-T	Promega, 2007
SP6	GAT TTA GGT GAC ACT ATA G	pGEM-T	Promega, 2007
GC clamp	CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G		Muyzer et al., 1993
U968-f	AAC GCG AAG AAC CTT AC	Bacterial 16S	Nübel et al., 1996
L1401-r	CGG TGT GTA CAA GAC CC	Bacterial 16S	Nübel et al., 1996
Arch109-f	ACK GCT CAG TAA CAC GT	Archaeal 16S	Großkopf et al., 1998
515-r	ATC GTA TTA CCG CGG CTG CTG GCA C	Universal 16S	Lane, 1991

6.2.9. Cloning and sequencing

PCR amplicons were purified with the Qiaquick PCR purification kit according to manufacturer's instructions and cloned into *E. coli* JM109 by using the Promega pGEM-T easy vector system (Promega, Madison, WI). After blue white screening, positive colonies were transferred to 200 µl ampicilin containing LB medium and grown overnight at 37 ^{o}C. To lyse the cells, 10 µl overnight culture plus 90 µl Tris-EDTA buffer was incubated at 95 ^{o}C for 10 min. Archaeal and bacterial DGGE PCR and DGGE were performed as described above and band profiles were analyzed.

Clones with unique band profiles were selected and amplification products of the complete inserts were sequenced by BaseClear (Leiden, The Netherlands) and used to test the specificity of Q-PCR primers designed for the *Syntrophobacter* spp. reference gene ("16SrRNA-fw" and "16SrRNA-rv"). Therefore, Q-PCR was performed as described above by using 21 different PCR amplicons of complete bacterial clone inserts with equal DNA concentration as template.

6.2.10. Chemical analyses

VFA (Volatile Fatty Acids) were determined using gas liquid chromatography as described by Weijma et al. (2000). The total suspended solids (TSS) and volatile suspended solids (VSS) concentrations were determined according to Standard Methods (APHA/AWWA, 1998). All chemicals were of analytical or biological grade and purchased from E. Merck AG (Darmstadt, Germany), unless otherwise stated.

6.3. RESULTS

6.3.1. Reactor operation

The overall performance of the UASB reactor during the whole operation was reasonable, not optimal. Almost no propionate removal was observed during the first 25 days of operation (Fig. 6.1A). Upon day 25 of operation propionate degradation rates started to increase. Although propionate removal was not complete (Fig. 6.1A), the OLR of the reactor was increased to 12 g COD·L^{-1}·day^{-1} (6 g COD-VFA·L^{-1} in the influent) on day 44 of operation. Propionate was degraded up to 70 % during the following 23 days of operation. The VFA effluent concentration started to increase after day 67, reaching a maximum value of 4.7 g COD-VFA·L^{-1} on day 81, corresponding to 60 % of the influent propionate concentration on that day (Fig. 6.1A). From day 81, the VFA effluent concentration decreased to 0 on day 116. From day 116 to day 140 no VFA accumulated in the effluent. From day 140 to day 225, the VFA effluent concentrations were highly variable but they did not exceed 3.5 g COD-VFA·L^{-1}. The average VFA effluent concentration during this period was 1.5 g COD-VFA·L^{-1}. From day 225 VFA started to accumulate, reaching a value of 7.4 g COD-VFA·L^{-1} on day 249. On day 249, 5 µM of

molybdenum, tungsten and selenium was supplied to the influent. The propionate effluent concentration decreased up to 1.4 g COD-VFA·L^{-1} on day 258. This could indicate a beneficial effect on the propionate degradation due to the metal addition, however, a further propionate effluent accumulation was observed reaching a value of 4 g COD-VFA·L^{-1} on day 271.

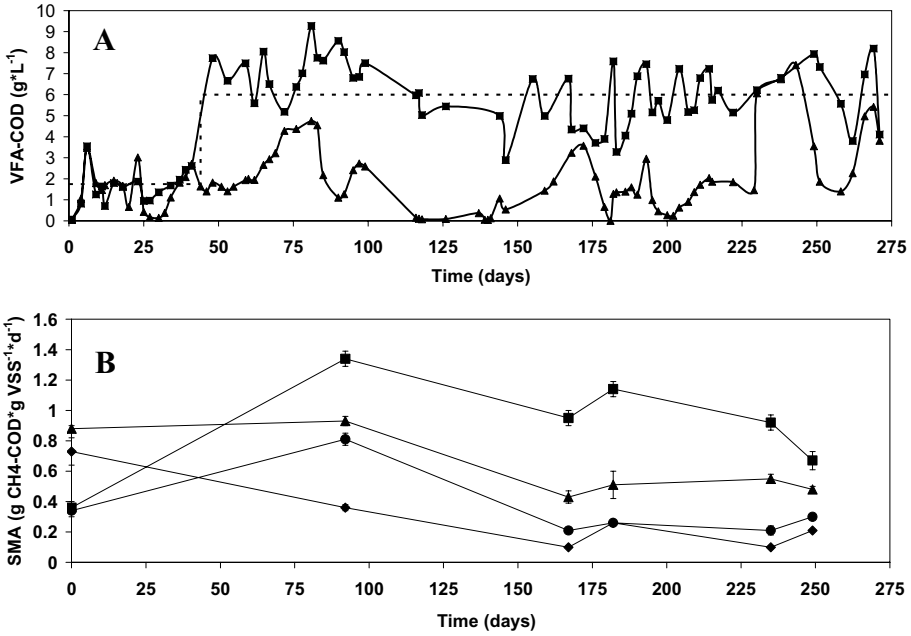

Figure 6.1. (A) Evolution of reactor performance with time. Influent COD (■), effluent COD (▲). (B) Evolution of SMA with time and with propionate (♦), formate (■), H_2 (●) and acetate (▲) as substrates.

6.3.2. Evolution of SMA over time

The SMA assays did not reveal that molybdenum, tungsten and selenium limitation were induced during the complete reactor run with any of the substrates used (Table 6.3). From day 0 to day 92 of operation, the SMA with acetate as the substrate was constant, the SMA with H_2 and formate as the substrate increased twice and three times respectively. However, the SMA with propionate as the substrate decreased around 50 % between day 0 (0.74 g CH_4-COD·g VSS^{-1}·d^{-1}) and day 92 (0.35 g CH_4-COD·g VSS^{-1}·d^{-1}). These trends suggest that propionate oxidisers decreased their activity, but methanogens did not.

Table 6.3. Evolution of the specific methanogenic activity (g CH_4-COD·g VSS^{-1}·d^{-1}) as a function of time, organic source and trace metal content of the medium.

Specific maximum methanogenic activity [g CH_4-COD·g VSS^{-1}·d^{-1}]		Operation days 0	92	167	182	235	249
Propionate	No trace elements	0.74	0.37	0.10	0.28	0.09	n.d.
	Complete trace element solution except oxyanions	0.60	0.38	0.11	0.27	0.10	0.21
	Complete trace element solution	0.82	0.35	0.10	0.25	n.d.	n.d.
	Oxyanions solely	0.75	0.33	0.11	0.25	n.d.	n.d.
Formate	No metals	0.33	1.39	0.89	1.18	0.96	n.d.
	Complete trace element solution except oxyanions	0.38	1.27	0.99	1.15	0.91	0.67
	Complete trace element solution	0.31	1.36	n.d.	1.08	0.85	n.d.
	Oxyanions solely	0.39	1.34	0.97	n.d.	0.97	n.d.
Hydrogen/CO_2	No metals	0.39	n.d.	0.22	0.26	0.19	n.d.
	Complete trace element solution except oxyanions	0.34	0.86	0.23	n.d.	0.21	0.30
	Complete trace element solution	0.35	0.79	0.20	n.d.	0.25	n.d.
	Oxyanions solely	0.29	0.77	0.20	0.25	0.21	n.d.
Acetate	No metals	0.89	0.85	0.46	0.63	0.59	n.d.
	Complete trace element solution except oxyanions	0.97	n.d.	0.42	0.53	0.51	0.48
	Complete trace element solution	0.93	0.70	0.38	0.45	0.53	n.d.
	Oxyanions solely	0.91	1.10	0.46	0.44	0.55	n.d.

n.d.: not determined

From day 92 to day 167, the SMA with propionate as the substrate further decreased till 0.10 g CH_4-COD·g VSS^{-1}·d^{-1} (Table 6.3). The SMA with H_2, acetate or formate as the substrate also decreased. From day 167 to day 235, the SMA with H_2, propionate or formate followed a slightly decreasing trend, the SMA with acetate as the substrate followed a slightly increasing trend (Fig. 6.1B). From day 235 to day 249, the SMA with propionate as the substrate increased from 0.10 g CH_4-COD·g VSS^{-1}·d^{-1} to 0.21 g CH_4-COD·g VSS^{-1}·d^{-1}, as well as the SMA with H_2 the substrate increased from 0.20 g CH_4-COD·g VSS^{-1}·d^{-1} to 0.30 g CH_4-COD·g VSS^{-1}·d^{-1}. The SMA with formate as the substrate decreased around 40 %. This suggests that the SMA increase with propionate as the substrate was mainly due to an increase in the activity of H_2 using methanogens.

6.3.3. Metal composition of the sludge

The method quantification limit (MQL) in the anaerobic granular sludge for tungsten, selenium and molybdenum was 20 µg ·g TSS^{-1},10 µg ·g TSS^{-1} and 5 µg ·g TSS^{-1}, respectively.

Tungsten and selenium concentrations were below these values in all the sludge samples investigated (Data not shown). No wash out of molybdenum was observed (Data not shown). Molybdenum concentrations were constant through all the sludge samples tested (20 ± 2 µg ·g TSS^{-1}).

6.3.4. FDH gene expression

The relative gene expression of the tungsten and selenium containing FDH-1 decreased over 15 times from day 81 to 231 and relative gene expression of the W- and Se-containing FDH-2 decreased almost 10 times from day 40 to 231 (Fig. 6.2A, B). Relative gene expression of the W- or Mo-containing FDH-3 could not be determined because expression values were below the detection limit. Relative gene expression of the W-or Mo- and Se-containing FDH-4 was more or less constant during 250 days (Fig. 6.2C).

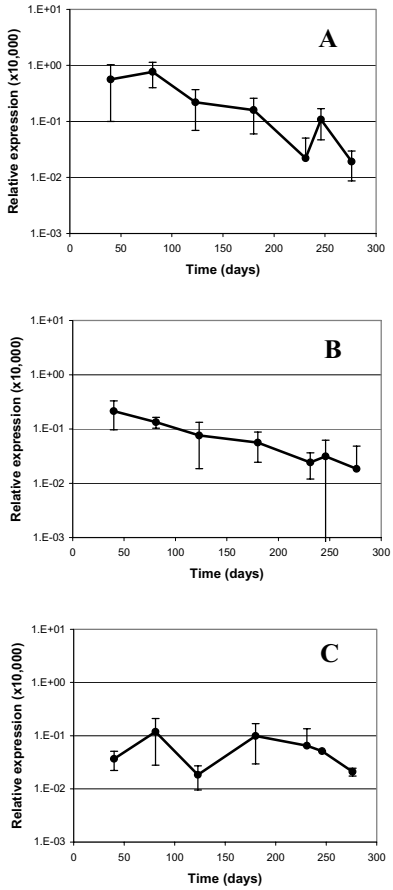

Figure 6.2. Evolution of expression levels of FDH coding genes in *Syntrophobacter* spp. over time. (A) FDH-1. (B) FDH-2. (C) FDH-4.

6.3.5. Microbial community analysis

DNA extraction and subsequent DGGE analysis of 16S rRNA genes were successful for all samples. Visual comparison of the DGGE profiles revealed that the archaeal and bacterial community composition hardly changed (Fig. 6.3A, B). Clone sequencing revealed the presence of *Syntrophobacter sulfatireducens* (92-99 % similarity), *Syntrophobacter fumaroxidans* (94 %), *Smithella propionica* (99 %), *Pelotomaculum* strain MGP (94 %) and different types of uncultured bacterial clones. Methanogens identified were *Methanosaeata concilli* (94 - 99 %) and *Methanospirillum hungatei* (93 – 99 %) (Fig. 6.3; Table 6.4).

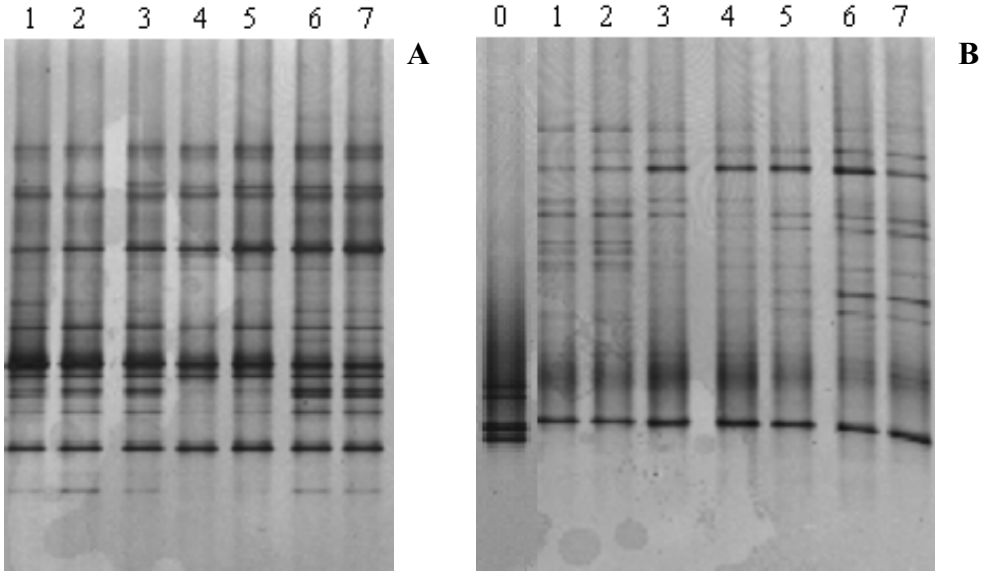

Figure 6.3. Evolution of the (A) archaeal and (B) bacterial community over time. (0) pure culture of *Syntrophobacter fumaroxidans*, (1) day 40, (2) day 81, (3) day 123, (4) day 180, (5) day 231, (6) day 246 and (7) day 276.

6.3.6. 16S rRNA gene primer specificity

Q-PCR with primers "16SrRNAfw" and "16SrRNArv" especially amplified 16S rRNA genes from *Syntrophobacter* spp. The four clones affiliated with *Syntrophobacter* spp. (Table 6.4) were all amplified in high quantities. Six other clones affiliated with uncultured bacteria were amplified as well, however to a 100-1000 fold less extent than *Syntrophobacter* spp. These six clones are not suspected to be present in the sludge with 100-1000 fold higher amounts than *Syntrophobacter* spp. One out of three clones affiliated with *Pelotomaculum* sp. MGP was amplified with the *Syntrophobacter* specific primers as well, however over 1000 fold less than *Syntrophobacter* spp. affiliating clones. The other two clones affiliated with *Pelotomaculum* sp MGP, the clone affiliated with *Smithella propionica* and seven clones affiliated with uncultured

bacteria/clones were not amplified with these primers at all. Therefore, transcription levels of 16S rRNA gene in *Syntrophobacter* spp. are most likely not biased by bacteria not belonging to the *Syntrophobacter* spp.

Table 6.4. Affiliation of the retrieved 21 bacterial and 12 archaeal 16S DNA clones.

Number of clones	Closest relative	Similarity (%)
3	*Syntrophobacter sulfatireducens strain* TB8106	92-99
3	*Pelotomaculum* sp. MGP	94
3	Uncultured bacterial clone affiliating with *Clostridiales*	93-95
3	Uncultured bacterial clone affiliating with unclassified *Bacteroidetes*	91-95
2	*Smithella propionica*	99
2	Uncultured bacterial clone affiliating with *Chlorobiales*	99
1	*Syntrophobacter fumaroxidans* MPOB	94
1	Uncultured bacterial clone affiliating with *Rubrobacterales*	93
1	Uncultured bacterial clone affiliating with *Proteobacteria*	98
1	Uncultured bacterial clone affiliating with *Spirochaetales*	98
1	Uncultured bacterial clone affiliating with *Thermotogae*	98
6	*Methanosaeta concilli*	94 - 99
6	*Methanospirillum hungatei JF-1*	93 - 99

6.4. DISCUSSION

Recognition of metal limitation on propionate degradation was achieved in the present study, even if the SMA was not limited by the absence of oxyanions in the test medium on any of the days tested. Indirect methods were used for the recognition of metal limitation. Transcription levels of genes, important to keep a stable anaerobic reactor operation, are possibly regulated by the metals present in the reactor in a similar way as was described for *Desulfovibrio vulgaris.* Selenium and nickel regulate [FeNiSe]-, [FeFe]- and [FeNi]-hydrogenase gene transcription in *D. vulgaris* Hildenborough (Valente et al., 2006). Transcription level determination in bioreactors was previously performed by Ciesielski et al (2007), who determined amoA and 16SrRNA gene transcription of ammonia and nitrite oxidizing bacteria in a nitrifying sequencing batch reactor with reverse transcription PCR. Quantification of transcription levels can be studied with Q-PCR. which is already often used in medical diagnostics (Bergqvist et al., 2007) as well as in other research fields, such as plant pathogens diagnosis (Okubara et al., 2005) or water quality measurement (Noble and Weisberg, 2005; Khan et al., 2007). Q-PCR can thus be applied to follow gene expression levels in bioreactors.

Community analysis of the metal-deprived sludge revealed a well adapted propionate degrading population. *Syntrophobacter sulfatireducens*, *S. fumaroxidans*, *Smithella propionica* and *Pelotomaculum* sp. (Table 6.4) are propionate metabolising bacteria (Liu, 1999; Chen et al., 2005; Imachi et al., 2007). *Methanospirillum hungatei* and *Methanosaeta* were detected as the most important methanogens. The whole population is thus in principle capable of converting propionate ultimately to methane and CO_2. Both methanogens keeps the hydrogen, formate and acetate concentration low, to allow propionate oxidation. The propionate oxidizers *Syntrophobacter* spp. and the methanogen *Methanospirillum hungatei* are common micro-organisms present in UASB reactors (Harmsen et al., 1998; Roest et al., 2005; Fernandez et al., 2007). Lueders et al. (2004) showed that the *Syntrophobacter* spp., *Smithella* spp. and *Pelotomaculum* spp., and archaeal species *Methanobacterium* and *Methanosarcina* spp were responsible for the syntrophic propionate oxidation in anoxic paddy soil.

In defined cocultures of *Syntrophobacter fumaroxidans* and *Methanospirillum hungatei*, grown on propionate, molybdenum and tungsten limitation lowered the methane production rate and the FDH activity in cell extracts of each organism (Jiang, 2006). Based on the results of Jiang (2006), FDH was the selected enzyme to study in methanogenic propionate degradation under metal limitation. In present study, FDH-1 and FDH-2 gene transcription in *Syntrophobacter* spp., determined by Q-PCR, was found to decrease (Fig. 6.2A, B) while no molybdenum, tungsten and selenium were present in the influent. The presence of molybdenum in the sludge (20 ± 2 μg $\cdot$g TSS^{-1}) suggests that the presence of metal in the granular matrix system is not necessarily correlated to its presence in the catalytic centers of the intracellular enzymes. In a similar way, Zandvoort et al. (2006) showed that total metal content in a methanol fed granular sludge did not correlate with the metal limitation in methanol biodegradation.

The decrease of propionate oxidizing activity from day 0 to day 92 suggests that metal limitation affected the activity of propionate oxidizers. The slight increase in activity of the acetate reducers observed from day 167 to day 182 suggests an increasing activity of *Smithella propionica* which is known to produce more acetate in coculture with *Methanospirillum hungatei* compared to *Syntrophobacter* (Liu, 1999). The further decrease of FDH-1 and FDH-2 gene transcription coupled to the increased H_2 usage and decreased formate usage by methanogens at the end of the reactor run (Fig. 6.2A, B) suggested that propionate degradation proceeded mainly via interspecies H_2 transfer instead of formate transfer. FDH-4 gene transcription shows low levels during the reactor run compared to FDH-1 and FDH-2 (Fig. 6.2), therefore, the effect of metal limitation on FDH-4 was negligible. FDH-4 is not expected to be a good marker for metal limitation (Fig. 6.2C). FDH-3 gene transcription was below the detection limit and certainly is not capable of marking metal limitation.

In the anaerobic treatment of complex wastewater, propionate accumulation leads to unstable UASB reactor operation, which will cause further operational problems, e.g a pH drop

or wash out of biomass from the reactor (Defour et al., 1994). The transcription of FDH-1 and FDH-2 are promising indicators for molybdenum, tungsten and selenium limitation in propionate fed UASB reactors that can cause propionate accumulation. According to our results, Q-PCR is a suitable technique to follow gene expression levels and therefore a very promising technique for recognition of trace metal limitation in wastewater treatments.

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Effect of tungsten, molybdenum and selenium limitation on propionate degradation in anaerobic methanogenic granular sludge

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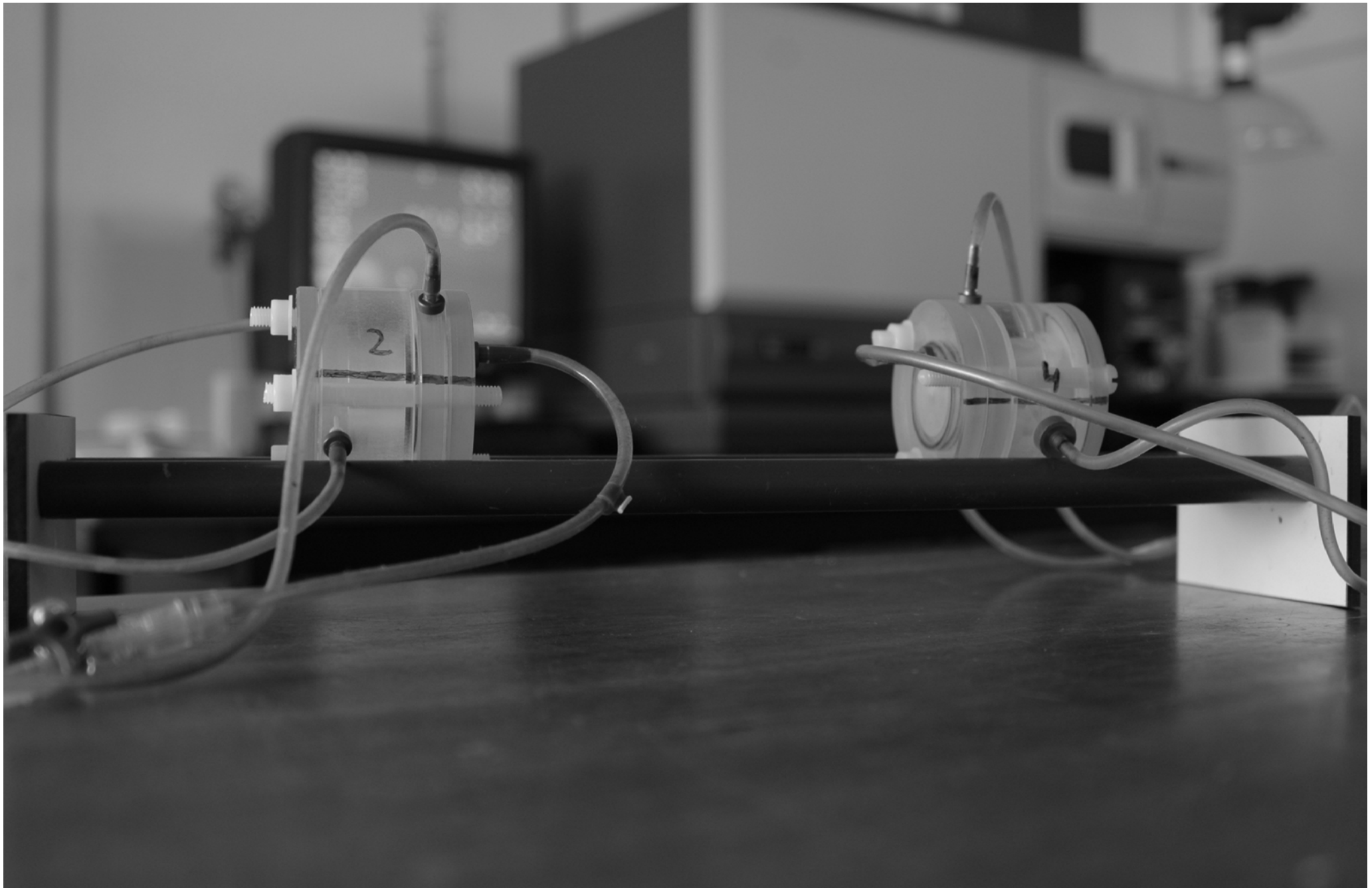
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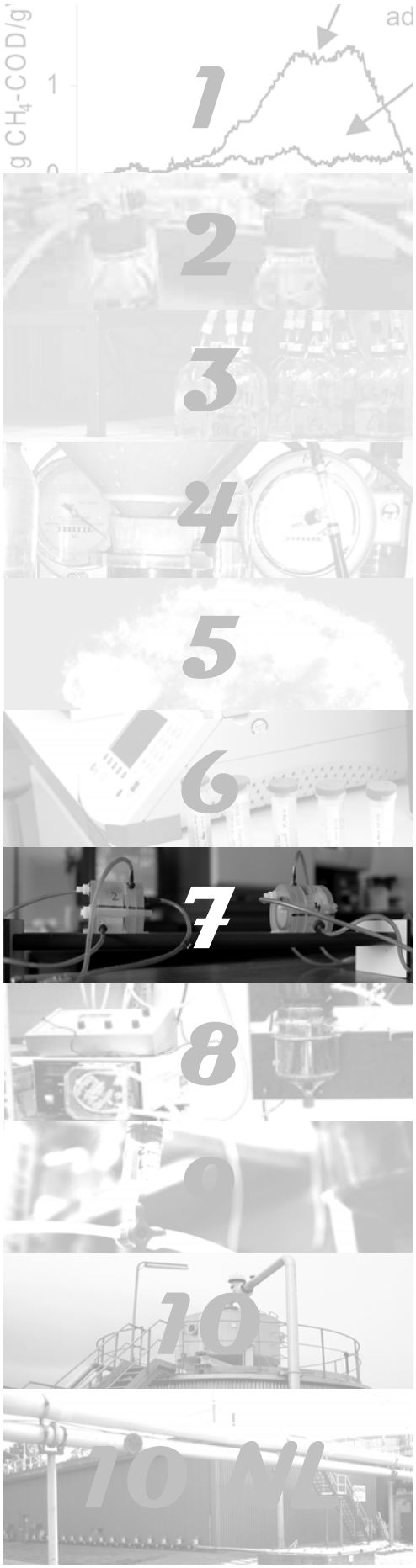
COBALT TOXICITY IN ANAEROBIC GRANULAR SLUDGE: INFLUENCE OF CHEMICAL SPECIATION COBALT SPECIATION



ABSTRACT

The influence of cobalt speciation on the toxicity of cobalt to methylotrophic methanogenesis in anaerobic granular sludge was investigated. The cobalt speciation was studied with three different media, that contained varying concentrations of complexing ligands (carbonates, phosphates and EDTA). Three fractions (total, dissolved and free) of cobalt were determined in the liquid media and were correlated with data from batch toxicity experiments. The average IC50 (for free Co2+) in the three sets of measurements was 13 µmol/L with a standard deviation of only 22% and a similarity of 71% between the data obtained in the three different media for the range of cobalt concentrations investigated. The standard deviation of the IC50 for the other two fractions was much higher, i.e. 85 and 144% for the total cobalt added and dissolved cobalt, respectively, and the similarity was almost 0% for both fractions. Complexation (and precipitation) with EDTA, phosphates and carbonates was shown to decrease the toxicity of cobalt on methylotrophic methanogenesis. The free cobalt concentration is proposed to be the key parameter to correlate with cobalt toxicity. Thus, the toxicity of cobalt to granular sludge can be estimated based on the equilibrium free cobalt concentration.

7.1. INTRODUCTION

Trace metals play a key role in the anaerobic conversion of methanol to methane in Upflow Anaerobic Sludge Bed (UASB) reactors (Lettinga et al., 1979). Cobalt, nickel, iron, copper, zinc and manganese are considered as the most important trace metals for methanogens (Florencio et al., 1993; Singh et al., 1999; Paulo et al., 2004; Chapter 3, Chapter 4, Chapter 5). They are dosed in excess in practice in industrial wastewater treatment plants to overcome problems associated with the lack of micro-nutrients. Although this method is effective, it is a waste of natural resources and it results in an unnecessary release of heavy metals in the environment, which leads to an increase of the operation costs (Burgess et al., 1999). Moreover, dosing trace metals at a too high concentration can cause inhibition of the methanogenic process (Ram et al., 2000). Although heavy metal toxicity in UASB reactors has been studied abundantly, reported values of toxic concentrations vary considerably between different authors (e.g. (Fang, 1997; Lin and Chen, 1997; Karri et al., 2006)). This is due to the fact that most authors report total cobalt concentration in the liquid phase, not considering cobalt speciation. Therefore, the observed toxic concentrations are affected by differences in medium composition, which causes changes in metal speciation (Worms et al., 2006).

The free metal ion has been most often used as the base of models describing metal uptake by microorganisms or higher organisms (Hassler Christel, 2003), such as the Free Ion Activity Model (FIAM) and the Biotic Ligand Model (BLM) (Hassler et al., 2004). Although the free metal concentration strongly depends on precipitation (Jansen et al., 2007), sorption (van Hullebusch et al., 2005a) and speciation (Weng et al., 2001), the free metal ion is the supposed most important chemical species for bacterial uptake (Hassler et al., 2004). The presence of organic complexing ligands such as citrate (that decrease the free metal concentration) clearly influences the cobalt and nickel toxicity to bacteria, as e.g. *Pseudomonas aeruginosa* (Chen et al., 2006) or *Bacillus subtilis* (Krom, 2002). These toxicity models were, however, established and verified for natural waters and higher organisms such as freshwater algae *Chlorella kesslerii* (Hassler et al., 2004), urchin larvae (Lorenzo et al., 2006) or mussels (*Mytilus galloprovincialis*) (Beiras et al., 2003). Thus far, this type of models has never been applied to the anaerobic consortia present in biofilms or granules from wastewater treatment reactors.

The present study focus on cobalt speciation and its influence on the specific methanogenic activity of granular sludge. Cobalt was chosen as a model for essential trace metals for methanogenesis. The borders between its nutritional requirement (Bhattacharya et al., 1996; Zandvoort et al., 2002; Chapter 3) and toxicity is very narrow; although so far no toxic concentrations have been reported for anaerobic granular sludge. Therefore, this study aimed to determine cobalt toxicity data for methanol fed anaerobic granular sludge based on changes in specific methanogenic activity (SMA) upon exposure to various cobalt concentrations. The

influence of cobalt speciation was evaluated as well by adding various concentrations of complexing ligands (EDTA, phosphate and carbonate) to the medium.

7.2. MATERIAL AND METHODS

7.2.1. Medium composition

To evaluate the role of the free cobalt (Co^{2+}) ion in toxicity, three sets of measurements of the specific maximum methanogenic activity (SMA) with different basal medium composition were performed (Table 7.1). Table 7.2 lists the chemical composition of the medium, prepared with ultra-pure water (MILLIPORE, resistivity of 18 MΩ·cm) as the dilution water. Although EDTA is usually not present in the UASB reactor influent, this ligand was chosen as an example of an extremely strong, but poorly degradable ligand (Oviedo and Rodriguez, 2003). The other compounds (chloride, phosphate and carbonate) were chosen as weak ligands naturally present in the UASB reactors. Cobalt chloride was used as the cobalt source in set I and III. Cobalt-EDTA (prepared from $CoCl_2$ and Na_2H_2EDTA; molar ratio 1:1.5) was used in set II. Carbonates and phosphates were added to the medium in set I and II. The pH of the medium was set to 7.0 with sodium bicarbonate (sets I and II) or with MOPS (3-(N-Morpholino)-propanesulfonic acid) (set III). The end pH values in activity tests in set III (around 6.5) were slightly lower than in the other sets (around 7.0) which was caused by differences in the buffering capacity of the media. It should be noted that the free cobalt concentration is affected insignificantly (± 5% – confirmed by Visual MINTEQ) in this range of pH changes.

Table 7.1. Sets of experiments used in the work.

	Phosphate addition (mM)	Carbonate addition (mM)	EDTA addition (mM)
Set I	1.44	30	
Set II	1.44	30	1.5 times Co addition
Set III	0.14	0	

7.2.2. Source of biomass

Mesophilic methanogenic granular sludge was obtained from a full-scale upflow anaerobic sludge bed (UASB) reactor treating alcohol distillery wastewater at Nedalco (Bergen op Zoom, The Netherlands). The total suspended solids (TSS) and volatile suspended solids (VSS) concentration of the wet sludge were 6.93 (± 0.02)% and 6.47 (± 0.03)%, respectively. Set III was performed 9 months before sets I and II. The sludge was stored without substrate at 4 ºC during the time between the experiments. This led to differences in SMA of the inoculum in the absence of cobalt.

Table 7.2. Composition of the basal medium used in the three measurement series.

	Set I and II		**Set III**	
Macronutriens	**mg/L**	**mmol/L**	**mg/L**	**mmol/L**
NH_4Cl	280	5.24	280	5.24
K_2HPO_4	250	1.44	25	0.144
$MgSO_4 \cdot 7H_2O$	100	0.406	100	0.406
$CaCl_2 \cdot 2H_2O$	10	0.068	14.7	0.100
$NaHCO_3$	2520	30	---	---
MOPS (pH buffer)	---	---	2091	10
Micronutriens	**mg·L^{-1}**	**µmol·L^{-1}**	**mg·L^{-1}**	**µmol·L^{-1}**
$CuCl_2\ H_2O$	0.862	5	0.862	5
$ZnCl_2$	0.681	5	0.681	5
$MnCl_2\ 4H_2O$	0.988	5	0.988	5
$NiCl_2\ 6H_2O$	1.173	5	1.173	5
$(NH_4)_6Mo_7O_{24}\ 4H_2O$	0.888	5	0.888	5
$Na_2SeO_3\ 5H_2O$	1.321	5	1.321	5
$Na_2WO_4\ 2H_2O$	1.649	5	1.649	5
$FeCl_2\ 4H_2O$	9.937	50	9.937	50

---: not supplied

7.2.3. Specific maximum methanogenic activity tests

The SMA with methanol as the substrate was determined in duplicate at 30 (± 2) ºC using on-line gas production measurements as described by Zandvoort et al. (2002). Granular sludge specimens (approximately 1 g wet weight) were transferred to 120 ml serum bottles containing 50 ml basal medium with methanol (4 g COD/L), along with different cobalt concentrations. The SMA data were plotted in a rate versus time curve, using moving average trend lines with an interval of 15 data points.

7.2.4. SMA inhibition

Inhibition of specific methanogenic activity (Eq. 7.1) is defined as the decrease in SMA under certain conditions (SMA_i) in comparison to the SMA under optimal conditions (SMA_{max}):

$$I = 1 - \frac{SMA_i}{SMA_{\max}} \tag{7.1}$$

In order to obtain the main characteristics of the inhibition curves (SMA_{max}, IC_{50}), the Hormesis model was used to fit the experimental data (Eq. 7.2), Hormesis is the term for generally-favorable biological responses to low exposures to stressors. A stressors showing

hormesis thus has the opposite effect in small doses than in large doses.. The parameters of the curve were calculated in accordance with Schabenberger et al. (1999):

$$I = \delta + \frac{\alpha - \delta + \gamma x}{1 + \theta \exp[\beta \ln(x)]} \tag{7.2}$$

where β, γ and θ are parameters and x is the metal concentration, α was considered as a constant that gives the SMA value when x goes to zero. δ was considered as a constant that gives the SMA value at x going to infinity (∞).γ is responsible for the Hormesis effect. β and θ are responsible for the slope of increase and decrease of the curve. The parameters were calculated using nonlinear regression in MS Excel.

7.2.5. Statistics

The obtained toxicity data were related to the cobalt concentration. For each set, a single toxicity curve for each cobalt fraction (added, dissolved, free) was plotted. To decide whether the three curves obtained for a certain fraction are similar among all three sets of experiments, the F test was used. The method was based on comparison of a full model (three curves for three sets) with a reduced model (one common curve for all three sets). The null hypothesis (H_0, Eq. 7.3) that all the data points obtained for a certain cobalt fraction in all the three experimental sets can be fitted with one common curve was compared to the alternate hypothesis (H_1, Eq. 7.4)) that at least one curve deviates:

H_0: $\beta_I = \beta_{II} = \beta_{III} = \beta_r$ and $\gamma_I = \gamma_{II} = \gamma_{III} = \gamma_r$ and $\theta_I = \theta_{II} = \theta_{III} = \theta_r$ (7.3)

H_1: H_0 is not valid (7.4)

Indexes I, II and III specify the sets for which the parameters β, γ and θ were determined. Index "r " means the reduced model. Finally, the null hypothesis was accepted or rejected based on the P-value obtained from the F test.

7.2.6. Dissolved cobalt concentration

The dissolved cobalt concentration was measured in filtrated (pore size of 0.45 μm) samples taken from the system (granular sludge – liquid medium) after four days of incubation (no substrate added, 30 (± 2) ºC, anaerobic conditions, shaking at 75 rpm). This incubation time is required to reach chemical equilibrium (Osuna et al., 2004).

7.2.7. Free cobalt ion (Co^{2+}) concentration

The free cobalt (Co^{2+}) concentrations related to particular cobalt concentrations in the given system (measurement sets I, II, and III) were measured under the same conditions as in the SMA test, but without substrate (Fig. 7.1). The free cobalt concentration was determined directly in the granular sludge/medium mixture under anaerobic conditions at 30 (± 2) °C using

the Donnan Membrane Technique (DMT), as described by (Temminghoff et al., 2000) (Fig. 7.2).

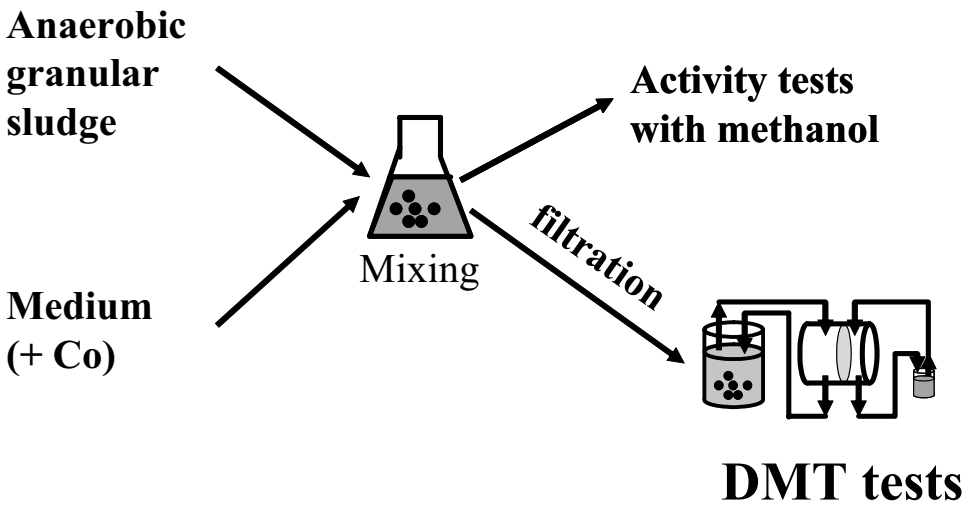


Figure 7.1. Experimental design used in this study to link the SMA measurement to solid phase and liquid phase speciation.

Prior to the DMT measurements, the granular sludge/medium mixture was shaken (75 rpm) for 4 days to reach equilibrium. The donor solution had the same composition as the medium used for activity tests (Table 7.2). As the DMT technique requires equilibrium conditions, no substrate was added to the medium to avoid methanogenic activity during the DMT test. The acceptor solution (before the start of experiments) contained 1 mmol/L $CaCl_2$ and 10 mmol/L MOPS as pH buffer.

The volumes of donor and acceptor solutions were 1 L and 17 ml, respectively. The sampling volume of the acceptor solution (1 ml) was always replaced with a fresh solution. The flow of both solutions was approximately 10 ml/min. Each DMT experiment was finished after at least 5 days (after reaching a plateau in the acceptor cobalt concentration).

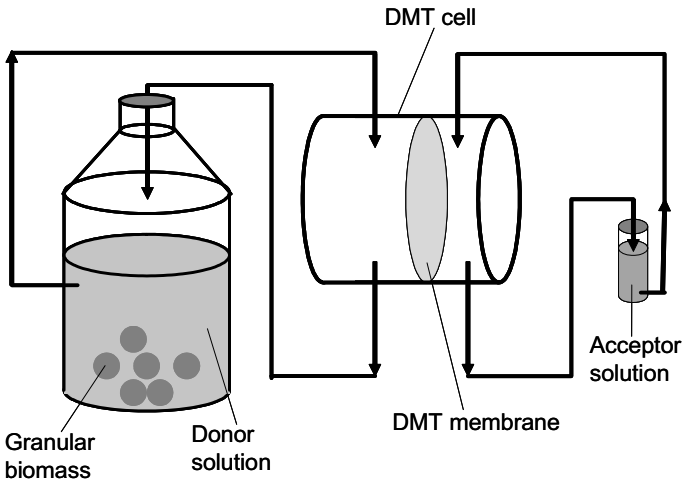


Figure 7.2. Experimental set-up of the DMT to measure the free metal concentration.

When the Donnan equilibrium is reached, samples are taken from both the donor and the acceptor. By measuring potassium, a correction can be made for Donnan potential differences (Eq. 7.5). This correction is based on the theory of the Donnan equilibrium, which implies that the charge corrected ratios of all cationic activities in the solutions on both sides of the membranes are equal (Helfferich, 1962.):

$$\left(\frac{C_{don.,i}}{C_{acc.,i}}\right)^{1/z_i} = \left(\frac{C_{don.,j}}{C_{acc.,j}}\right)^{1/z_j} \tag{7.5}$$

$C_{don.,i}$ and $C_{acc.,i}$ mean concentration of the targeted cation in donor and acceptor solution, respectively, and z_i is its charge. $C_{don.,j}$, $C_{acc.,j}$ and z_j are the same quantities for the reference cation. Potassium is chosen to correct for differences in ionic strength, because it hardly forms any complexes, and thus exists almost completely as 'free' ions (Weng et al., 2001). Therefore, only free K^+ is measured on both sides of the membranes.

7.2.8. Chemical analyses

The cobalt concentration was determined with ICP-OES (Varian, Australia) as described by Zandvoort et al. (2002). The total suspended solids (TSS) and volatile suspended solids (VSS) concentrations were determined according to Standard Methods (APHA/AWWA 1998). All chemicals were of analytical or biological grade and purchased from E. Merck AG (Darmstadt, Germany).

7.3. RESULTS

7.3.1. Validation of DMT

To assess the accuracy of the DMT for free cobalt concentration measurements, three experiments in a synthetic medium (ultra-pure water, $CaCl_2$ 1 mmol/L, MOPS 10 mmol/L) with three different ligands (chloride, carbonate and citrate) were performed. The results were compared with the data calculated by equilibrium modeling software (Visual MINTEQ Ver. 2.51). The experimental results were in a good agreement (maximum 6% deviation) with the calculated data (Table 7.3). Therefore, DMT was chosen to be applicable for the free cobalt concentration measurements in the matrixes under investigation.

Table 7.3. Validation of DMT results for free cobalt (Co^{2+}) ion to theoretical calculations.

	Ligand present		
	Cl^-	CO_3^{2-}	citrate
Total cobalt concentration ($\mu mol \cdot L^{-1}$)	5.02	3.94	5.35
Measured Co^{2+} concentration ($\mu mol \cdot L^{-1}$)	4.68	3.09	1.60
Calculated Co^{2+} concentration ($\mu mol \cdot L^{-1}$)	5.00	2.95	1.66

7.3.2. Influence of cobalt on methanogenic activity

Fig. 7.3 shows the methane production and evolution of SMA by the methanol fed granular sludge from the three experimental sets. Methanol (0.2 mg COD in each bottle) was completely transformed to methane in most cases. Incomplete methanol to methane conversion occurred only when higher amounts of cobalt were added (almost 100% inhibition): above 2000 $\mu mol \cdot L^{-1}$ for sets I and II and above 200 $\mu mol \cdot L^{-1}$ for set III.

Cobalt limitation occurred when cobalt was added at sub-optimum amounts (Table 7.4). The observed decrease of the SMA at suboptimal cobalt concentrations were 39% (5 $\mu mol \cdot L^{-1}$ cobalt addition), 18% (5 $\mu mol \cdot L^{-1}$ cobalt addition), and 58% (0 $\mu mol \cdot L^{-1}$ cobalt addition) in set I, II, and III, respectively, in comparison with the SMA_{max}.

There is a difference between the SMA_{max} in sets I and II (2.28 and 2.06 g COD/(g VSS·d), respectively) on the one hand, and set III (0.60 g COD/(g VSS·d)) on the other hand (Fig. 7.3B). This difference was accompanied by lower end pH values in set III (approximately 6.5), which was probably caused by the different pH buffering system required for the experimental setup (Table 7.2). The difference in SMA_{max} could be also caused by the storage of the granular sludge between performing set III experiments and sets I and II experiments.

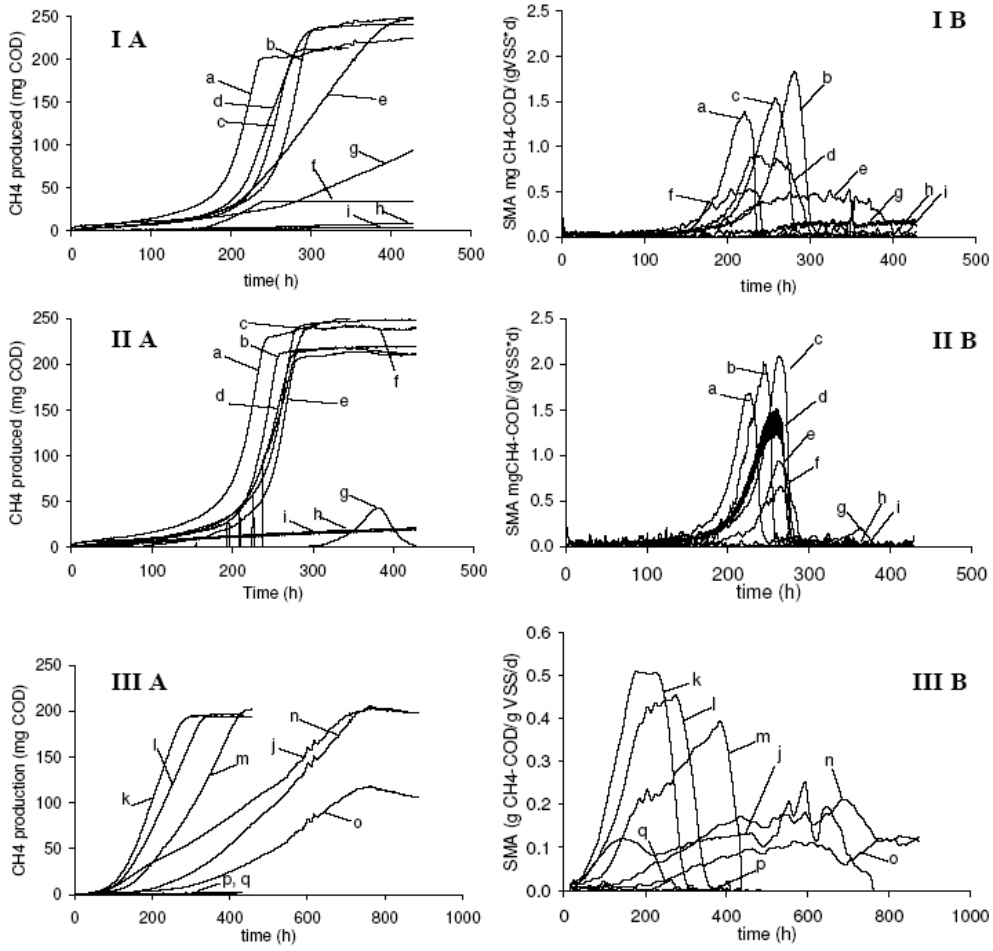


Figure 7.3. Methane production (A) and SMA (B) for various cobalt concentrations in sets I – III. The curves show activities with the following cobalt concentrations (in □mol/L); (sets I and II) a – 5, b – 75, c – 100, d – 300, e – 600, f – 1200, g – 2000, h – 32000, i – 5000, and (set III) j – 5, k – 25, l – 50, m – 75, n – 100, o – 200, p – 500, q – 1000.

Table 7.4. Inhibition values obtained at different cobalt concentrations.

	Inhibition of SMA		
Addition of cobalt	Set I	Set II	Set III
$\mu mol \cdot L^{-1}$	%	%	%
0	---	---	58
5	39	18	15
25	---	---	24
50	---	---	36
75	20	2	34
100	33	-2	65
200	---	---	79
300	60	33	---
500	---	---	99
600	77	54	---
1000	---	---	96
1200	77	70	---
2000	87	90	---
3000	94	98	---
5000	96	100	---
SMA_{max} g COD/(g VSS·d)	2.28	2.06	0.60

---: not determined

The toxicity curves showed different shapes depending on the media composition in the three sets and differences between IC_{50} values were large (Table 7.5, Fig. 7.4A). The highest IC_{50} (478 $\mu mol \cdot L^{-1}$ in set II) was almost 7 times higher than the lowest one (69 $\mu mol \cdot L^{-1}$ in set III). Moreover, the P-value of the statistical test was almost zero (Table 7.5) and therefore the hypothesis that all three data sets can be fitted with one common curve was rejected. The applied cobalt concentration affected also the time when the maximal SMA occurred (t_{max}). The influence of Co-EDTA was rather negligible in set II and the t_{max} was slightly increasing with higher $CoCl_2$ addition in set I (from 200 to 350 h). In contrast, a steep increase of t_{max} was observed in set III (from 200 to 600 h).

Table 7.5. The maximal SMA and IC_{50} values obtained from the batch-experiments. The measured data are supplemented with standard deviation of IC_{50} between the three sets (for each cobalt fraction) and the probability that the three toxicity curves are identical.

Set	IC_{50} ($\mu mol \cdot L^{-1}$)	Average IC_{50} ($mol \cdot L^{-1}$)	Standard deviation IC_{50}	P-value of the statistical test
Total added cobalt				
I ($CoCl_2$ + carbonate and phosphate)	186			
II (Co-EDTA)	478	245	85 %	0.000
III ($CoCl_2$)	71			
Dissolved cobalt				
I ($CoCl_2$ + carbonate and phosphate)	36			
II (Co-EDTA)	442	166	144 %	0.000
III ($CoCl_2$)	19			
Free cobalt				
I ($CoCl_2$ + carbonate and phosphate)	15			
II (Co-EDTA)	14	13	22 %	0.716
III ($CoCl_2$)	10			

7.3.3. Dissolved cobalt and toxicity

The toxicity curves obtained for dissolved cobalt also varied significantly among different measurement sets (Fig. 7.4B, Table 7.5). While the IC_{50} values for sets I and III were similar (IC_{50} of 36 and 18 $\mu mol \cdot L^{-1}$, respectively), the IC_{50} for set II (Co-EDTA) was approximately ten times higher (442 $\mu mol \cdot L^{-1}$). The IC_{50} value for set II obtained for dissolved cobalt was as high as the IC_{50} for the total cobalt addition in set II (478 $\mu mol \cdot L^{-1}$). The P-value of the statistical test was almost zero (Table 7.5) and therefore the hypothesis that all three data sets can be fitted with one common curve was rejected.

Fig. 7.5 shows the differences in the cobalt distribution between the solid and liquid phase at equilibrium within the three systems. Indeed, almost all (more than 90%) cobalt was present in the liquid phase when it was added as Co-EDTA (set II – except the cobalt concentration of 100 $\mu mol \cdot L^{-1}$). In contrast, a much lower amount of cobalt stayed in the liquid phase when it was supplied as $CoCl_2$. If carbonate and phosphate were present in the system in high concentrations (set I), around 70% of the cobalt precipitated (or sorbed) and only around 30% of it remained in the liquid phase. Even more (90 – 100%) cobalt precipitated/sorbed at lower amounts of cobalt added (5 and 75 $\mu mol \cdot L^{-1}$). When phosphates and carbonates were not present in the system (set III), a substantial part of the cobalt was also present in the solid phase. In contrast with set I, the distribution between the solid and liquid phase in set III depended on

the amount cobalt added (Fig. 7.5). Fig. 7.6 shows that the equilibrium concentration in bulk solution upon finishing an activity test was equal to that measured after a DMT experiments.

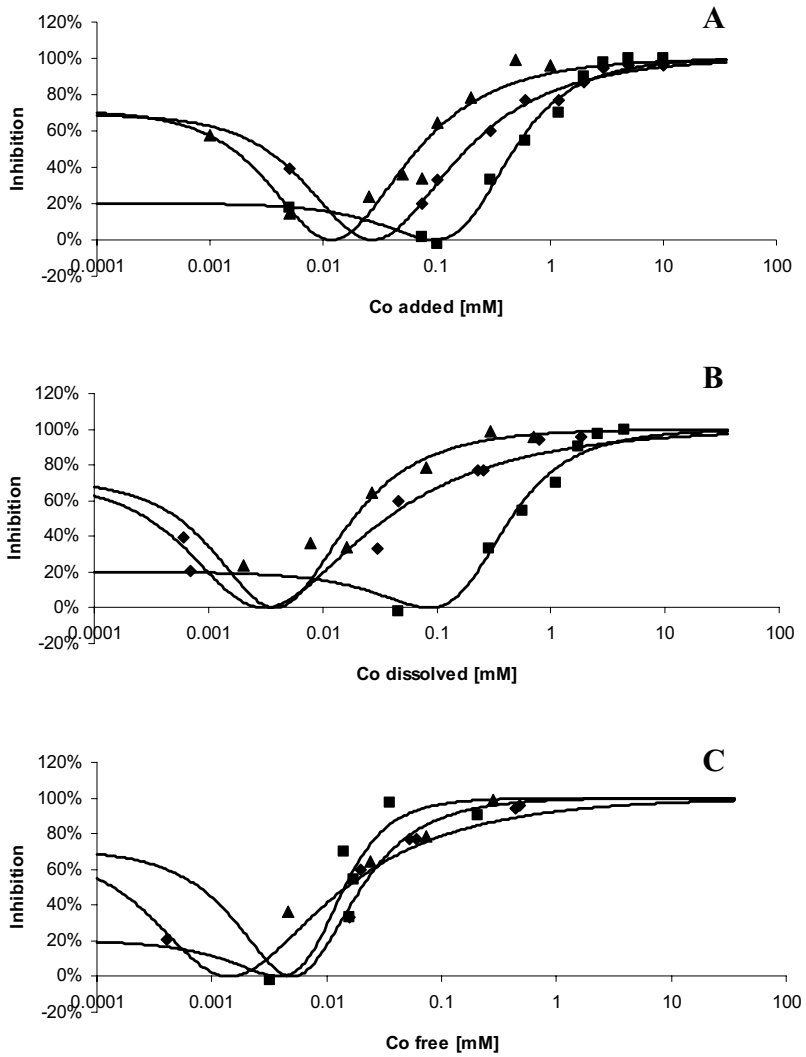


Figure 7.4. SMA inhibition with various total cobalt additions (A), dissolved concentrations (B) and free cobalt (Co^{2+}) concentrations (C). Cobalt was added in the form of $CoCl_2$ – set I (♦), CoEDTA – set II (■), and $CoCl_2$ without the presence of carbonates and phosphates – set III (▲). The curves were calculated using equations (1) and (2).

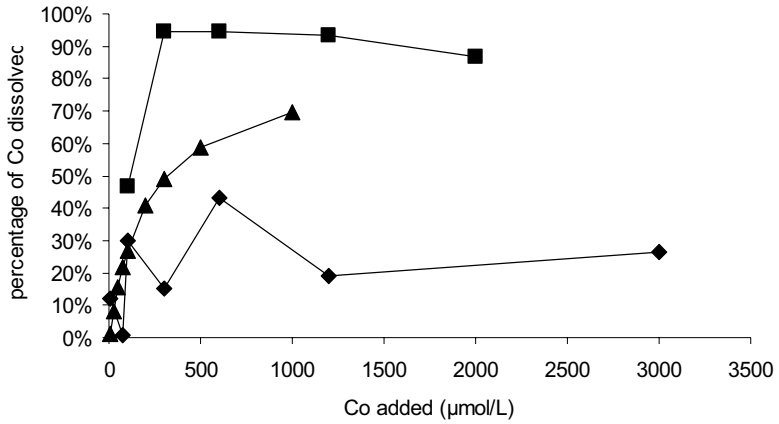


Figure 7.5. Percentage of dissolved cobalt at various cobalt additions in sets I (♦), II (■), and III (▲).

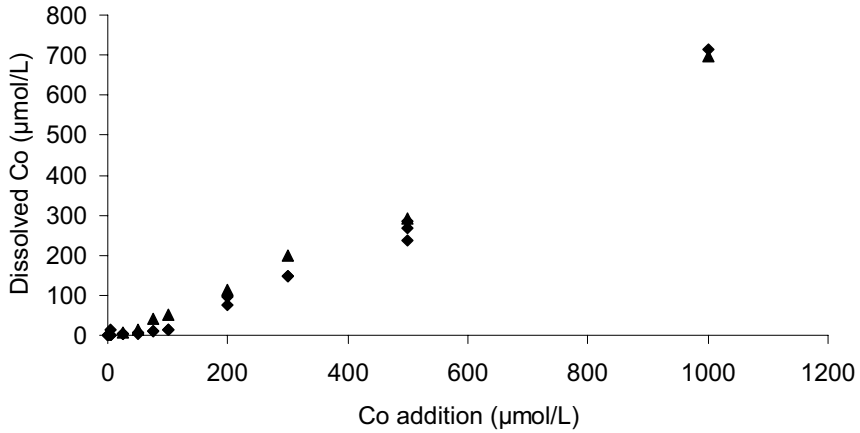


Figure 7.6. Dissolved cobalt concentrations in set III obtained at equilibrium after activity tests (♦) and after DMT measurements (▲).

7.3.4. Free metal content and toxicity

The inhibition curves are very similar for the three systems when the free cobalt concentration is plotted versus inhibition (Fig. 7.4C). The IC_{50} values for all three systems ranged from 14 to 20 $\mu mol \cdot L^{-1}$, with a standard deviation of only 20% (Table 7.5). The P-value of the statistical test was 0.72 (Table 7.5), confirming the hypothesis that all three data sets can be fitted with one common curve.

Fig. 7.7 shows free cobalt concentrations at various dissolved cobalt concentrations in the three sets. There was a steep increase in the free cobalt concentration with increasing dissolved

cobalt concentrations in set III and a slightly slower increase in set I. The free cobalt concentration reached typically 30-50, 10 and 90% of the total dissolved cobalt concentration in sets I, II and III, respectively.

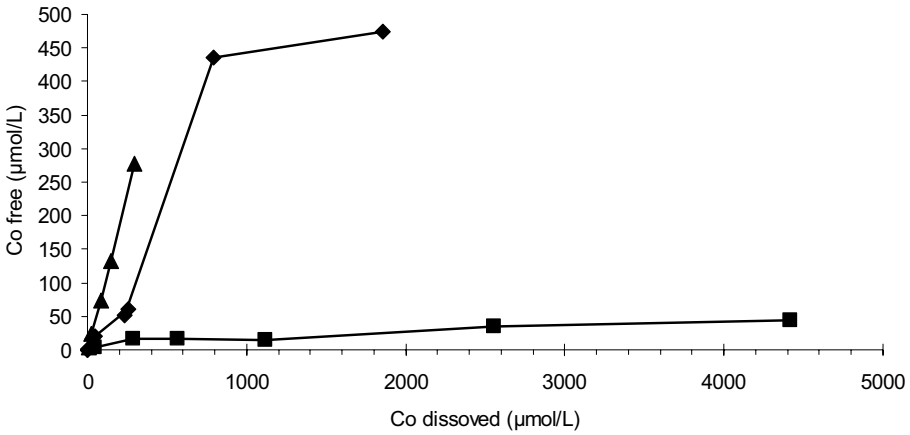


Figure 7.7. Free cobalt concentrations in sets I (♦), II (■), and III (▲) at various dissolved concentrations.

7.4. DISCUSSION

7.4.1. Role of speciation in cobalt toxicity

This study shows that the toxicity of cobalt cannot be evaluated solely based on the total cobalt concentration in the liquid phase, but requires also the determination of the amount of cobalt precipitated/sorbed in methanogenic granular sludge and the speciation of cobalt present in the bulk solution (Fig. 7.4C). Moreover, the results suggest that cobalt toxicity can be assessed based on the free cobalt concentration. The latter statement is only valid when all important conditions such as pH, concentration of competing metals (namely calcium or magnesium) etc. are kept constant. Although not only the free cobalt species can be transported into a microbial cell (also e.g. vitamin B_{12} (Ferguson and Deisenhofer, 2004), Co-citrate (Krom, 2002) and siderophores (Worms et al., 2006) are taken up by the methanogenic cell), similar IC_{50} values for all three measurement sets (standard deviation of only 22%) were obtained when taking only the free cobalt species (Co^{2+}) concentration into account (Table 7.5). This is due to the fact that when equilibrium conditions are established, metal toxicity can be predicted based on any metal species (Worms et al., 2006).

Although the IC_{50} values for the dissolved cobalt fraction obtained for sets I and III are similar, the value from set II (Co-EDTA) is more than ten times higher (Table 7.5, Fig. 7.4B). It means that although the presence of ligands (EDTA, but also phosphates and carbonates) keeps

cobalt in solution (Fig. 7.7), the bioavailability of cobalt is decreased. These results are in agreement with the conclusions of Chen et al. (2006), who observed a significant decrease of cobalt toxicity to *Pseudomonas aeruginosa* in the presence of citrate and carbonate, indicating a decrease of the metal bioavailability of cobalt due to complexation. EDTA was also reported to decrease nickel and cadmium toxicity to nitrificants (Hu et al., 2002) or copper, nickel and zinc toxicity to *Thiothrix* sp. (Shuttleworth and Unz, 1991). Similarly Kungolos (2006) demonstrated a decrease of bioavailability (and therefore also toxicity) of heavy metals in the presence of increasing amounts of humic acids. For dissolved cobalt, the IC_{50} values reported in this study (Table 7.5) increased with the stability of the complexes formed, which increases (at pH around 7) in the following order: $CoCO_3(aq)$ (pK 4.28) < $CoHCO_3^+$ (pK 12.22) < $CoHPO_4(aq)$ (pK 15.43) < $CoEDTA^{2-}$ (pK 18.16) (VisualMINTEQ thermodynamic database).

Bhattacharya et al. (1995) studied cobalt toxicity to anaerobic sludge in semi-continuous reactors under different experimental conditions: suspended enrichment culture, different medium composition (higher concentrations of inorganic salts) and different substrates (acetate and glucose). They observed an approximately 50 times higher IC_{50} value for the free cobalt ion (approximately 750 and 950 $\mu mol \cdot L^{-1}$ for glucose and acetate, respectively) compared to the present study. Also the IC_{50} values obtained for cobalt addition (both approx. 14500 $\mu mol \cdot L^{-1}$) were substantially higher than in the present study. The higher value for the supplied cobalt is probably due to higher salt concentrations (e.g. 60 mM HCO_3^-) applied by Bhattacharya et al. (1995), which lead to more precipitation and complexation. The high free cobalt IC_{50} value is likely due to toxicity prevention by the presence of higher calcium and magnesium concentration (0.17 mM Ca^{2+}, 5.25 mM Mg^{2+}), because calcium as well as magnesium competes with cobalt for metal transporters (Hassler et al., 2004). As cobalt toxicity is mainly due to interaction with physiological ions, mainly Fe^{2+} (Nies, 1999), the higher iron concentration (0.2 $mmol \cdot L^{-1}$) used by Bhattacharya et al. (1995) probably also decreased the free cobalt toxicity. The different methods for free cobalt concentration measurement (a combination of dialysis and ion exchange methods) used by Bhattacharya et al. (1995) could also affect the conclusions.

Table 7.5 and Fig. 7.4 show that there is only a small difference between the optimal and toxic free cobalt concentration: the optimal concentration was shown to be approximately 7 $\mu mol \cdot L^{-1}$ with an apparent K_M value of 0.9 $\mu mol \cdot L^{-1}$ (based on total cobalt addition) (Zandvoort et al., 2002), whereas a free cobalt concentration of only around 18 $\mu mol \cdot L^{-1}$ is already significantly toxic (50% inhibition). This means that in case only a small amount of complexing agents is present in the medium, a relatively low total cobalt concentration can cause operational problems with anaerobic reactors.

Zinc, chromium, nickel, cadmium and copper are usually reported as the most toxic heavy metals that may occur in UASB reactors (Fang, 1997; Lin and Chen, 1997; Karri et al., 2006; Tiwari et al., 2006). Their reported toxic concentrations (based on total metal added) are,

however, substantially higher (order of magnitude of mmol·L^{-1}) than the IC_{50} values documented in this study for cobalt (Table 7.5). Also Chen et al. (2006) showed that the EC_{50} (effective concentration of metallic ion to provoke 50% response) for the aerobic bacterium *Pseudomonas aeruginosa* for cobalt was more than 100 times lower than the EC_{50} measured with cadmium and manganese. Therefore, a higher attention should be paid to induced cobalt toxicity.

7.4.2. Role of precipitation/sorption

Fig. 7.5 shows that there are significant differences in the solubility of cobalt in the three systems studied. Set I (cobalt in the presence of inorganic ligands) shows the highest accumulation of cobalt in the solid phase (granules). This accumulation can be caused by or adsorption or absorption on/in the granules or by cobalt precipitation with the inorganic ions contained in the liquid medium (van Hullebusch et al., 2006). Fig. 7.5 shows a similar level of cobalt precipitation/sorption at the lower cobalt concentrations in sets I and III. However, in the case of set I (in contrast to set III), the portion of dissolved cobalt cannot be increased by addition of more cobalt. Since the only difference between the conditions of these two sets was the presence of carbonates and phosphates in set I, the difference between cobalt solubility in both systems was due to $CoCO_3$ and/or $Co_3(PO_4)_2$ precipitation. The formation of these precipitates under the applied conditions was also predicted by calculations with Visual MINTEQ (data not shown). Those precipitates are known to be present in granular sludge in significant amounts and have to be taken into account for interpretation of data when the solid-state speciation is investigated in granular sludge (van Hullebusch et al., 2005b).

The cobalt distribution between the solid and liquid phase in set III seems to be a typically adsorption-like type (shown e.g. by van Hullebusch et al. (2006)). Thus, cobalt adsorption plays a key-role in the cobalt distribution in this system. However, it is almost impossible to distinguish between precipitation and adsorption in the granular sludge matrix (van Hullebusch et al., 2005b). Therefore, precipitation of part of the cobalt added (e.g. as CoS) cannot be excluded.

In the system studied in set II, there seems to be almost no cobalt transport into the solid phase. As Fig. 7.5 shows, almost all cobalt was always (expect at concentrations as low as 100 µmol·L^{-1}) present in the liquid phase. This is caused by the high affinity of EDTA to cobalt, the strong Co-$EDTA^{2-}$ complex causes the very low precipitation/sorption of cobalt in this system.

7.4.3. Comparison of DMT with other available methods

Up to now, DMT has been most often used for measurement of the free species of various metals in synthetic media (Temminghoff et al., 2000; Weng et al., 2005; Kalis et al., 2007), surface water (Kalis et al., 2006), soil solutions (Weng et al., 2001) and manure (van der Stelt et al., 2005). In this study, DMT was successfully used for measurement of the free cobalt

(Co^{2+}) concentration in anaerobic granular sludge. Prior to the measurements on real samples, the suitability of this technique for cobalt was determined (Table 7.3). Similarly, Temminghoff et al. (2000) obtained a good agreement between calculated and measured (with the DMT) free metal concentrations for copper and cadmium. The only problem is the high time required to obtain experimental data (4 days for equilibrium establishment, five days for DMT measurement), which disables DMT for measurements under dynamic conditions of bioreactors.

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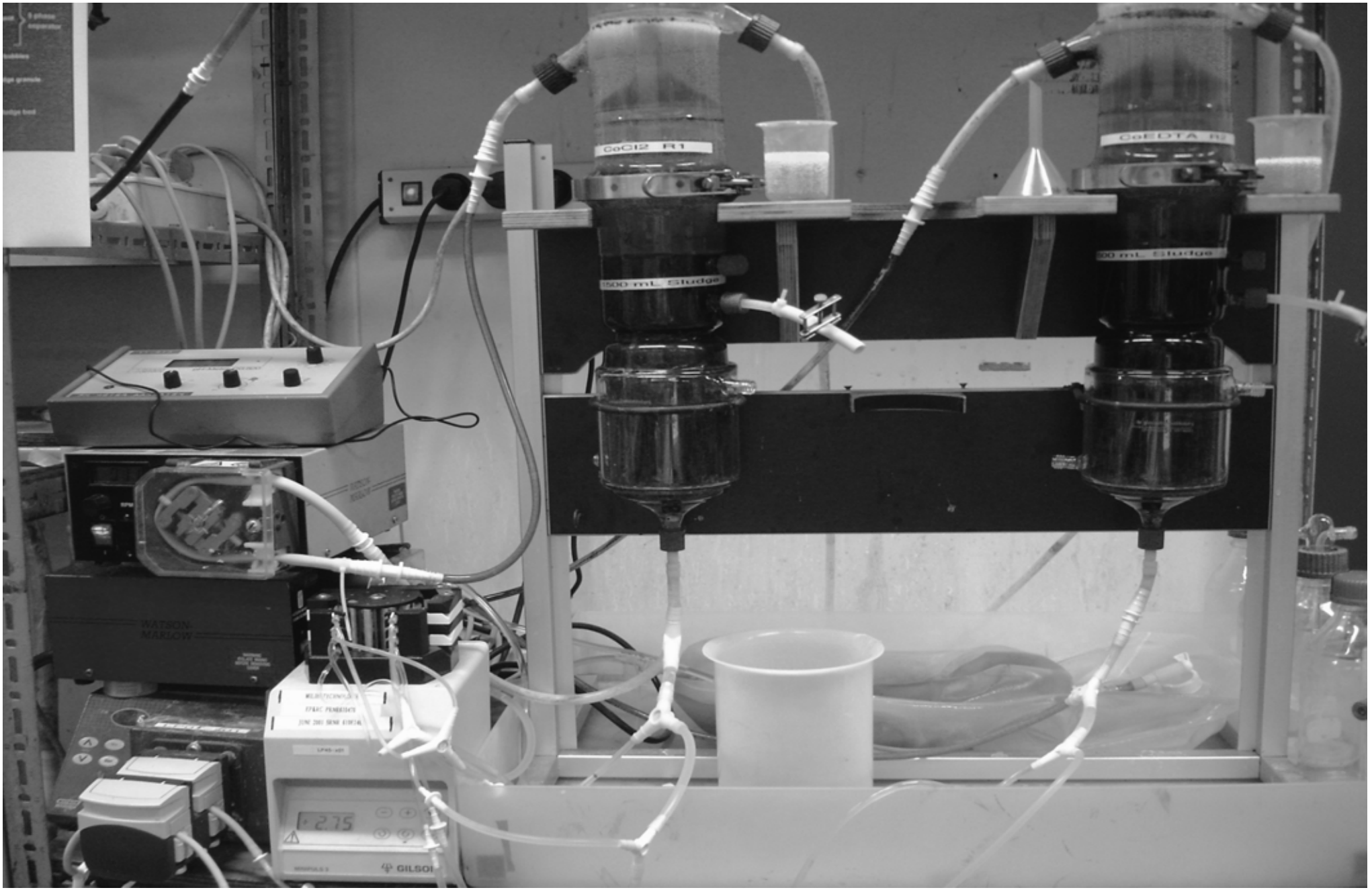
CoCl2 R1
CoEDTA R2
1500 mL Sludge
WATSON MARLOW
GILSON

EFFECT OF CHELATION ON COBALT DOSING STRATEGY IN METHANOL FED ANAEROBIC GRANULAR SLUDGE BIOREACTORS

ABSTRACT

The effect of cobalt chelation on dosing cobalt to restore the performance of a cobalt limited methanol fed bioreactor was investigated. Three upflow anaerobic sludge bed (UASB) reactors (30°C, pH 7.0) were operated at an organic loading rate of 8.5 g COD-$CH_3OH \cdot L^{-1} \cdot d^{-1}$. One UASB reactor was supplied with several pulses of cobalt bound to EDTA, and its operation was compared to that of another UASB reactor to which several pulses of $CoCl_2$ were given. The addition of cobalt (5 µmoles cobalt per litre of reactor volume) in the form of $CoCl_2$ creates a pool of cobalt in the granular sludge matrix due to the high cobalt retention (around 90 %). The methanogens present in the granular sludge are able to use that cobalt pool for stable methanol conversion during more than 15 days. Only around 8 % of the cobalt added is retained when added as Co-$EDTA^{2-}$. The small amount of retained cobalt in case of Co-$EDTA^{2-}$ addition does not support the methylotrophic methanogenesis process for more than a few operation days. Furthermore, the side-effects EDTA can have on the granule matrix or microbial cells make EDTA an unreliable ligand to be used for cobalt dosage in real applications.

8.1. INTRODUCTION

Cobalt plays a key role in anaerobic methanol degradation, as it regulates the methanol degradation pathway by affecting the different trophic groups (acetogens and methanogens) involved in methanogenic methanol conversion (Florencio et al., 1993; Zandvoort et al., 2002; Chapter 3). In case of lack of cobalt in the influent, cobalt will deplete from the sludge. This leads to a decrease in the specific methanogenic activity (SMA) of the sludge, resulting in methanol accumulation, and later acidification and ultimately complete process failure of methanol-fed UASB reactors (Chapter 3). On the another hand, too high influent cobalt concentrations can result in acetate build-up due to stimulated acetogenic activity (Florencio et al., 1993) or can even induce cobalt toxicity (Chapter 7). Thus, appropriate cobalt dosing strategies need to be developed that balance the nutritional requirements and the deterioration of bioreactor operation.

Dosing strategies have been studied for methanol fed UASB reactors using $CoCl_2$ as the cobalt species dosed, including continuous addition (Zandvoort et al., 2002), pre-loading of the granular sludge (Zandvoort et al., 2004) or pulse addition of high amounts of cobalt (Zandvoort et al., 2004). All these dosing protocols have as drawback that high amounts of cobalt are lost with the effluent. An alternative to these dosing strategies might be repeated pulse additions of low amounts of cobalt, which can be expected to minimize these losses. Moreover, dosing cobalt as a more bioavailable species might be more effective. Dosing cobalt bound to a strong ligand as EDTA is an interesting option to improve the bioavailability of the dosed cobalt, and making the cobalt more mobile within the granular sludge matrix. EDTA is a strong ligand that prevents metals to precipitate and keeps them in solution. In some cases, EDTA is used to mobilise the metals from contaminated biofilms, soils or sediments, making them more bioavailable for metal biouptake (Humez et al., 1997; Grcman et al., 2001; Oviedo and Rodriguez, 2003).

The aim of this study was, therefore, to evaluate the pulse addition of cobalt bound to EDTA and compare it to $CoCl_2$ pulsing. Moreover, the pulse addition of $CoCl_2$ to restore methanogenesis in an acidified bioreactor is investigated. The evaluation was carried out by determination of the effect of the cobalt speciation on the performance of the UASB reactors, cobalt retention into the granular sludge, the evolution of the specific activity profile of the biomass present in the bioreactor over time and the dynamics of the key microbial populations present in the sludge.

8.2. MATERIALS AND METHODS

8.2.1. Source of biomass

The UASB reactors were inoculated with 1.5 L anaerobic granular sludge obtained from a full-scale UASB reactor treating alcohol distillery wastewater of Nedalco (Bergen op Zoom,

the Netherlands). The total suspended solids (TSS) and volatile suspended solids (VSS) content of the wet sludge amounted to 7.2 (± 0.1) % and 6.7 (± 0.1) %, respectively. The initial TSS and VSS content in the UASB reactors amounted to 7.5 (± 0.3) $g \cdot L_{Reactor}^{-1}$ and 6.5 (± 0.1) $g \cdot L_{Reactor}^{-1}$.

8.2.2. Influent composition

The reactors were fed a basal medium consisting of methanol, macronutrients and a trace metal solution (Table 8.1). Methanol was fed to the reactors at an organic loading rate (OLR) of 8.5 (± 1.6) g $COD \cdot L^{-1}_{reactor} \cdot d^{-1}$. The inorganic macronutrients contained (in milligrams per litre of basal medium): NH_4Cl (280), K_2HPO_4 (250), $MgSO_4 \cdot 7H_2O$ (100) and $CaCl_2 \cdot 2H_2O$ (10). To ensure pH stability around 7.0, 2.52 g (30 mM) of $NaHCO_3$ was added per litre of basal medium. To avoid precipitation in the storage vessels, the influent was composed of 3 streams: (1) macronutrients and trace metal solutions without K_2HPO_4 and $NaHCO_3$, (2) methanol with $NaHCO_3$ and K_2HPO_4 and (3) dilution water. Demineralised water was used to prepare the influent and as dilution water.

Table 8.1. Composition of trace metal solution added to reactor influent and activity tests media.

Compound	Metal	Conc. [µM]
$FeCl_2 \cdot 4H_2O$	Fe (II)	5
$CuCl_2 \cdot 2H_2O$	Cu (II)	0.5
$ZnCl_2$	Zn (II)	0.5
$MnCl_2 \cdot 4H_2O$	Mn (II)	0.5
$NiCl_2 \cdot 6H_2O$	Ni (II)	0.5
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	Mo (VI)	0.5
$Na_2SeO_2 \cdot 5H_2O$	Se (VI)	0.5
$Na_2WO_4 \cdot 2H_2O$	W (VI)	0.5

8.2.3. Experimental set-up

The experiments were performed using 3.25 L glass cylindrical UASB reactors as described in Chapter 4. The reactors were operated at 30 (±2)°C, at a hydraulic retention time (HRT) of 8 hours and a superficial liquid upflow velocity of 0.5 $m \cdot h^{-1}$.

8.2.4. Experimental design

Three UASB reactors were run to which cobalt was added in pulses. Every pulse addition consisted of 17.5 µmoles of cobalt, corresponding to 5 µmoles cobalt per litre of reactor volume. This value supports stable methylotrophic methanogenic UASB bioreactors inoculated with the same inoculum sludge as used in this study (Zandvoort et al., 2003). The solution of cobalt

bound to EDTA (Co-EDTA^{2-}) was prepared by mixing respective amounts of $CoCl_2$ and Na_2H_2EDTA to a molar relation of 1:1.5 for cobalt and EDTA, respectively. This results in an excess of 0.5 μM $EDTA^{4-}$.

One reactor (R1) was supplied with two pulse additions of cobalt chloride ($CoCl_2$). Its operation was compared to that of another UASB reactor (R2) to which four pulses of Co-EDTA^{2-} were given. The effect of $CoCl_2$ pulsing in an acidified reactor was investigated in R3.

In Chapter 3 was showed that acidification of methanol fed UASB reactors proceeds via a first phase of methanol accumulation due to the reduced SMA on methanol of the sludge. The elevated methanol concentration than triggers the acidogens, resulting in elevated VFA concentrations and acidification, accompanied with a decay of the abundance of the *Methanosarcina* population in the granular sludge. Therefore, methanol accumulation prior to acidification (i.e. VFA accumulation) was chosen as the time for the pulse dosing in this study. The timing for a cobalt pulse addition in R1, R2 and the first pulse in R3 was set when methanol accumulation started (up to 2000 mg COD-$CH_3OH \cdot L^{-1}$), but prior to VFA accumulation (below 300 mg COD-VFA$\cdot L^{-1}$) in the reactor effluent. The second $CoCl_2$ pulse in R3 was done after both methanol and VFA accumulated in the effluent, i.e. when the reactor was acidified.

8.2.5. Specific maximum methanogenic activity tests

The SMA of the sludge was determined in duplicate at 30 (±2) ◦C using on-line gas production measurements as described by (Zandvoort et al., 2002). Approximately 1 g (wet weight) of granular sludge was transferred to 120 mL serum bottles containing 50 mL of basal medium with the same composition as the reactor basal medium, supplemented with methanol (4 g COD$\cdot L^{-1}$) as the substrate. SMA values reported refer to measurements done in the absence of cobalt in the test medium, unless specified otherwise. Gas production measurements were plotted in a rate versus time curve, using moving average trend lines with an interval of 15 data points. The samples were always taken from the same place (the mid-height) in the sludge bed.

8.2.6. Fluorescent in situ hybridization

Anaerobic granules from operation days 63, 70 and 81 in R1, 12 and 27 in R2 and 63, 81, 90, 94 and 108 in R3 were prepared as described in Chapter 4. Fluorescent *in situ* hybridization (FISH) was performed as described in Chapter 5 using Cy5 and Cy3 labeled 16S rRNA-targeted oligonucleotide probes (Biomers.net, Germany): *Sarci551* (Sorensen et al., 1997), *Eury498* (Burggraf et al., 1994) and *Eub338* (Stahl and Amann, 1991), specific for the genus *Methanosarcina*, the phylum *Euryarchaeota* and the kingdom *Eubacteria*, respectively.

8.2.7. Metal composition of the sludge

The metal composition of the sludge was determined after destruction with Aqua regia (mixture of 2.5 mL 65% HNO_3 and 7.5 mL 37% HCl) as described in Chapter 4.

8.2.8. Other analytical procedures

Methanol and VFA concentrations were determined using gas liquid chromatography as described by Weijma et al. (2000). Total dissolved metal concentrations in the influent and effluent were determined by ICP-OES (Varian, Australia) in samples acidified with 0.1 M HNO_3. The samples were centrifuged at 10000 rpm to remove particles from the liquid phase. The total suspended solids (TSS) and volatile suspended solids (VSS) concentrations were determined according to Standard Methods (APHA/AWWA, 1998). All chemicals were of analytical or biological grade and purchased from E. Merck AG (Darmstadt, Germany).

8.3. RESULTS

8.3.1. Pulse addition of cobalt to methanogenic UASB reactors

8.3.1.1. Cobalt chloride pulses to a methanogenic UASB reactor

R1 started-up within 7 days, after which its methanol removal efficiency achieved 95 %. Due to the cobalt omission from the influent, the methanol effluent concentration rose to 734 mg COD-MeOH·L^{-1} within 12 days (70 % methanol removal). Therefore, the first $CoCl_2$ pulse was applied to R1 on day 12 (Fig. 8.1A). Following the $CoCl_2$ pulse, the methanol removal efficiency achieved nearly 100 % over 14 days. The SMA of the R1 sludge (in the absence of cobalt in the SMA medium) did not increase subsequent the pulse (Fig. 8.1D, Table 8.2).

On day 30, the SMA had decreased to 336 mg COD-CH_4·g VSS^{-1}·d^{-1} (Fig. 8.1D). Due to this low SMA, methanol R1 effluent concentrations reached 1200 mg COD-MeOH·L^{-1} on day 31 (Fig. 8.1A). The second cobalt pulse to R1 was applied on this day, which increased the SMA of the R1 sludge after the second pulse to 794 mg COD-CH_4·g VSS^{-1}·d^{-1} on day 32 (Fig. 8.1D, Table 8.2). This lead to an increase of the methanol removal efficiency from 60% to 100 % over the 12 days following this pulse (Fig 8.1A).

No further pulse was dosed to R1 anymore to confirm that in that case, the reactor acidifies due to the accumulation of methanol and subsequent VFA formation. Indeed, effluent methanol and VFA concentrations as high as 900 mg COD-MeOH·L^{-1} and 220 mg COD-VFA·L^{-1}, respectively, were observed in the R1 effluent on day 45 (Fig. 8.1A). The SMA decreased abruptly from 1056 mg COD-CH_4·g VSS^{-1}·d^{-1} on day 40 to 83 mg COD-CH_4·g VSS^{-1}·d^{-1} three days before the end of experiment (day 47). On day 50 (end of the experiment), methane formation was absent and the pH dropped gradually from 6.8 on day 45 to 5.5 on day 50 (Fig. 8.1B). The reactor had completely acidified and the reactor run terminated.

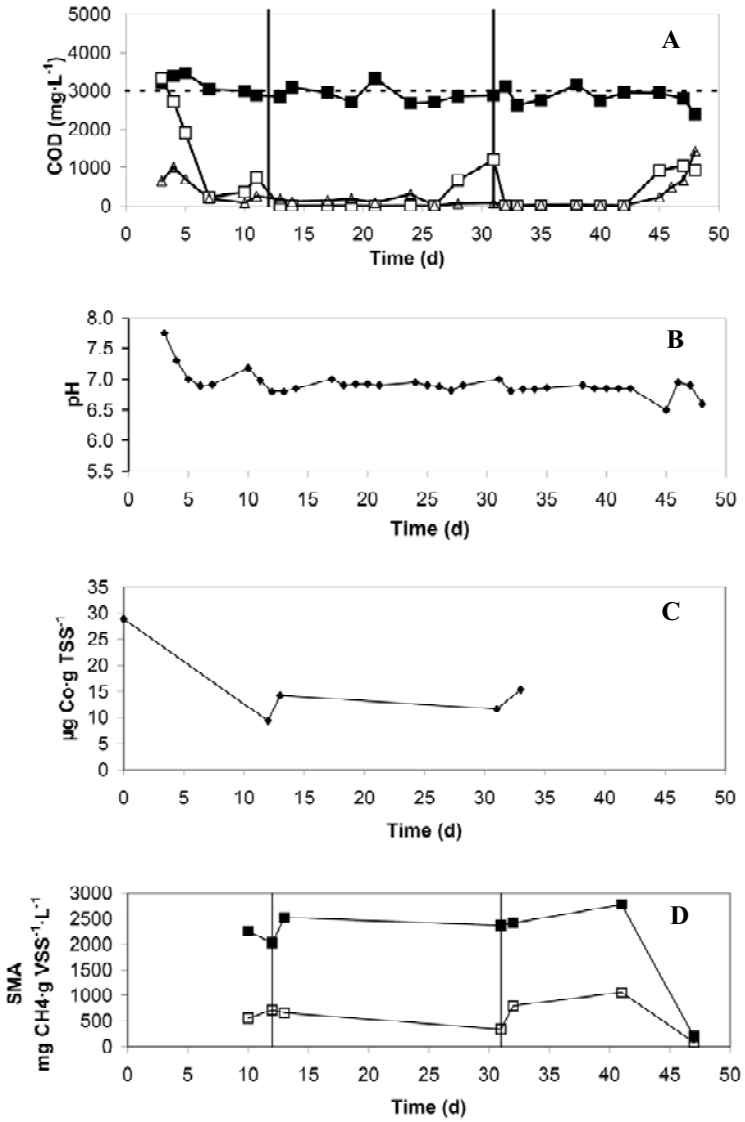

Figure 8.1. Evolution of R1 performance with time. (A) Calculated influent methanol (---), measured influent methanol (■), effluent methanol (□) and effluent VFA (Δ) concentrations. (B) pH (♦) in reactor. (C) Total cobalt concentration. (D) SMA measured without (□) and with (■) cobalt in the medium.

Table 8.2. Characteristics of the cobalt pulse given in the three UASB reactors.

Added Cobalt specie	Pulse	% washed out cobalt after 24 hours	Maximum cobalt concentration in the effluent during 24 hours after the pulse [µM]	Cobalt content in the sludge before the pulse [µg Co*(g TSS)$^{-1}$]	Cobalt content in the sludge after the pulse [µg Co*(g TSS)$^{-1}$]	SMA[a] before the pulse [mg COD-CH_4/(g VSS*d)$^{-1}$]	SMA[a] after the pulse [mg COD-CH_4/(g VSS*d)$^{-1}$]
Co-EDTA (R1)	1	94	5.54	11.1	8.8	717	1675
	2	83	5.76	7.6	8.4	273	442
	3	90	5.67	9.1	7.9	391	433
	4	95	6.38	9.2	9.0	356	400
$CoCl_2$ (R2)	1	8	0.69	9.3	14.2	711	656
	2	8	0.72	11.6	15.4	336	794
$CoCl_2$ (R3)	1	10	0.82	13.4	21.8	300	860
	2	8	1.26	12.8	29.8	246	159

a: SMA measured in medium without cobalt

8.3.1.2. Pulse addition of Co-EDTA^{2-} to a methanogenic UASB reactor

R2 started-up within 20 days, after which a methanol removal efficiency of 97 % was achieved. After 24 days of operation, methanol started to accumulate in the effluent of R2, resulting in a decrease of the methanol removal efficiency from 97 % to 90 % (Fig. 8.2A). Following this increase in effluent methanol concentration to 275 mg COD-MeOH·L^{-1}, a first Co-EDTA^{2-} pulse addition was given to R2 on day 28. This re-stabilised the methanol removal efficiency to 99 % during 7 days till day 35. Following the Co-EDTA^{2-} addition, the SMA of the reactor sludge (without cobalt in the SMA medium) doubled from day 28 to day 31 (717 mg COD-CH_4·g VSS^{-1}·d^{-1} and 1675 mg COD-CH_4·g VSS^{-1}·d^{-1}, respectively), and even reached a value as high as 1862 mg COD-CH_4·g VSS^{-1}·d^{-1} on day 35 (Fig. 8.2D). Remarkably, the SMA increased with 233 % after the Co-EDTA^{2-} addition between day 28 and day 31, whereas the SMA of the R1 sludge did not increase after the first $CoCl_2$ pulse addition. This suggests a higher bioavailability or more efficient transport of cobalt bound to EDTA compared to $CoCl_2$.

The effect of the Co-EDTA^{2-} pulse dosing was only temporarily, and the SMA of the reactor sludge was as low as 273 mg COD-CH_4·g VSS^{-1}·d^{-1} on day 44, which is around 6 times lower than that on day 35 (Fig. 8.2D). The lower activity lead to incomplete methanol removal, with methanol effluent concentrations reaching 2800 mg COD-MeOH·L^{-1} on day 46 (Fig. 8.2A). The second Co-EDTA^{2-} pulse addition was applied to R2 on day 46, but the methanol removal efficiency increased only from 22 % on day 46 to 34 % upon the second addition. The SMA increased only up to 442 mg COD-CH_4·g VSS^{-1}·d^{-1} on day 49 and no further increase of the methanol removal efficiency was observed during the next 10 days. The SMA increase of the UASB sludge (without cobalt in the SMA tests medium) in this pulse was much lower (160 %) than in the first pulse (233 %) even though a completely similar cobalt pulse of 17.5 µmoles of Co-EDTA^{2-} was given. The main difference between both pulses was the accumulated methanol concentration prior the pulses: in the first pulse only 275 mg COD-MeOH·L^{-1} accumulated, whereas 2800 mg COD-MeOH·L^{-1} had accumulated in the second pulse.

A third Co-EDTA^{2-} pulse was given on day 63 to restore the UASB reactor operation, when the R2 methanol effluent concentrations had stabilized at 2000 mg COD-MeOH·L^{-1}. The methanol removal efficiency restored from 25 % to 99 % and lasted approximately for 24 days (till day 87). Following the third pulse, no increase in SMA of the sludge sampled could be detected at the time points the SMA was recorded (Fig. 8.2D, Table 8.2).

From day 87 to day 90, the methanol effluent concentration started to increase again up to 700 mg COD-MeOH·L^{-1} (Fig. 8.2A). The fourth Co-EDTA^{2-} pulse was given on day 90. This pulse stabilized the methanol conversion capacity of R2 over 3 days. The measured SMA on day 94 was slightly higher compared to the measured SMA prior to the forth pulse (Fig.8.2D, Table 8.2). From day 94, methanol and three days later VFA started to accumulate rapidly and

achieved a maximum value up to 2000 mg COD-MeOH·L^{-1} and 1800 mg COD-VFA·L^{-1}, respectively (Fig. 8.2A). This led to a pH drop from 6.8 down to 5.5 (Fig. 8.2B). The SMA of the R2 sludge dropped down to 209 mg COD-CH_4·g VSS^{-1}·d^{-1} on day 111.

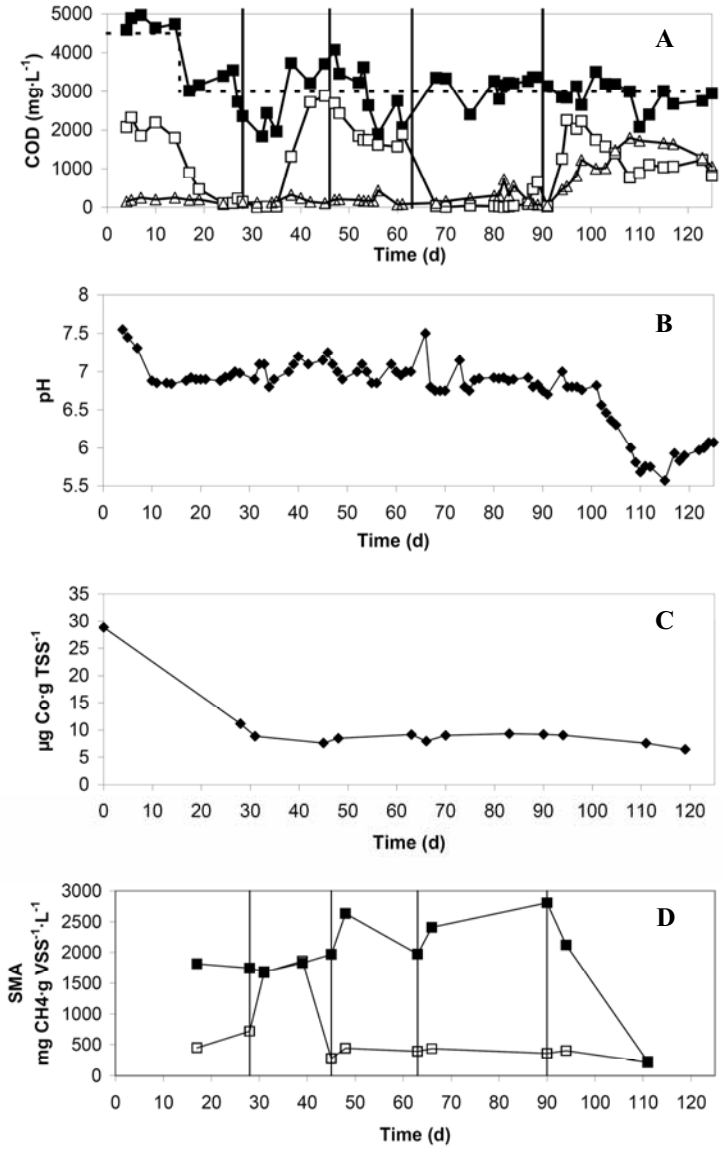

Figure 8.2. Evolution of R2 performance with time. (A) Calculated influent methanol (---), measured influent methanol (■), effluent methanol (□) and effluent VFA (Δ) concentrations. (B) pH (♦) in reactor. (C) Total cobalt concentration. (D) SMA measured without (□) and with (■) cobalt in the medium.

8.3.1.3. Cobalt retention by the granule sludge

Cobalt was highly retained in the UASB granular sludge matrix when added as $CoCl_2$. Following the 5 µmoles $CoCl_2$ per litre of reactor volume pulse on day 12 in R1, the maximum cobalt concentration measured in the R1 effluent amounted to 0.69 µM 1 hour after the cobalt pulse was given (Fig. 8.3B, Table 8.2). The cobalt concentration in the effluent decreased to 0.05 µM after 25 hours. The amount of cobalt washed out after this 25 hours amounted to 7.9 % of the total added cobalt (Fig. 8.3B, Table 8.2). Similar to the first $CoCl_2$ pulse addition, 8 % of the total added cobalt washed out within 25 hours after the second pulse to R1. The cobalt retention by the UASB reactor and, therefore, cobalt accumulation in the UASB sludge granules was also reflected in the total cobalt content of the R1 sludge. One day after the first cobalt pulse addition, the cobalt content of the R1 sludge increased from 9.3 µg·g TSS^{-1} on day 12 up to 14.2 µg·g TSS^{-1} on day 13 (Fig. 8.1C, Table 8.2). Similarly, two days after the second pulse to R1, the cobalt content of the R1 sludge increased from 11.6 µg·g TSS^{-1} on day 31 to 15.3 µg·g TSS^{-1} on day 33 (Table 8.2).

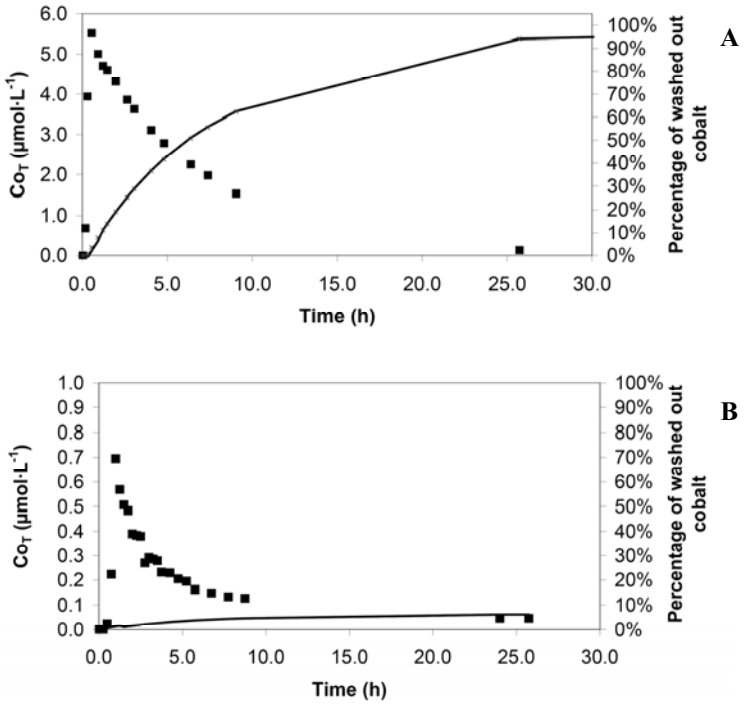

Figure 8.3. (A) Evolution of first cobalt bound to EDTA pulse in R2 with time. Washed out cobalt when added as cobalt EDTA (■), percentage of the total added cobalt washed out (─). (B) Evolution of first cobalt chloride pulse in R1 with time. Washed out cobalt when added as cobalt chloride (■), percentage of the total added cobalt chloride washed out (─).

Cobalt supplied bound to EDTA was poorly retained by the R2 granular sludge. After the 5 µM Co-EDTA^{2-} pulse on day 28 to R2, the maximum cobalt concentration measured in the R2 effluent amounted to 5.54 µM (Fig. 8.3A, Table 8.2) and 94 % of the total added cobalt (Fig. 8.3A) washed out within 25 hours. Similarly, the cobalt wash out within 25 hours after the cobalt pulses amounted to 83, 90 and 95 % of the total amount of supplied Co-EDTA^{2-} for the second, third and fourth pulse in R2, respectively (Table 8.3). This is an about 10 times lower cobalt retention than for any of the $CoCl_2$ pulse additions given to R1 (Fig. 8.3A, Table 8.2). The addition of Co-EDTA^{2-} even decreased the cobalt content of the sludge in the first pulse addition to R2 from 11.5 µg·g TSS^{-1} on day 28, prior to the first cobalt pulse addition, down to 8.8 µg·g TSS^{-1} three days after the pulse addition (Fig. 8.2C, Table 8.2). The cobalt content of the sludge kept constant (8.6 ± 0.6 µg·g TSS^{-1}) during the subsequent reactor operation and thus independent of the three cobalt pulse additions given to R1 between day 31 and the end of the experiment on day 119.

Table 8.3. Comparison of different $CoCl_2$ dosing strategies in methanol fed UASB reactors.

Cobalt dosing strategy	Added Cobalt	Maximum activity reported [mg COD-CH_4*(g VSS*d)$^{-1}$]	Maximum cobalt content in the sludge [µg Co*(g TSS)$^{-1}$]	Cobalt losses from sludge [µg*(g TSS*d)$^{-1}$]	OLR [g COD*(L*d)$^{-1}$]	Reference
Continuos	0.62 µmoles per litre of reactor volume and per day during 31 days	215	32	0.1	2.6	Zandvoort et al. 2002
Pre-load	Direct contact with 1 mM during 24 hours	1200	1667	22	10	Zandvoort et al. 2004
Pulse	77.5 µmoles per litre of reactor volume	613	107	3.5	10	Zandvoort et al. 2004
Pulse	5 µmoles per litre of reactor volume	860	21.8	0.1	8.5	This study

8.3.2. Pulse addition of cobalt chloride to an acidified UASB reactor

Methanol effluent concentrations in R3 were below 2000 mg COD-VFA·L^{-1} during the first 28 days of operation. A first $CoCl_2$ pulse was applied to R3 on day 28, when methanol removal stabilized at 53 % and no VFA were present in the R3 effluent. Similar to both $CoCl_2$ pulse additions in R1, the methanol removal efficiency achieved nearly 100 % over the 14 operation days following the $CoCl_2$ pulse addition (Fig. 8.4A).

R3 was further operated till it acidified. On day 42, methanol started to accumulate and VFA increased up to 700 mg COD-VFA·L^{-1} on day 56. On day 60, the methanol and VFA effluent concentrations reached 2200 mg COD-MeOH·L^{-1} and 550 mg COD-VFA·L^{-1}, respectively (Fig. 8.4A). The second cobalt pulse addition to R3 was applied on day 63 in the presence of VFA (540 mg COD-VFA·L^{-1}) in the effluent (Fig. 8.4A). The $CoCl_2$ pulse addition induced a rapid increase of VFA in the R3 effluent, from 550 mg COD-VFA·L^{-1} on day 62 to 1770 mg COD-VFA·L^{-1} on day 70. The pH dropped from 7.0 on day 63 up to 6.5 on day 70 (Fig. 8.4B) and no methane was produced in this period.

Also in the presence of high VFA concentrations (540 mg COD-VFA·L^{-1}), cobalt was highly retained when added as $CoCl_2$ (Fig. 8.4A). A maximum cobalt effluent concentration of 1.26 μM was measured 1.25 hours after the second cobalt pulse to R3, almost the double of the maximum concentration measured when the pulse was given with only methanol accumulated in the effluent, 0.7 μM in both pulses in R1 and 0.8 μM in the first pulse in R3 (Table 8.3). Interestingly, the amount of cobalt washed out 25 hours after the second pulse to R3 amounted to 8 % of the total added cobalt. This is similar to the total amount of cobalt washed out in both cobalt pulses to R1 (Table 8.2) and in the first cobalt pulse to R3 (Table 8.2). Also similar to the first cobalt pulse to R3 and both pulses to R1, the cobalt content of the sludge increased from 12.8 μg·g TSS^{-1} on day 63 to 29.8 μg·g TSS^{-1} on day 66 (Table 8.2).

In order to study if a continuous cobalt addition could restore methylotrophic methanogenesis, continuous $CoCl_2$ feeding (5 μM) was supplied to R3 from day 80 onwards. The R3 effluent cobalt concentrations prior to the continuous cobalt addition were very low (< 0.02 μM). Continuous cobalt supplementation to the R3 influent led to cobalt accumulation in the R3 effluent concentrations up to 0.27 μM (Data not shown). Effluent VFA concentrations increased up to 2400 mg COD-VFA·L^{-1} on day 84 (the end of the run), accompanied by a further drop of the effluent pH from 6.5 on day 70 to 5.5 on day 84 (Fig. 8.4B).

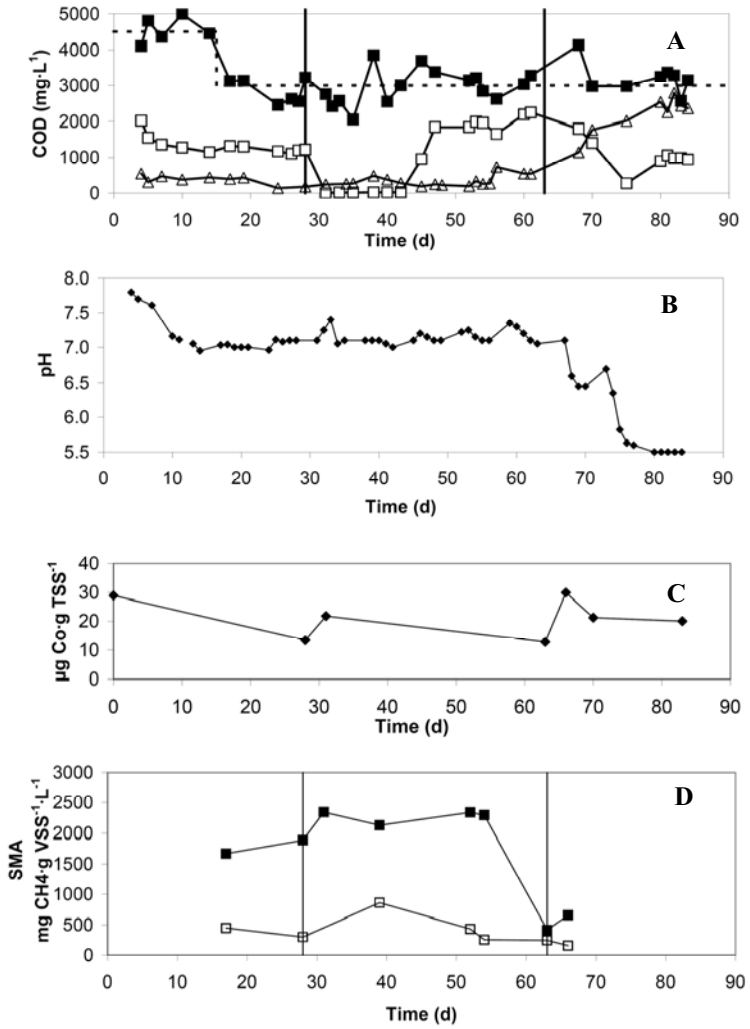

Figure 8.4. Evolution of R3 performance with time. (A) Calculated influent methanol (---), measured influent methanol (■), effluent methanol (□) and effluent VFA (Δ) concentrations. (B) pH (♦) in reactor. (C) Total cobalt concentration. (D) SMA measured without (□) and with (■) cobalt in the medium

8.3.3. Evolution of key microbial populations

The abundance of the *Methanosarcina* population in the R1 granules did not change between day 12, when the first $CoCl_2$ pulse was given, till day 27, just before the second cobalt pulse addition and just before methanol started to accumulate in the R1 effluent. However, their spatial orientation changed: *Methanosarcina* cells were present in more dense clusters on day 12 compared to 27 (Fig. 8.5c,f). The *Euryarchaeota* population increased between day 12 and day 27. On day 12, the *Euryarchaeota* population was mainly present in small clusters around the periphery of the granule (Fig. 8.5a). On day 27, the *Euryarchaeota* population was observed in small clusters around the periphery of the granule as well, and additionally, dense clusters of *Euryarchaeota* cells were observed in the core of the granule (Fig. 8.5d). The abundance of the *Eubacteria* population in R1 was stable between day 12 and day 27. Opposite to the *Methanosarcina* population, *Eubacteria* cells were presented in more dense clusters on day 27 compared to day 12 (Fig. 8.5b,e).

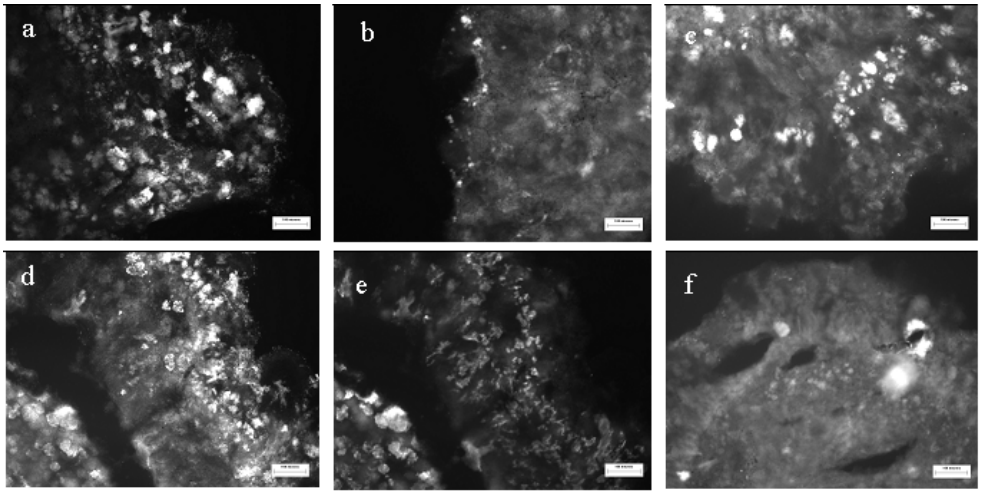

Figure 8.5. In situ hybridizations using cross-sections of anaerobic granules in R1 illustrating (a) Eury498, (b) Eub338 and (c) Sarci551 in day 12; (d) Eury498, (e) Eub338 and (f) Sarci551 in day 27. Scale bar = 100 μm.

The R2 granules sampled on day 63 contained very few *Methanosarcina* cells (Fig. 8.6c), which coincides with the high methanol effluent concentrations (Fig. 8.2A). On day 90, however, when no methanol was detected in the R2 effluent, the *Methanosarcina* population of the R2 granules had increased compared to day 63. *Methanosarcina* cells were mainly observed in dense clusters throughout the granule section on day 90 (Fig. 8.6f). Only 4 days later (day

94), after the Co-EDTA^{2-} pulse and high methanol and R2 effluent VFA concentrations were measured, the *Methanosarcina* population decreased in R2 granules (Fig. 8.6i). *Methanosarcina* cells were present in dense clusters, similar to day 90, but in smaller numbers (Fig. 8.6i). A temporal evolution of the *Euryarchaeota* population was observed in the R2 run. On day 63, they were allocated mainly in the periphery of the granular section (Fig. 8.6a). On day 90, the *Euryarchaeota* population was observed in clusters throughout the whole granule section (Fig. 8.6d), but 4 days after the Co-EDTA^{2-} pulse (day 94), a slight decrease of the *Euryarchaeota* population was observed (Fig. 8.6g). The *Eubacteria* population followed an opposite trend than the *Methanosarcina* population. On day 63, the abundance of the *Eubacteria* population was much higher than on day 90 (Fig. 8.6b,e), but on day 94, the *Eubacteria* population present was again much larger compared to 4 days before (day 90) (Fig. 8.6e,h).

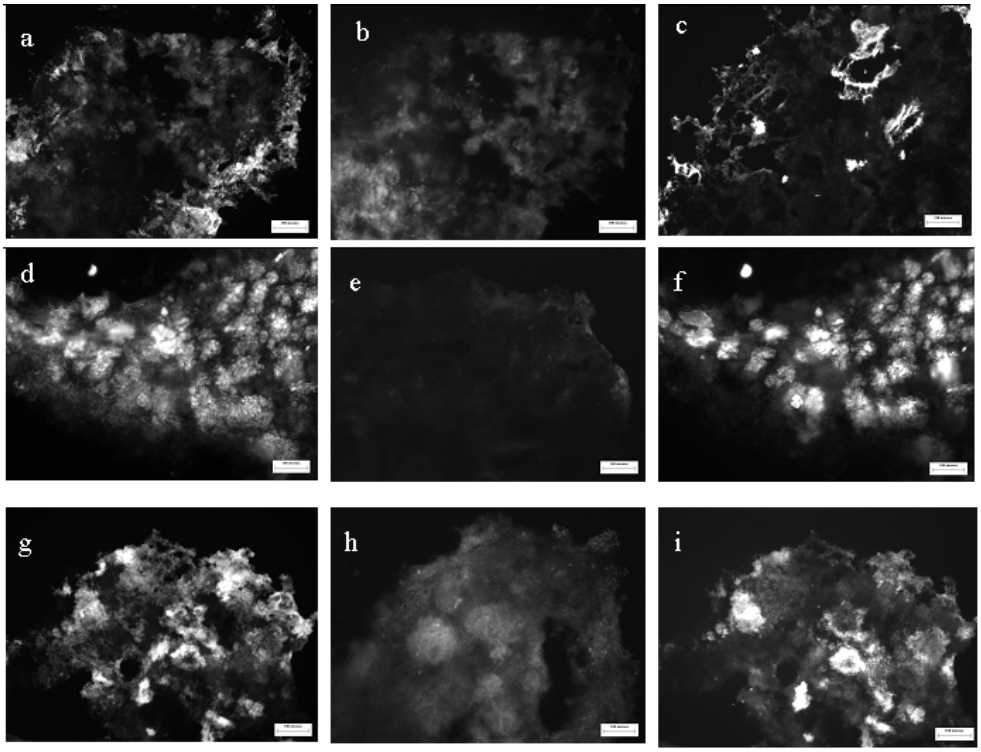

Figure 8.6. In situ hybridizations using cross-sections of anaerobic granules in R2 illustrating (a) Eury498, (b) Eub338 and (c) Sarci551 in day 63; (d) Eury498, (e) Eub338 and (f) Sarci551 in day 90; (g) Eury498, (h) Eub338 and (i) Sarci551 in day 94. Scale bar = 100 μm.

The *Methanosarcina* and *Euryarchaeota* population in R3 granules were present in densely packed clusters on day 63 (Fig. 8.7a,c). The *Methanosarcina* population was more abundant on this day, with VFA effluent concentrations of 540 mg COD-VFA·L^{-1}, compared to day 82 with more acidification (VFA effluent concentration 2400 mg COD-VFA·L^{-1}). On the latter day, the *Methanosarcina* population was mainly present in the core of the granule. The *Euryarchaeota* population was less dispersed through the granule section on day 63 than on day 81 (Fig. 8.7f). Opposite to the *Methanosarcina* population, the abundance of the *Eubacteria* population in R3 granules increased between days 63 (Fig. 8.7b) and 81 (Fig. 8.7e).

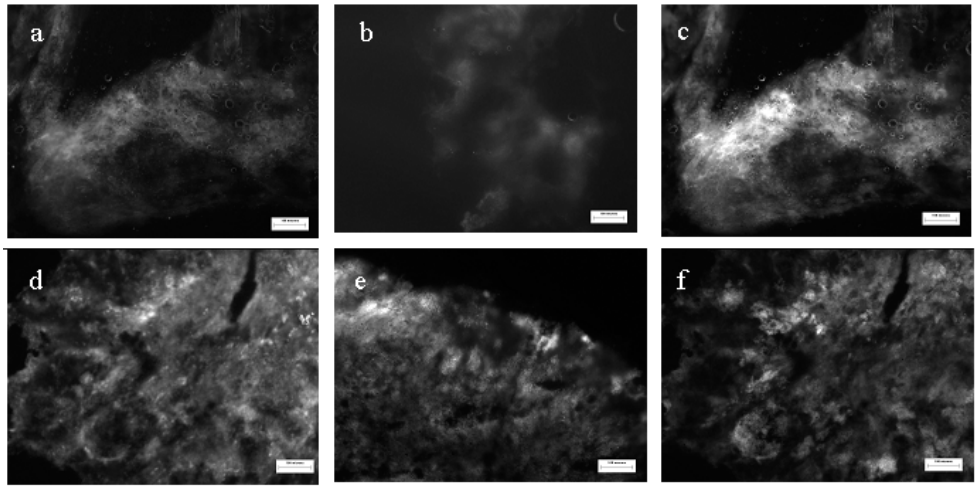

Figure 8.7. In situ hybridizations using cross-sections of anaerobic granules in R3 illustrating (a) Eury498, (b) Eub338 and (c) Sarci551 in day 63; (d) Eury498, (e) Eub338 and (f) Sarci551 in day 81. Scale bar = 100 µm.

8

8.4. DISCUSSION

8.4.1. Effect of cobalt speciation on the capacity of cobalt pulses to restore the reactor performance

8.4.1.1. Cobalt chloride pulsing

This study shows that repeated pulsing of cobalt chloride is an efficient dosing strategy to maintain a stable methanogenic methanol fed UASB reactor. Pulse addition of cobalt (5 µmoles cobalt per litre of reactor volume) in the form of $CoCl_2$ creates a pool of cobalt into the granular sludge matrix due to the high cobalt retention (around 90 %). In contrast, a much smaller cobalt pool is formed when cobalt is dosed as Co-$EDTA^{2-}$: only 8 % of the supplied Co-$EDTA^{2-}$ is retained (Fig. 8.3, Table 8.2). Additionally, cobalt wash-out was much less compared to the

pulse addition of high amounts of cobalt or the pre-loading cobalt strategies reported in previous studies with methanol fed UASB reactors (Table 8.3). The cobalt pool formed upon $CoCl_2$ dosing was enough to keep stable methanogenesis at an OLR of 8.5 g $COD \cdot L^{-1}_{reactor} \cdot d^{-1}$ for more than 15 days between the pulses.

Sequential extraction of metals showed that the pool formed upon $CoCl_2$ dosing in anaerobic granular sludge is mainly through cobalt precipitation in the sulfidic/organic matter fraction (Van Hullebusch et al., 2005). Using the acid volatile sulfide (AVS) technique, sulfides were shown to dominate the bonding form distribution of essential metals (Co and Ni) in anaerobic methanogenic granular sludge and have a strong influence on the metal bioavailability (Chapter 2). Cobalt sulfide dissolution was, nevertheless, proposed to provide enough Co^{2+} to supply the cobalt requirements of a *Methanosarcina bakeri* enrichment (Jansen et al., 2007). These methanogens are commonly present in the methanol fed granular sludge investigated (Fig. 8.5c,f, Fig. 8.6c,f,i, Fig 8.7c,f).

8.4.1.2. Co-EDTA^{2-} pulsing

Co-EDTA^{2-} is less vulnerable to metal precipitation into the solid phase compared to $CoCl_2$ and can thus travel more through the granular matrix than $CoCl_2$. Similarly, in case of nickel, the nickel retention is lower when Ni-EDTA^{2-} is added to a granular sludge compared to $NiCl_2$ (Chapter 4). As the stability constant for Co-EDTA^{2-} and Ni-EDTA^{2-} are quite similar (log K: 18.16 and log K: 20.11, respectively), the retention of cobalt bound to EDTA in granular sludge is, as for nickel, likely to be low as indeed shown in the present study (Fig. 8.2C, Table 8.3). EDTA can prevent metals from precipitation or sorption in soils (Grcman et al., 2001; Wang et al., 2007) or UASB reactor sludge (Osuna et al., 2004; Virkutyte et al., 2005). EDTA has been shown to enhance phytoextraction of heavy metals from contaminated soils by increasing their metal bioavailability, thus enhancing plant uptake, and translocation of metals from the root system to the green parts of plants (Grcman et al., 2001).

The high mobility of complexed metals to EDTA was expected to help to increase the diffusion of metals into the granular matrix and the internalization of the metal cations into the methanogenic cells. When Co-EDTA^{2-} was dosed in the first cobalt pulse dose, the cobalt limitation was clearly overcome immediately after dosage as shown by the high increase of SMA (233 %) after the pulse was given (Fig. 8.2D, Table 8.3). However, the small amount of retained cobalt did not support the methylotrophic methanogenesis process more than a few operation days after the first Co-EDTA^{2-} pulse dose (Fig. 8.2A, Table 8.3).

The different response in the reactor performance upon the second, third and fourth Co-EDTA^{2-} pulse addition (Fig. 8.2A) might be due to the different effects that EDTA has on the biofilm matrix and microbial cells. Free EDTA^{4-} has been shown to be toxic for biological iron reduction in anaerobic granular sludge (Van Der Maas et al., 2005). However, the reported

dissociated $EDTA^{4-}$ concentrations (5 mM) are much higher than the dissociated $EDTA^{4-}$ concentrations (7.5 µM Na_2H_2EDTA per pulse addition) in the present study. Moreover, the EDTA added in excess will likely bind to other cations present in the liquid phase (Table 8.1), so that most likely no free $EDTA^{4-}$ is present. EDTA could cause metal extraction from the metal active sites of the enzymes involved in methylotrophic methanogenesis. However, the metal content of the sludge (zinc, nickel and iron) was not affected by the Co-$EDTA^{2-}$ pulse (Data not shown). In contrast, the calcium content of the granule decreased. Calcium is a structural ion for the granular matrix and thus decreased calcium concentration lead to a decrease granular strength (Morgan et al., 1990; Shen et al., 1993), as also observed in the present study (Data not shown). (Chen and Stewart, 2000) tested the abilities of various chemical treatments to remove mixed *P. aeruginosa* - *Klebsiella pneumoniae* biofilms. EDTA treatment (10 mM) resulted in a 49% reduction in biofilm viable cell areal density. They presented evidence that this was due to dispersal of biofilm bacteria. Similarly, Fig. 8.6f,i suggests that dispersal of the *Methanosarcina* population present in the periphery of the granules occurred especially after the fourth Co-$EDTA^{2-}$ pulse addition to R2. It should be noted, however, that the Na_2H_2EDTA concentrations applied in this work (7.5 µM) were much lower than those applied by (Chen and Stewart, 2000).

8.4.2. Timing of cobalt dosing

This study showed that the $CoCl_2$ addition can not be done at any stage of the reactor operation. When acetate was already accumulating to high concentrations, i.e. the acidifying population had developed, both the cobalt pulse dosage and continuous cobalt dosing stimulated the acidification. This is agreement with Chapter 3, where was showed, working with a methylotrophic methanogenic UASB reactor initially fed without trace metal supply, that continuous cobalt addition of an already acidified reactor further enhanced the acidification. This is due to the cobalt dependence of the enzymatic system of acetogenesis (Bainotti and Nishio, 2000). Alternatively, toxic effects of cobalt suppress the methylotrophic methanogenic population, the IC_{50} of free cobalt amounted to 13 µM for methylotrophic methanogenic anaerobic granular sludge (Chapter 7). Therefore, cobalt addition has to be done prior to the VFA accumulation in the effluent (Chapter 3; Zandvoort et al., 2002). It should be noted that also acetate is able to complex metals, and most likely also complexed some of the supplied cobalt facilitating cobalt wash out from the acidifying reactor, thus leading to a double cobalt effluent concentration when added to the acidified R3 compared to the methanogenic R1.

8.4.3. Quantification of the required amount of cobalt to be dosed

Calculation of the required amount of cobalt needed per day to support adequate methanogenesis is very important for practice. With the data provided in this study, the

calculation of the mg of cobalt needed to be dosed per litre of reactor and per day can be obtained empirically. In the present study, considering a stable methanol removal period of 15 days after the $CoCl_2$ pulse addition and an OLR of 8.5 g $COD \cdot L^{-1}_{reactor} \cdot d^{-1}$, the required cobalt amounted to 0.36 $\mu moles \cdot L_{reactor}^{-1} \cdot d^{-1}$ of $CoCl_2$. In case of Co-$EDTA^{2-}$ pulse, considering a stable methanol removal period of 7 days upon the pulse, the required cobalt amounted to 0.77 $\mu moles \cdot L_{reactor}^{-1} \cdot d^{-1}$ of $CoCl_2$. In a methanol fed UASB reactor with an OLR of 2.6 g $COD \cdot L^{-1}_{reactor} \cdot d^{-1}$, Zandvoort et al. (2002) estimated that 0.15 $\mu moles \cdot L_{reactor}^{-1} \cdot d^{-1}$ would be required in order to maintain a stable methanol removal capacity when $CoCl_2$ was supplied continuously.

A much more accurate quantification of the required amount of cobalt to be dosed can be obtained when a mechanistic model for the $CoCl_2$ dosing to methanol fed UASB reactors is developed. The mechanistic modelling of the cobalt uptake rate is, however, rather complicated because of the complexity of the system under study, where the relation between chemical speciation, biological uptake, biomass growth, hydrodynamic of the reactor and biofilm characteristics has to be taken into account (Jansen, 2004). Some of these aspects are described in literature for other biofilm systems than granular sludge (Flemming, 1995; Arican et al., 2002; Flynn, 2003; Wang et al., 2003; Weng et al., 2003; Worms et al., 2006), but the aspects of greatest importance, i.e. chemical interactions between cobalt and the granular sludge matrix, as well as cobalt uptake and biomass growth kinetics are not yet quantified (Van Hullebusch et al., 2003).

8.4.4. Cobalt dosing strategies

Several strategies have been adopted to dose $CoCl_2$ to methylotrophic methanogenic UASB reactors (Table 8.3). In batch methanol degradation tests, supplying cobalt continuously was shown to be more effective in overcoming cobalt limitation than dosing the total amount of cobalt at once to the batch medium at the beginning of the test (Gonzalez-Gil et al., 1999). In continuous reactor operation, continuous addition of a low $CoCl_2$ concentration (0.62 µmoles per litre of reactor volume and per day) to a methanol fed UASB reactor only enhanced the SMA of the sludge up to 215 mg $COD\text{-}CH_4 \cdot g\ VSS^{-1} \cdot d^{-1}$ (Zandvoort et al., 2002). The same author studied the possibility of pre-loading sludge by pre-incubating the inoculum in a 1 mM $CoCl_2$ solution for 24 hours. Pre-loading clearly overcame cobalt limitation in the methanol fed UASB reactor, but high amounts of cobalt washed-out with the reactor effluent (Zandvoort et al., 2004). Pulse dosing of $CoCl_2$, comprising an intermediate dosing strategy between pre-loading and continuous dosing, is effective in overcoming almost acute cobalt limitations in reactor operation (Zandvoort et al., 2004). The pulse of high amounts of cobalt (77.5 µmoles of cobalt per litre of reactor volume) overcame cobalt limitation (Zandvoort et al., 2004). However, cobalt losses from the sludge with this dosing procedure were considerably higher compared to the continuous dosing strategy: 3.5 $\mu g \cdot g\ TSS^{-1} \cdot d^{-1}$ and 0.1 $\mu g \cdot g\ TSS^{-1} \cdot d^{-1}$, respectively (Table

8.3). In the present study, repeated pulse doses of low quantities of $CoCl_2$ (5 µmoles per litre of reactor volume) overcame the metal limitation, without the need for continuous cobalt addition (Zandvoort et al., 2002).

This study shows that the pulse addition of $CoCl_2$ presents several advantages compared to Co-EDTA^{2-}. First of all, as cobalt added as chloride is much more retained in the granular sludge compared to cobalt bound to EDTA, its addition frequency would be much lower, i.e. once per 15 days for $CoCl_2$ versus once per 7 days for Co-EDTA^{2-}. The side-effects that EDTA causes to the granular sludge matrix and to the microbial cells make EDTA an unreliable ligand to use in real applications for trace metal supplementation. Moreover, cobalt chloride hexahydrated is cheaper than cobalt bound to EDTA: 345 €·kg^{-1} and 450 €· kg^{-1}, respectively (Merck, 2005).

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COBALT DOSING TO ANAEROBIC GRANULAR SLUDGE BIOREACTORS: QUANTIFICATION OF METAL RETENTION TO PREDICT METHANOGENIC ACTIVITY

ABSTRACT

Cobalt dosing to methanol fed upflow anaerobic sludge bed (UASB) reactors has been shown previously to be an excellent model system to study trace metal deficiencies of methanogenic bioconversions. This study quantified the correlation between methanogenic activity and metal retention in anaerobic granular sludge bioreactors. The metal retention upon cobalt pulse dosing showed a significant correlation ($p \leq 0.001$) with the bioavailability of the retained cobalt in the subsequent 16 and 35 days. This correlation can be used to minimize the amount of cobalt supplied as a trace metal, and at the same time maximize the organic removal efficiency of methanol fed UASB reactors. Moreover, the correlation between metal retention and metal bioavailability is proposed to be a tool to implement metal dosing strategies for other biofilm based bioprocesses.

9.1. INTRODUCTION

The solid and liquid retention times in high-rate anaerobic wastewater treatment systems, like the upflow anaerobic and expanded granular sludge-bed bioreactors (UASB and EGSB, respectively), are uncoupled (Gonzalez-Gil et al., 2002). This is accomplished by self-immobilization of the methanogenic consortia in the form of granules. These granules consist of methanogenic consortia, contain different bacterial species and typically contain millions of organisms per gram of biomass. The extracellular polysaccharides (EPS) produced by these organisms play an important role in maintaining the structural integrity of granules as EPS form a strong and sticky non deformable polymeric gel-like matrix (Liu and Tay, 2004). The granules have a high porosity and a large surface, are onto which metals can adsorb more to specific sites (Liu et al., 2003). The effect of interactions between metals and the sludge matrix on metal bioavailablity and transport has thus far hardly been investigated.

Cobalt plays a key role in anaerobic methanol degradation, as it regulates the methanol degradation pathway by affecting the activity of acetogens and methanogens involved in methanogenic methanol conversion (Florencio et al., 1993; Zandvoort et al., 2002; Chapter 3). Cobalt dosing to methanol fed UASB reactors was previously shown to be an excellent model system to study trace metal deficiencies (Chapter 3). This study aims to correlate the cobalt retention upon a pulse dosing of cobalt to the influent of a UASB reactor with the reactor performance in the time period following the trace metal pulse dosing. The amount of retained metal within the granule was varied by dosing different chemical species of cobalt. This correlation will facilitate the metal addition strategy to anaerobic granular sludge bioreactors in practice as it can predict the time when a new metal pulse dosing needs to be done.

9.2. MATERIALS AND METHODS

9.2.1. Source of biomass

The UASB reactors were inoculated with anaerobic granular sludge originating from a full-scale UASB reactor treating alcohol distillery wastewater of Nedalco (Bergen op Zoom, the Netherlands). The total suspended solids (TSS) and volatile suspended solids (VSS) content of the wet sludge amounted to 7.2 (± 0.1) % and 6.7 (± 0.1) %, respectively.

9.2.2. Influent composition

The reactors were fed a basal medium consisting of methanol as the substrate, macronutrients and a trace metal solution dissolved in demineralised water. The inorganic macronutrients contained (in milligrams per litre of basal medium): NH_4Cl (280), K_2HPO_4 (250), $MgSO_4 \cdot 7H_2O$ (100) and $CaCl_2 \cdot 2H_2O$ (10). The trace metal contained (in micromoles per litre of basal medium): $FeCl_2 \cdot 4H_2O$ (5), $CuCl_2 \cdot 2H_2O$ (0.5), $ZnCl_2$ (0.5), $MnCl_2 \cdot 4H_2O$ (0.5), $NiCl_2 \cdot 6H_2O$ (0.5), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (0.07), $Na_2SeO_2 \cdot 5H_2O$ (0.5) and $Na_2WO_4 \cdot 2H_2O$ (0.5).

To ensure pH stability around 7.0, 2.52 g (30 mM) of $NaHCO_3$ was added per litre of basal medium. To avoid precipitation in the storage vessels, the influent was composed of 3 streams: (1) macronutrients and trace metal solution without K_2HPO_4 and $NaHCO_3$, (2) methanol with $NaHCO_3$ and K_2HPO_4 and (3) dilution water. Demineralised water was used to prepare the influent and as dilution water.

9.2.3. Experimental design

Ten mesophilic (30°C) UASB reactors were run at organic loading rates (OLR) between 8.5 g $COD \cdot L^{-1} \cdot day^{-1}$ and 12 g $COD \cdot L^{-1} \cdot day^{-1}$ (Table 9.1) to which cobalt was supplied as pulses. The pH of the reactors was 7.0, unless specified otherwise. A hydraulic retention time (HRT) of 8 hours was applied in all UASB reactors. They had the same recirculation flow rate of 3.9 litre per hour (superficial liquid upflow velocity of 0.5 $m \cdot h^{-1}$), around 10 times higher than the influent flow rate.

Each cobalt pulse addition consisted of injecting 17.5 µmoles of cobalt from a concentrated cobalt solution (1 mM) to the reactor influent, corresponding to 5 µmoles cobalt per litre of reactor volume. This value was previously shown to support stable methylotrophic methanogenesis in UASB bioreactors inoculated with the same inoculum sludge as used in this study (Zandvoort et al., 2003; Chapter 3).

Two UASB reactors received a single cobalt pulse dose in the cobalt chloride form (chloride_1 and chloride_2) and two other UASB reactors (citrate_1 and citrate_2) were supplied once with cobalt bound to citrate. Cobalt bound to EDTA was pulse dosed to two UASB reactors: once to one UASB reactor (EDTA_1) and three times to another UASB reactor (EDTA_2, EDTA_3 and EDTA_4). Cobalt was also dosed as vitamin B_{12}, a complex organic macromolecule with cobalt ion as catalytic center (Stupperich et al., 1990). It was supplied once to two UASB reactors, operating at pH 7 (vitamin B_{12}_1) and pH 5.8 (vitamin B_{12}_2).

The Co-$Citrate^-$ solution was prepared by mixing respective amounts of $CoCl_2$ and $C_6H_5O_7Na_3 \cdot 2H_2O$ to a molar ratio of 1:1.5 for cobalt and citrate, respectively. The Co-$EDTA^{2-}$ solution was prepared by mixing respective amounts of $CoCl_2$ and Na_2H_2EDTA to a molar ratio of 1:1.5 for cobalt and EDTA, respectively.

9.2.4. Hydrodynamic characteristics

The hydrodynamic characteristics of the UASB reactors were determined by a tracer experiment with fluoresceine as the tracer. The type of flow with the tracer measurements was characterized by the morril dispersion index (MDI), according to Metcalf and Eddy (Metcalf and Eddy, 2003).

$$MDI = \frac{P_{90}}{P_{10}} \quad (9.1)$$

Where: P_{90}: 90 percentile value, P_{10}:10 percentile value.

A MDI value of 1 indicates that the reactor behaves as an ideal plug flow reactor, whereas a MDI value of 22 indicates that the reactor behaves as an ideal completely stirred tank reactor (CSTR). The UASB reactors in this study had a MDI of 19.3, indicating a CSTR behaviour of the liquid phase.

Table 9.1. Characteristics of the UASB reactor performance prior to the cobalt pulse dosing.

Ligand	OLR ($g\ COD \cdot L^{-1} \cdot d^{-1}$)	MeOH accumulated ($mg\ COD \cdot L^{-1}$)	VFA accumulated ($mg\ COD \cdot L^{-1}$)	Co before pulse ($\mu g \cdot gTSS^{-1}$)	SMA before pulse ($mg\ COD\text{-}CH_4 \cdot g\ VSS^{-1} \cdot L^{-1}$)
Chloride_1	8.5	3	100	17.1	800
Chloride_2	8.5	734	250	9.3	711
Citrate_1	10	2	285	21.8	750
Citrate_2	8.5	3	35	18.6	764
EDTA_1	12	1	5	25.8	882
EDTA_2	8.5	275	120	11.1	717
EDTA_3	8.5	2000	87	9.1	391
EDTA_4	8.5	700	71	9.2	356
Vitamin B_{12}_1	10	195	35	22.9	600
Vitamin B_{12}_2	12	705	2082	23.0	0

Mass balance for a CSTR including a term for metal retention in the sludge

There are several mechanisms involved in the immobilisation of metals in the sludge, such as ion exchange, chelation, adsorption, reaction and precipitation (van Hullebusch et al., 2003). In order to include this retention phenomenon in a CSTR mass balance, a retention constant (K_r) is introduced as a sum parameter of all metal retention mechanisms. The metal retention was assumed to be a first order mechanism in order to simplify the calculation (Eq. 9.2):

$$\frac{dC}{dt}V = QC_o - QC + K_r C \quad (9.2)$$

Where: C: cobalt concentration in the effluent at time t, C_0: cobalt concentration in the effluent at time 0, K_r: retention constant, Q: influent flow and V: reactor volume.

After the integration between the limits $C = C_0$ to C = C, and $t_0 = 0$ to t = t, the resulting expression is:

$$LnC = LnC_0 - (\frac{Q + K_r}{V})t \quad (9.3)$$

C_0 and K_r can be obtained easily from Eq. 9.3 when representing Ln C versus time. The profile of cobalt wash out from the reactor can be represented by a set of data (C, t) for every pulse addition of cobalt by Eq. 9.4 (Fig. 9.1):

$$C = C_0 e^{-(\frac{Q+K_r}{V}).t} \quad (9.4)$$

9.2.5. Calculation of the amount of retained cobalt upon a cobalt pulse

The total amount of retained cobalt after the pulse addition to a reactor can be measured as the difference between the total amount of cobalt added and the amount of cobalt washed out during 24 hours after the cobalt pulse. The total amount of retained cobalt can be separated in two retained cobalt fractions (A0 + B0). The first fraction (A0) is the amount of cobalt retained during the 24 hours upon a pulse, assuming that the amount of dosed cobalt is equal to C_0 and the reactor behaves as a perfect CSTR. A0 is calculated by the area between the model of washed out cobalt defined by Eq. 9.4 and the model of washed out cobalt without retention term (K_r) (Fig. 9.1). The second fraction (B0) is the amount of cobalt that is retained instantaneously upon a pulse. B0 is calculated by the difference between the total retained cobalt in the reactor and A0. An interaction term between A0 and B0, A0·B0, was defined as well and calculated as well for every pulse. The term A0·B0 is a measure for possible interactions between the two fractions A0 and B0 (Schabenberger and Pierce, 2002).

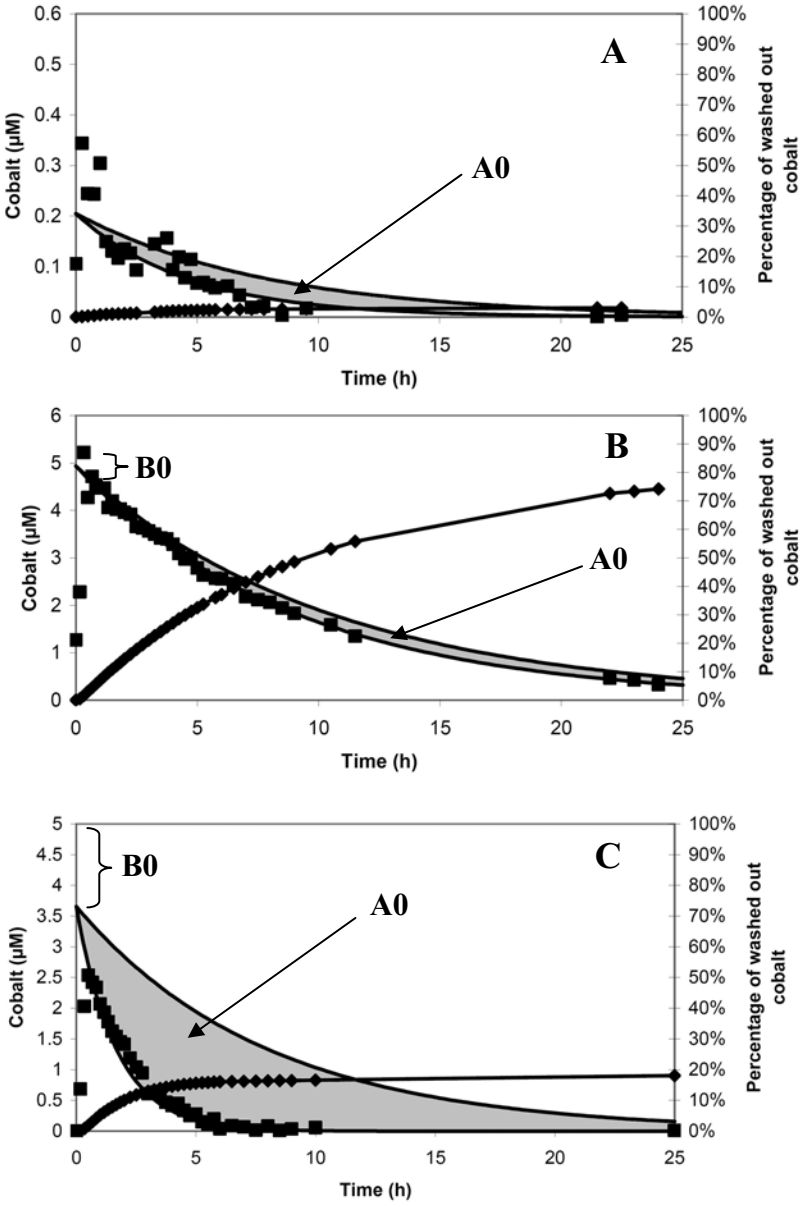

Figure 9.1. Wash out and retention of cobalt upon the cobalt pulse dosing. A0 is the amount of cobalt retained during the 24 hours upon a pulse. B0 is the amount of cobalt that is retained instantaneously upon a pulse. (A) Cobalt chloride pulse. (B) Cobalt bound to EDTA pulse. (C) Cobalt bound to vitamin B_{12} pulse.

9.2.6. Quantification of reactor performance

Long term metal deprivation results in acidification of methanol fed UASB reactors. This proceeds via a first phase of methanol accumulation due to the diminishing SMA on methanol of the sludge because of lack of cobalt for the methanogens. The restoration of the reactor performance upon a cobalt pulse was quantified by determining the evolution of the SMA of the sludge with methanol as the substrate in the absence of cobalt over time (Fig. 9.2). Three parameters were deducted from the SMA profile: (1) SMA_{max}, that is the maximum SMA value recorded following the cobalt pulse addition, it is usually observed 2-3 days after the cobalt pulse, (2) Area16, the area below the SMA-time profile 16 days after the pulse was done and (3) Area35, the area below the SMA-time profile 35 days after the pulse (Fig. 9.2).

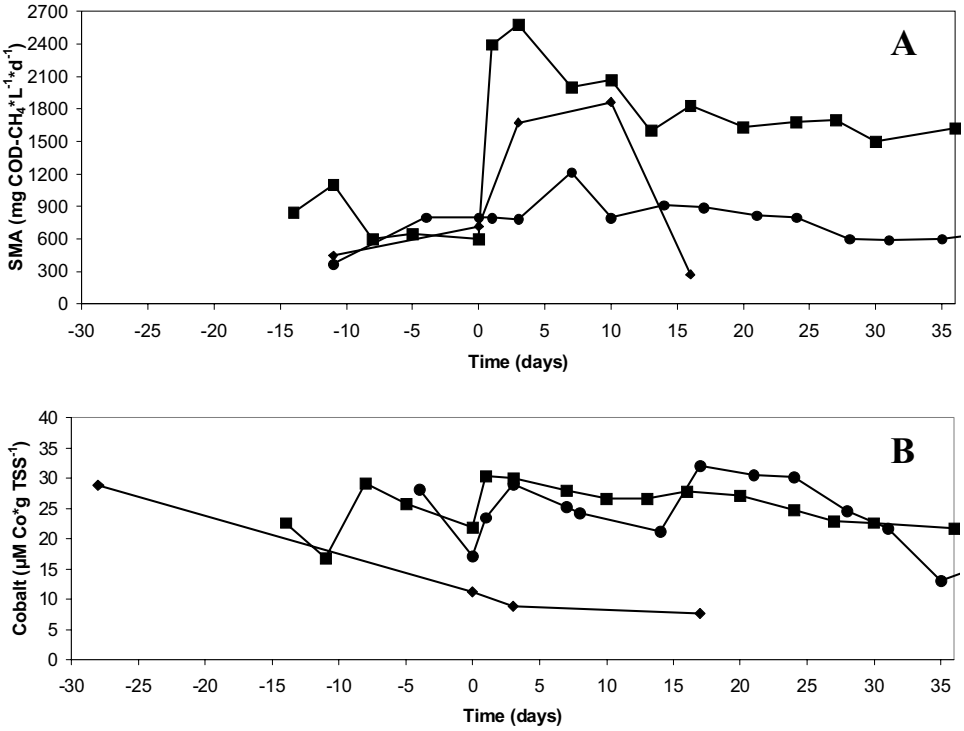

Figure 9.2. Evolution of (A) specific methanogenic activity with methanol as the substrate of the sludge and (B) total cobalt content of the sludge during time. (●) Reactor supplied with cobalt chloride pulse. (♦) Reactor supplied with cobalt bound to EDTA pulse. (■) Reactor supplied with vitamin B_{12}.

9.2.7. Specific maximum methanogenic activity tests

The SMA of the sludge was determined in duplicate at 30 (±2) ◦C using on-line gas production measurements as described by (Zandvoort et al., 2002). The SMA tests were

supplemented with methanol (4 g COD·L^{-1}) as the substrate and in the absence of cobalt. The granular sludge samples were always taken from the same place (the mid-height) in the sludge bed.

9.2.8. Metal uptake kinetics

The effect of the cobalt complexes under study (cobalt bound to EDTA, cobalt bound to citrate, $CoCl_2$ and vitamin B_{12}) on the apparent affinity constant (K_M) and maximum Specific Methanogenic Activity (V_{max}) of cobalt-deprived granular sludge with methanol as the substrate was determined. To calculate the K_M and V_{max} values, a Monod – type equation (Eq. 9.5) was modelled to the SMA values obtained for different cobalt concentrations ranging from 0 µM to 10 µM (0, 0.01, 0.1, 0.5, 1, 10 µM).

$$SMA = (V_{\max} - V_0)\frac{[Co]}{K_M + [Co]} + V_0 \qquad (9.5)$$

Where: V_{max}: Maximum Specific Methanogenic Activity, V_0: Specific Methanogenic Activity without cobalt in the medium, K_M: apparent affinity constant.

9.2.9. Metal composition of the sludge

The metal composition of the sludge was determined by ICP-OES (Varian, Australia) after destruction with Aqua regia (mixture of 2.5 ml 65% HNO_3 and 7.5 ml 37% HCl) as described in Chapter 4.

9.2.10. Chemical analyses

Methanol and VFA were determined using gas liquid chromatography as described by Weijma et al. (2000). Total dissolved metal concentration in the influent and effluent were determined by ICP-OES (Varian, Australia) in samples acidified with 0.1 M HNO_3. The samples were centrifuged at 10000 rpm to remove particles from the liquid. The total suspended solids (TSS) and volatile suspended solids (VSS) concentrations were determined according to Standard Methods (APHA/AWWA, 1998). All chemicals were of analytical or biological grade and purchased from E. Merck AG (Darmstadt, Germany).

9.2.11. Correlation Analysis

Statistical data analysis was performed with the statistical software package SPSS for Windows (Rel. 12.0.1. 2003; SPSS Inc., Chicago, IL, USA). The Pearson correlation coefficient (R), that is a measure of linear association of two variables, and its statistical significance (p) was used for the correlation analysis.

9.3. RESULTS

9.3.1. Ligand characteristics

The four selected ligands yield cobalt complexes with completely different properties (Table 9.2). The molecular weight ranges from 94 $g \cdot mol^{-1}$ for $CoCl^+$ up to 1355 $g \cdot mol^{-1}$ for vitamin B_{12}. The log K ranges from -0.35 for $CoCl^+$ up to 27.4 for vitamin B_{12}. The different properties result in different kinetic parameters V_{max} and K_M of the cobalt deprived inoculum sludge (Table 9.2). Especially the K_M value of the cobalt deprived sludge is different: the K_M with vitamin B_{12} and $CoEDTA^{2-}$ is 0.16 µM, whereas it is higher with $CoCl^-$ and $Co\text{-}Citrate^-$: 0.62 µM and 0.82 µM, respectively. The same group association can be made with the values of Log K. Cobalt complexes with a high log K also show a low K_M. This indicates that the stronger the cobalt complex is, the more effective is the cobalt utilization by the microorganisms present in the anaerobic granular sludge.

Table 9.2. Characteristics of the cobalt complexes.

Ligand	Compound	Molecular weight ($g \cdot mol^{-1}$)	K_M[a] (µM)	V_{max} (mg COD-$CH_4 \cdot g$ $VSS^{-1} \cdot d^{-1}$)	Log K $CoL^{2-z} \leftrightarrow Co^{2+} + L^{z-}$	Log K in sea water
Chloride	$CoCl^+$	94.38	0.62	1177	-0.35	----
Citrate	$Co\text{-}Citrate^-$	248.03	0.82	1355	6.19	----
EDTA	$Co\text{-}EDTA^{2-}$	347.14	0.16	1438	18.16	7.6
Vitamin B_{12}	Vitamin B_{12}	1355.40	0.17	1653	27.4[b]	16.4

a: Calculated based in cobalt addition.
b: Calculated by assuming that the ratio K_{CoEDTA} / K_{vitB12} is the same than $K_{CoEDTA}^{sea\ water} / K_{vitB12}^{sea\ water}$.

9.3.2. Cobalt retention

The cobalt retention upon pulse dosing was strongly affected by the type of cobalt complex supplied. Cobalt bound to EDTA was hardly retained in the granular sludge (Fig. 9.3) compared to the high cobalt retention for cobalt chloride, vitamin B_{12} and cobalt bound to citrate (Fig. 9.3). Cobalt bound to chloride and citrate was mainly retained in the B0 fraction. Cobalt incorporated in vitamin B_{12} was equally retained in the A0 and B0 fraction, whereas cobalt bound to EDTA was mainly retained in the A0 fraction (Fig. 9.3).

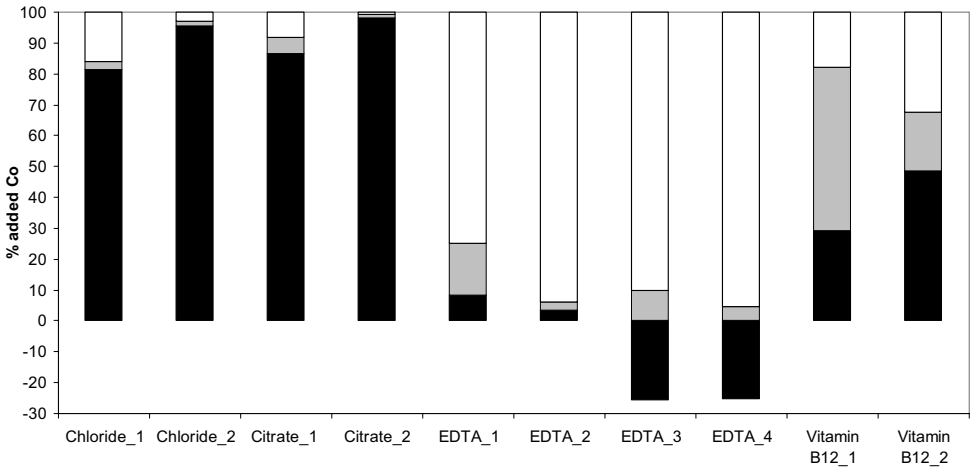

Figure 9.3. Fractions of added cobalt: □ not retained cobalt, ■ A0 and ■ B0.

9.3.3. Reactor operation200

The sludge from the UASB reactor supplied with vitamin B_{12} at pH 7.0 (vitamin B_{12}_1) showed the highest values for SMA_{max}, Area16 and Area35, whereas sludge from the Co-$EDTA^{2-}$ pulsed reactor (EDTA_4) showed the lowest values for SMA_{max}, Area16 and Area35 (Table 9.3). The different reactor conditions at the moment of the pulse influenced the cobalt retention (Table 9.1).

The methanol effluent concentration prior to cobalt dosing to the reactor was below 5 mg MeOH-COD·L^{-1} in four reactors (chloride_1, citrate_1, citrate_2, and EDTA_1). The SMA of the sludge of these four reactors prior to cobalt dosing to the reactor exceeded 750 mg CH_4-COD·g VSS^{-1}·d^{-1} (Table 9.1). In three other reactors (vitamin B_{12}_1, chloride_2 and EDTA_2), methanol had accumulated between 195 and 734 mg MeOH-COD·L^{-1}. The SMA of these sludges, just before cobalt dosing to the reactors, was slightly lower (between 600 and 717 mg CH_4-COD·g VSS^{-1}·d^{-1}).

The SMA of the sludge, just before the cobalt dosing, of the pulses to EDTA_3 and EDTA_4 was much lower, 391 and 356 mg CH_4-COD·g VSS^{-1}·d^{-1}, respectively. Methanol had accumulated in the EDTA_3 reactor to a much higher methanol concentration (2000 mg MeOH-COD·L^{-1}) compared to the EDTA_4 reactor (700 mg MeOH-COD·L^{-1}). In the vitamin B_{12}_2 reactor, 2085 mg VFA-COD·L^{-1} had accumulated in the effluent prior to the pulse and the pH had dropped to 5.3. In the other reactor runs, no VFA concentrations higher than 300 mg VFA-COD·L^{-1} were observed.

The vitamin B_{12}_2 reactor was not included in the calculations after the pulse due to the complete acidification of the reactor (pH dropped to 5.3) and the absence methanogenic activity of this sludge on methanol prior to the pulse.

Table 9.3. Cobalt retention and reactor performance after cobalt pulse dosing.

Ligand	Total cobalt retention (µM)	B0 (µM)	A0 (µM)	A0-B0	SMA_{max} (mg COD-CH_4·g VSS^{-1}·L^{-1})	Area16	Area35	Predicted SMA (mg COD-CH_4·g VSS^{-1}·L^{-1})	Predicted Area16	Predicted Area35
Chloride_1	14.70	14.21	0.49	6.94	1936	12963	15788	1537	13976	23211
Chloride_2	16.97	16.73	0.24	4.05	1215	9809	17823	1448	12896	21148
Citrate_1	16.07	15.17	0.90	13.51	1907	20660	29555	1738	16420	27883
Citrate_2	17.35	17.19	0.16	2.67	1329	11965	24727	1406	12385	20170
EDTA_1	4.37	1.49	2.88	4.30	1140	10294	21310	1456	12994	21334
EDTA_2	1.05	0.63	0.42	0.26	1862	17653	17653	1332	11491	18461
EDTA_3	1.75	-4.45	6.20	-27.58	433	1564	2948	479	1125	-1350
EDTA_4	0.79	-4.38	5.17	-2.68	400	712	712	629	2949	2135
Vitamin B_{12}_1	14.35	5.11	9.24	47.20	2576	27581	54331	2770	28964	51857
Vitamin B_{12}_2	11.9	8.5	3.36	28.56	-----	-----	-----	-----	-----	-----

9.3.4. Correlation analysis

The Pearson correlation coefficient R for the SMA before the pulse and the total cobalt content present in the sludge before the pulse amounted to 0.629 (n: 9). Determination of the total metal content of a sludge is thus insufficient to predict the SMA with methanol as the substrate before the pulse.

The Pearson correlation coefficient was calculated between the three parameters describing the SMA-Time curve (SMA_{max}, Area16 and Area35) and the parameters describing the metal retention (Total cobalt retention, A0, B0 and A0·B0), reactor performance (methanol and VFA accumulation), the sludge (cobalt content of the sludge and SMA on methanol before the pulse) and ligand (K_M, V_{max}, Log K and molecular weight of the different cobalt complexes) characteristics (Table 9.4).

Table 9.4. Pearson correlation coefficient (R) and p value (two tailed) (n: 9) for every measured parameter of each reactor run versus the three chosen parameters to describe the SMA-Time curve (SMA_{max}, Area16 and Area35).

	SMA_{max}	Area16	Area35
Total cobalt retention	R: 0.552	R: 0.499	R: 0.559
(A0 + B0)	p: 0.123	p: 0.172	p: 0.118
B0	R: 0.464	R: 0.400	R: 0.381
	p: 0.208	p: 0.286	p: 0.312
A0	R: -0.040	R: 0.049	R: 0.259
	p: 0.919	p: 0.900	p: 0.501
A0·B0	**R: 0.910**	**R: 0.929**	**R: 0.970**
	p: 0.001	**p: 0.000**	**p: 0.000**
MeOH accumulated	R: -0.659	R: -0.621	R: -0.548
	p: 0.054	p: 0.0.74	p: 0.127
VFA accumulated	R: 0.121	R: 0.143	R: -0.044
	p: 0.756	p: 0.714	p: 0.911
Co before pulse	R: 0.560	R: 0.611	R: 0.707
	p: 0.117	p: 0.080	p: 0.033
SMA before pulse	R: 0.540	R: 0.484	R: 0.403
	p: 0.133	p: 0.186	p: 0.282
K_M	R: 0.235	R: 0.191	R: 0.148
	p: 0.542	p: 0.622	p: 0.705
V_{max}	R: 0.160	R: 0.321	R: 0.440
	p: 0.681	p: 0.400	p: 0.235
Log K	R: 0.191	R: 0.314	R: 0.439
$CoL^{2-z} \leftrightarrow Co^{2+} + L^{z-}$	p: 0.623	p: 0.411	p: 0.238
Molecular Weight	R: 0.477	R: 0.574	R: 0.725
	p: 0.194	p: 0.106	p: 0.027

There is a significant positive correlation ($R \geq 0.910$, $p \leq 0.001$, n: 9) between A0·B0 and the three parameters used to describe the SMA profile curve (Table 9.4). These correlations allow to predict values for the SMA_{max}, area16 and area35 using the A0·B0 value only. The linear correlation was calculated for every pair (A0·B0-SMA_{max}, A0·B0-Area16 and A0·B0-Area35) (Fig. 9.4). Table 9.3 shows the predicted values for SMA_{max}, area16 and area35. As expected for the significant positive correlation, there are little discrepancies between the predicted and measured values. The A0·B0 value is thus an accurate parameter to predict the reactor performance after the cobalt pulse.

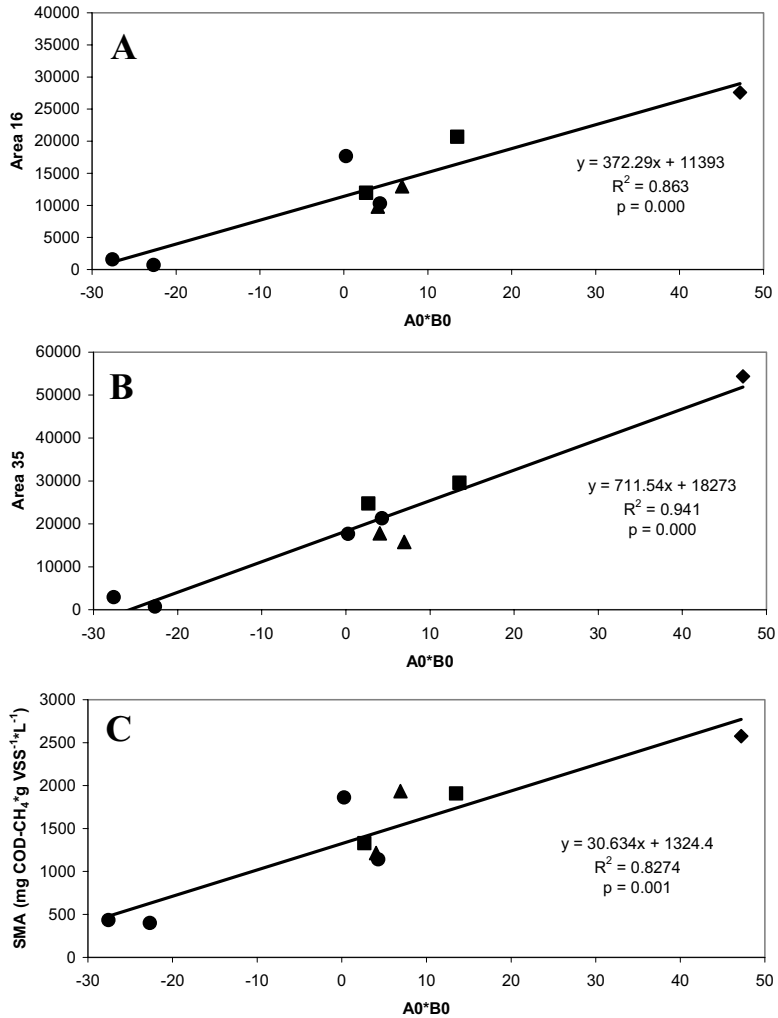

Figure 9.4. Linear correlation between: (A) A0·B0-Area16, (B) A0·B0-Area35 and (C) A0·B0-SMA_{max}. (▲) Cobalt chloride pulse. (■) Cobalt citrate pulse. (●) Cobalt bound to EDTA pulse. (♦) Cobalt bound to vitamin B_{12} pulse.

For all other parameters, the R values were below 0.8 and p values exceeded 0.01 (Table 9.4). This indicates that there is no significant correlation between these parameters and the parameters describing the SMA profile curve.

9.4. DISCUSSION

9.4.1. Correlation to assess reactor performance

This study shows how the retention of cobalt in the sludge during 24 hours following a cobalt pulse correlates with the decline of the SMA of the sludge present in methanol fed UASB reactors during the subsequent 16 and 35 days (Table 9.4; Fig. 9.4). This correlation shows that the quantification of the reactor performance upon a pulse not solely depends on the type of ligand used but also on other conditions (Table 9.3; Fig. 9.4), e.g. the methanol or VFA concentration in the reactor mixed liquor or the cobalt concentration in the sludge prior to the pulse (Table 9.1). Correlation studies are important in assessing metal bioavailability due to the wide range of factors that affect exposure, retention, bioavailability, and, ultimately, UASB operation (Aquino and Stuckey, 2007). It should be noted, however, that correlation does not imply a causal relationship. Thus, the retention-SMA correlation should not be overstretched, but it is the basis for further research on mechanisms of metal bioavailability (Davies et al., 1998; Mowat and Bundy, 2001).

The metal retention was evaluated by two retention fractions (A0 and B0) (Fig. 9.5). The B0 fraction contains the instantaneously retained metal during the first minutes following the cobalt pulse addition. The A0 fraction includes the retained metal in a later phase, when the cobalt concentration in the reactor effluent decreases up to the same level than before the pulse addition (Fig. 9.1). Therefore, A0 is related to slow retention processes whereas B0 to fast retention processes. It was shown that not only the single fractions (A0 or B0) correlate with the reactor performance. A significant correlation was found between their product (A0·B0), which indicates an interaction between the two fractions A0 and B0 (Fig. 9.5) and the reactor performance (Table 9.4). Thus, both of the above defined retention fractions influence the subsequent SMA of the sludge and thus, indirectly, the reactor performance. It was also shown that the single sum of these two fractions (A0 + B0) can not be correlated to the SMA after the cobalt pulse addition (Table 9.4). This justifies the approach to distinguish A0 and B0 as two separate fractions.

9.4.2. Fast cobalt retention (B0)

During the first few minutes upon the pulse, when the B0 fraction is retained, several retention mechanisms take place, such as precipitation and sorption (Fig. 9.5). Precipitates are formed via nucleation followed by growth (Manna et al., 2001). Nucleation plays an important role as the first step of precipitation (Manna et al., 2001). There are only few studies on

nucleation up to now since the nuclei are usually unstable because of their high surface energy and fast grow after their formation (Jiang et al., 2005; Ball et al., 2006). Al-Tarazi et al. (2004) modeled the crystallization kinetics of zinc sulfide and copper sulfide as model metals, in which a mass nucleation rate was calculated in order to include nucleation process into the crystallization kinetics. Using the equations to calculate the mass nucleation rate described by Al-Tarazi et al. (2004), a mass nucleation rate of 10^4 to 10^3 mol cobalt per liter of reactor volume and per second was calculated for a solution with 0.1 µmol cobalt per liter of reactor volume (minimum dissolved cobalt concentration observed in reactor effluent immediately after the cobalt pulses in all cases). A few picoseconds would be enough to precipitate all the cobalt present (Al-Tarazi et al., 2004). In reality, a time period of several minutes will be needed for the mass transfer of cobalt from the concentrated pulsed volume to the bioreactor medium (Omil et al., 1996).

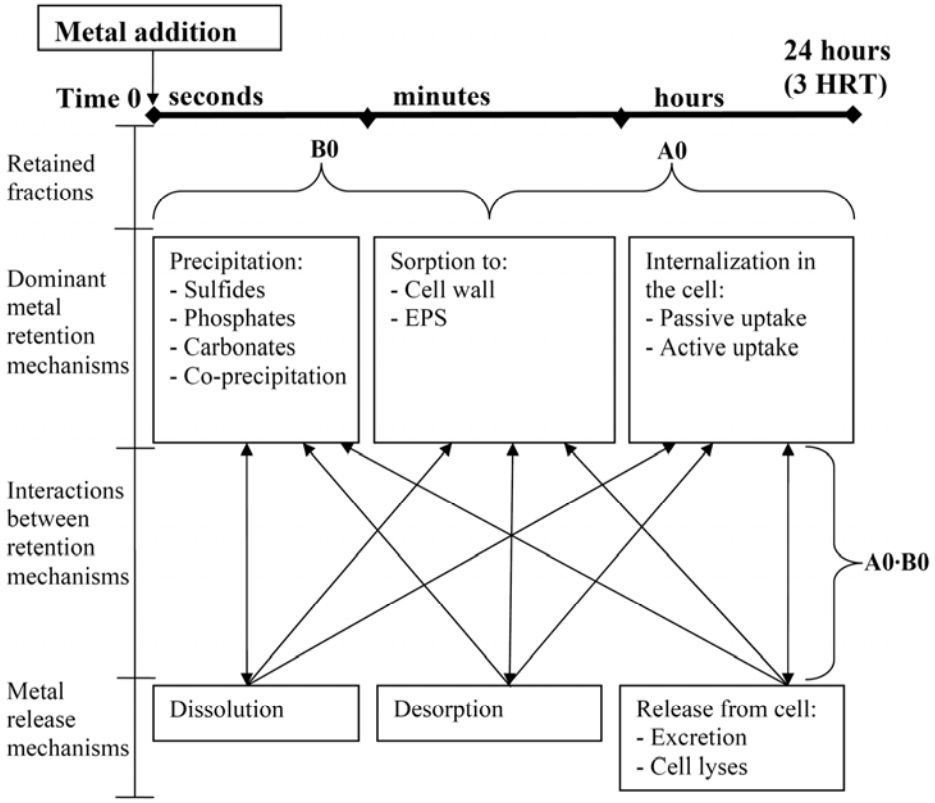

Figure. 9.5. Metal dynamics upon pulse dosing of Co^{2+} in an UASB reactor.

Sorption processes between the biofilm and the cobalt present in the liquid phase, such as physical sorption or ion exchange, may also be of significance for the retention of the B0 fraction. Extracellular polymeric substances (EPS) can serve as sorption sites in biofilms (Ozdemir et al., 2005). EPS typically contain a large number of anionic functional groups and may have a significant metal sorption capacity (van Hullebusch et al., 2003). Sorption of cadmium and cobalt ions in EPS from *Chryseomonas luteola* TEM05 reached the sorption equilibrium within 60 min (Ozdemir et al., 2005). Ion exchange on EPS plays an important role in metal sorption (Guibaud et al., 2008): calcium is the most abundant metal ion available in the EPS matrix (Wloka et al., 2004), thus the main cation from the EPS matrix to exchange with the divalent Co^{2+} metal ions present in the liquid phase. Cobalt retention in the EPS through ion exchange is unlikely due to the much higher dissolved calcium concentration in the system described in the present study (0.6 - 0.5 mM) compared with the dissolved cobalt concentration (maximal 5 μM) present in the liquid phase. Metals also accumulate in the biofilm by binding to negatively charged functional groups within the cells walls (Macfie and Welbourn, 2000). In a study using unicellular green algae, cobalt sorption kinetics in the cell wall of the algae showed that the sorbed cobalt was constant from 12 hours up to 24 hours after the addition (Macfie and Welbourn, 2000). Unfortunately, this study does not report when exactly the sorption equilibrium between the sorbed cobalt in the cell wall and the dissolved cobalt was achieved. In a study of copper biosorption in *Pseudomonas aeruginosa* the sorption equilibrium was reached in maximally 10 minutes (Li et al., 2007). Therefore, the B0 fraction most likely constitutes of cobalt precipitates and cobalt sorption on the cell wall and in the EPS.

Deeper insight of the B0 fraction in the anaerobic granular sludge could be achieved by further characterization of the solid phase speciation of the cobalt sinks, e.g. by sulfur or metal X-ray Absorption Near Edge Structure (XANES) spectroscopy which offers a unique non-destructive tool to identify, and to determine (with appropriate data analysis) *in situ* the relative amounts of functional sulfur or metal species down to low concentrations, about 0.05 mass % sulfur (Jalilehvand, 2006).

9.4.3. Slow cobalt retention (A0)

During the several hours upon the cobalt pulse, when the A0 fraction is retained, slower retention mechanisms take place, such as internalization into the cell (Foulkes, 2000) (Fig. 9.5). Microorganisms normally employ two types of metal internalization mechanisms. One is fast, unspecific and driven by chemiosmotic gradients, whereas the other has a high substrate specificity, is slower and inducible, often use adenosine triphosphate (ATP) hydrolysis as the energy source and is only used by the cell in times of starvation (Aquino and Stuckey, 2007). Few values of the cobalt uptake rate by biomass are reported in the literature. Jansen et al. (2007) calculated a cobalt biouptake rate for a solution of 0.6 g *Methanosarcina bakeri* per litre

(dry weight) of 10^{-13} to 10^{-12} mol cobalt per liter and per second. Assuming that all the biomass in the presently studied reactor has the same cobalt biouptake rate as reported by Jansen et al. (2007), the maximum cobalt biouptake rate amounts to 10^{-12} to 10^{-11} mol cobalt per liter of reactor volume and per second in the reactor systems. Thus, between 6 and 60 hours will be required to take up 0.1 μmol cobalt per liter of reactor volume. Therefore, cobalt internalization into the cell is most likely involved in the A0 fraction. To better characterize the A0 fraction further, determination of the cobalt uptake by the cell should be quantified, e.g. by mapping radioactive metal isotopes previously supplied to the sludge (Lombi et al., 2001).

The mass transfer of the metal inside the granule plays an important role in the metal dynamics and thus the size of A0 and B0 fractions. Gonzalez-Gil et al. (2001) suggest that external mass transfer limitation in UASB granules is negligible compared to the internal mass transfer limitation. The internal mass transfer limitation could be a strong limiting step in the metal internalization, first into the granule and subsequently into the cell. The use of microelectrode arrays could provide metal concentration profiles through the granule (De Marco et al., 2007) that can further characterize and quantify the mass transfer into the granule. Besides, metal transport within the anaerobic methanogenic granules can also be studied using magnetic resonance imaging (MRI) in case the metal ion has paramagnetic properties, as e.g. Co^{2+}, Ni^{2+} and Fe^{3+} (Lens et al., 2003).

9.4.4. Interactions between the retained cobalt fractions (A0·B0)

Dissolution (Jansen et al., 2007), desorption (Li et al., 2007), cell lyses and metal excretion by the cell (Rezanka and Sigler, 2008) will bring the metal back into the liquid phase, where metal precipitation, sorption or internalization can occur again. The system is highly dynamic and there is a high interrelation between the immobilization and retention mechanisms (Fig. 9.5). This is in agreement with our results where the interaction between the two defined fractions (A0·B0) positively correlates to the reactor performance.

Bioavailability is a characteristic of an active compound in a biological system. The compound is bioavailable when available at the site of action and produces a biological response (Shargel and Yu, 1999). After the metal pulse, the retained metal has already reached the biological system (Fig. 9.3). However, it is there not necessarily bioavailable at the site of action. In order to become bioavailable during the 35 days after the pulse, the mechanisms involved in the retention-immobilization interrelationships between metal speciation and enzymatic reactions take place (Fig. 9.5). These interactions are expressed as A0·B0, and include metal sulfide dissolution (Jansen et al., 2007), active metal uptake (Aquino and Stuckey, 2007), excretion of ligands to acquire metal (Roth et al., 1996; Shaked et al., 2005), translocation of metals across the membrane in bacteria (Koster, 2001) or metal allocation in different enzymes (Mulrooney and Hausinger, 2003).

9.4.4. Practical implications

The outcome of this work shows that the correlation between an initial response pulse of cobalt addition, and the reactor behavior in a long time period is a strong tool to further develop metal dosing protocols (Table 9.3, Fig. 9.4). The correlation between metal retention (A0 and B0, Fig. 9.3) and methanogenic activity (evolution of SMA during time, Fig. 9.2) is the key tool to minimize the cobalt addition to methanol fed UASB reactors, and at the same time maximize the organic removal efficiency of the bioprocess. Moreover, the correlation between the initial metal retention and further metal bioavailability (Fig. 9.4) is a promise tool for metal dosing strategies for other bioprocess using biofilms (Nicolella et al., 2000; Skiadas et al., 2003).

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9

SUMMARY AND GENERAL DISCUSSION

10.1. OBJECTIVE

The objective of this thesis is to optimize essential metal dosing in sludge bed based reactors like the Upflow Anaerobic Sludge Bed (UASB) reactor and the Expanded Granular Sludge Bed (EGSB) reactor, which are used for the anaerobic treatment of organically polluted wastewater. Optimization of essential metal dosing in UASB reactors is a compromise between achieving the maximum biological activity of the biomass present in the reactor, while minimizing the costs of the supplied metal and the metal losses into the environment. The boundary conditions for stable reactor operation vary between nutrient deficiency due to lack of essential metals and toxicity due to their excess (Fig. 10.1). The suitable range in which nutrient dosing in the UASB reactor system is required needs to be quantified. Based on this range a metal addition strategy is developed, which obviously affects the metal losses and the cost of the added metal, while achieving the optimal metal concentration inside the UASB reactor. The optimization of the metal dosing process was done by an environmental engineering approach. This chapter summarizes and discusses the main findings of this thesis.

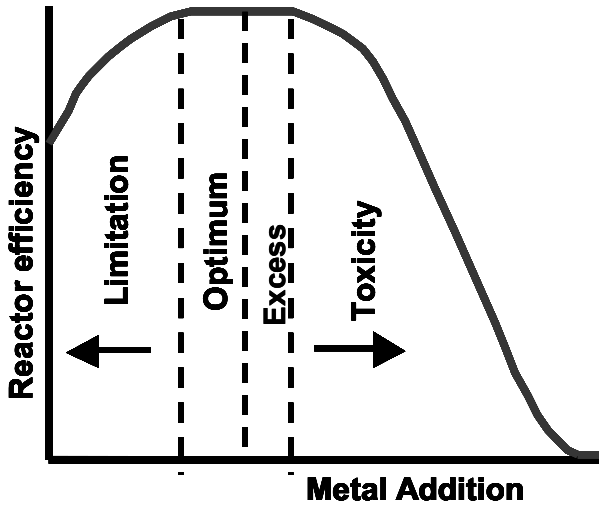

Figure 10.1. Boundary conditions for metal addition to keep the UASB reactor efficiency optimal.

10.2. ENVIRONMENTAL ENGINEERING APPROACH FOR PROCESS OPTIMIZATION

In our technology-oriented society, one of the competences that an environmental engineer needs to develop is to design and optimize biotechnological treatment processes for polluted solid waste, air and water streams. These processes use micro-organisms and enzymes to reach a more effective and cleaner treatment than possible with physical-chemical treatment

methods. The difference with most other technological fields, like chemical or civil engineering, is that the treatment process contains a sensitive, unstable, often living, biological component that requires special attention to ensure optimal functioning of the biocatalyst.

Because of the many choices that have to be made during the design process and the large personal influences of the designer on the design process, there are many possible results and there is no way to say what the optimal process configuration is. Making a design requires understanding and skills in fields relevant for bioprocess technology, such as microbiology, (bio)chemistry and physics (Fig. 1.3).

Process design is an open process, and there is more than one way to look at a problem. There is more than one "good" solution and it is not possible to determine one "best" solution (Sessink et al., 2007). Design is about making decisions and ranking trade-offs. When facing a design problem, there are in theory an infinite number of possible answers and it is impossible to make an evaluation to uniquely assign "the best" answer. For most design processes, there is a standard set of steps that can be used to structure the design process. The details of these methodological steps depend on the object and context of the design and can be different for each situation (Van Der Schaaf et al., 2003).

For the design of our research question: "What is the optimal metal dosage strategy in anaerobic granular sludge bioreactors?", the set of methodical steps given by Van Der Schaaf et al. (2003) was used. This comprises: Analyze the problem, generate concept solutions, choose a solution, work out the solution in detail, evaluate the solution, if necessary, change the design according the findings and present the solution (Fig 10.2).

10.3. ANALYZE THE PROBLEM AND PROPOSE CONCEPT SOLUTION FOR OPTIMAL METAL ADDITION TO UASB REACTORS

10.3.1. Environmental engineering problem

Speece et al. (1986) showed that the maximum potential acetate and propionate conversion rates of 9 out of 30 anaerobically digested sewage sludges could be improved by cobalt, nickel and iron supply. Metal deficiencies of anaerobic and aerobic treatment systems are, apparently not a rare phenomenon and a rational trace metal supply is required in wastewater treatment systems in order to achieve maximal substrate conversion rates, in both aerobic (Clark and Stephenson, 1998; Burgess et al., 1999a; Burgess et al., 1999b; Philips et al., 2003) and anaerobic (Speece et al., 1983; Takashima and Speece, 1989; Patidar and Tare, 2004; Zitomer et al., 2008) wastewater treatment plants.

Addition of micronutrients to anaerobic granular sludge bioreactors has to be studied from different research fields; i.e. from the biology of the cell, and the effect that a metal could have on a certain microorganism, such as gene expression and enzyme activity, to the full scale

bioreactor design and operation where advanced technology is used, e.g. full scale sludge bed reactors.

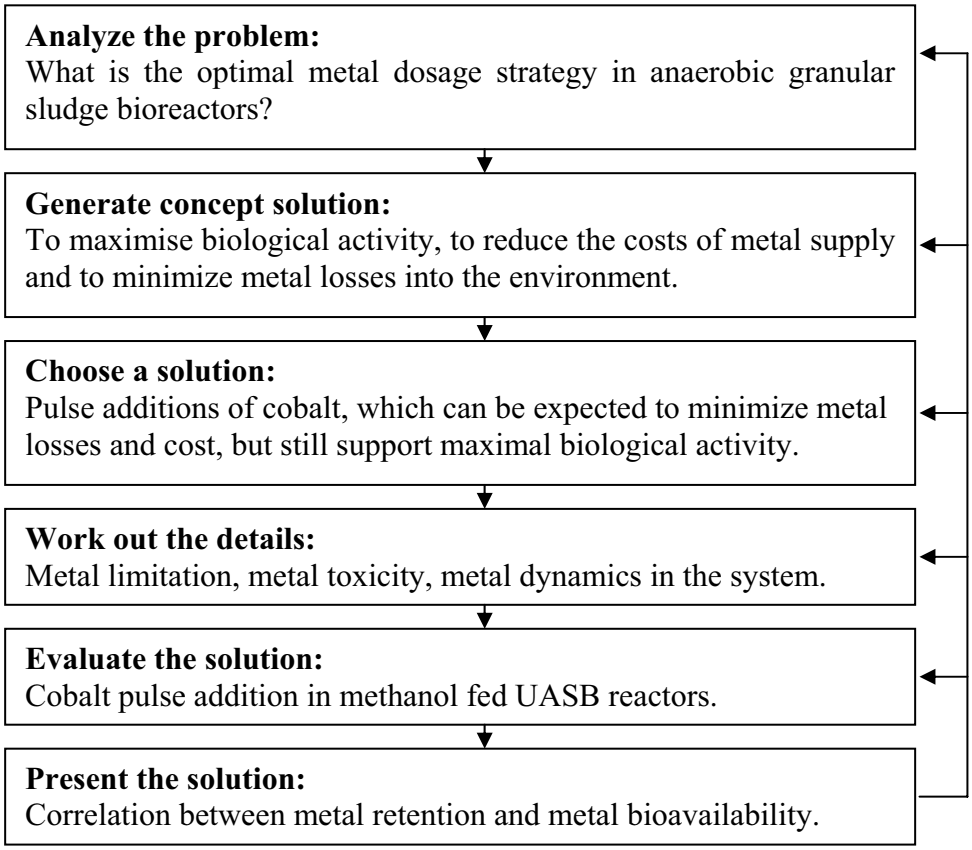

Figure 10.2. Design steps used in this thesis to answer the question: "What is the optimal metal dosage strategy in anaerobic granular sludge bioreactors?".

Cobalt has been shown to be an essential trace metal for methanol fed anaerobic reactors (Florencio et al., 1993; Florencio et al., 1994; Gonzalez-Gil et al., 1999; Zandvoort et al., 2002a; Zandvoort et al., 2006a; Zandvoort et al., 2006b; Zandvoort et al., 2006c; Chapter 3). In continuous reactor operation, cobalt supplementation is required to maintain a high specific methanogenic activity (SMA), and therefore, a stable methanogenic methanol-fed reactor in case of cobalt limitation (Zandvoort et al., 2003).

Several strategies have been adopted to dose cobalt to methylotrophic methanogenic UASB reactors (Table 8.3). Continuous addition of a low $CoCl_2$ concentration (0.62 μmoles per

litre of reactor volume and per day) to a methanol fed UASB reactor only enhanced the SMA of the sludge up to 215 mg COD-CH_4·g VSS^{-1}·d^{-1} (Zandvoort et al., 2002a). The same author studied the possibility of pre-loading sludge by pre-incubating the inoculum in a 1 mM $CoCl_2$ solution for 24 hours. Pre-loading clearly overcame cobalt limitation in the methanol fed UASB reactor, but high amounts of cobalt washed-out with the reactor effluent (Zandvoort et al., 2004).

Pulse dosing of $CoCl_2$, comprising an intermediate dosing strategy between pre-loading and continuous dosing, is effective in overcoming almost acute cobalt limitations in reactor operation (Zandvoort et al., 2004). The pulse of cobalt in high concentrations (77.5 µmoles of cobalt per litre of reactor volume) overcame cobalt limitation (Zandvoort et al., 2004). However, cobalt losses from the sludge with this dosing procedure were considerably higher compared to the continuous dosing strategy: 3.5 µg·g TSS^{-1}·d^{-1} and 0.1 µg·g TSS^{-1}·d^{-1}, respectively (Table 8.3).

10.3.2. Concept solution

An alternative to the above mentioned dosing strategies might be repeated pulse additions of cobalt to the influent at low concentrations, which can be expected to minimize metal losses from the sludge and at the same time overcome cobalt limitation. Therefore, this dosing strategy was chosen as a concept solution in this thesis.

Fig. 10.3 shows the structure of the thesis to study and optimise the pulse dosing of low amounts of cobalt in UASB reactors. First of all, the interactions between metal, biofilm and microbes in granular sludge were studied (Metal fractioning, Chapter 2). Two boundary conditions for stable operation were defined and studied: metal limitation (Chapters 3-6) and metal toxicity (Chapter 7). These conditions in fact determine when nutrient dosing is required into a anaerobic system. Limitation of anaerobic reactors by cobalt, nickel or zinc was studied. These metals are divalent cation metals, which are mainly controlled by the sulfur chemistry. Limitation of UASB reactors by oxyanions, which are not controlled by the sulfur chemistry, was studied as well. The pulse addition of low amounts of cobalt in methanol fed UASB reactors was evaluated in Chapter 8. Finally, the correlation between the metal retention upon pulse dosing to the influent of a UASB reactor and the reactor performance in the period following the trace metal pulse dosing was studied in Chapter 9.

10.4. WORK OUT THE DETAILS OF METAL ADDITION TO UASB REACTORS

10.4.1. Metal dynamics in granular sludge

The predominating role of sulfides in metal fixation in anaerobic systems is supported by the high acid volatile sulfide (AVS) content (Fig. 2.3) and the high metal content in the oxidizable fraction, containing both sulfidic and organic bonding forms (Fig. 2.4). Therefore, the

bioavailability or mobility of essential trace metals in the UASB reactors are mainly controlled by the sulfide chemistry. As metal sulfides have a very low solubility product, it would be expected that these metals are non-bioavailable to the methanogenic consortia. Jansen et al. (2007) showed nevertheless that in most cases, the dissolution rates of Co and Ni sulfides are not limiting the methanogenic activity in anaerobic wastewater treatment. It should, however, be noted that this was based on batch tests with methanogenic enrichments and freshly prepared metal sulfide precipitates. Ageing of sulfidic precipitates, which takes place in the sludge during reactor operation, will lower the dissolution rates and might therefore lower the metal bioavailability (Gonzalez-Gil et al., 2003).

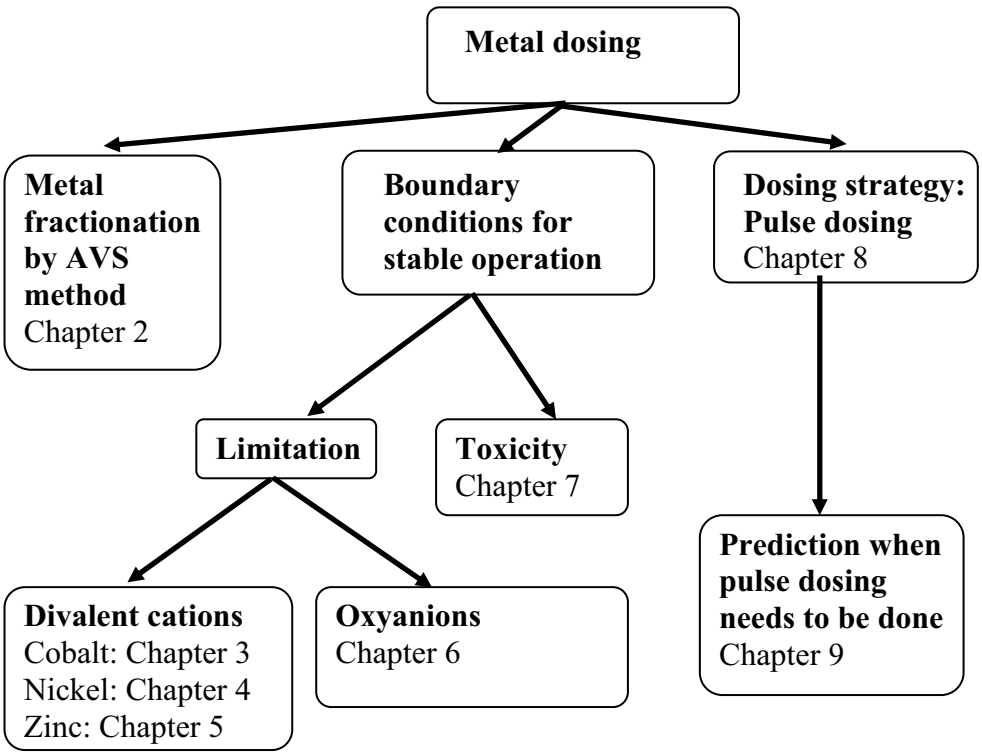

Figure. 10.3. Structure of the thesis. (AVS = Acid Volatile Sulfides).

The potential bioavailability of retained metals in granular sludge can be assessed from the bonding form distribution although the fractions are operationally-defined and not phase-specific. The fractions are extracted at different pH steps from pH 7.0, corresponding to the exchangeable fraction, pH 5.5, corresponding to the carbonates fraction, pH 2, corresponding to the organic matter/sulfide fraction and residual fraction extracted with *Aqua regia*. The fractions

possess a decreasing solubility/reactivity from the first to the last step. This decreasing solubility can be used as a measure of potential bioavailability (Jong and Parry, 2004), regarding the first fraction, *i.e.* adsorbed, the most bioavailable and the last fraction, *i.e.* residual, the least bioavailable. Since studies have shown that even sulfides are bioavailable for methanogenic bacteria under anoxic conditions (Gonzalez-Gil et al., 2003), the first three fractions can be added up for a ranking in potential bioavailability. Mn has the highest bioavailability in a sludge from a UASB treating paper-mill wastewater (Mn > Co > Ni >> Fe), while in a sludge from a UASB reactor treating alcohol distillery wastewater, Co and Ni have similar potential bioavailability (Co = Ni > Mn >> Fe). The effect on Fe bioavailability is small since its bonding form distribution is dominated by the residual fraction (Fig. 2.4).

10.4.2. Metal limitation in granular sludge

10.4.2.1. Limitation of methanogenesis by divalent cations

Omission of a divalent cation metal (cobalt, nickel or zinc) from the feed of methanol-fed anaerobic granular sludge bioreactors led to a reduced SMA on methanol and, consequently, to methanol accumulation in the effluent, followed by the enhanced formation of acetate as shown in Fig. 10.4 (Chapter 3-5). Zandvoort et al. (2006), working under cobalt limiting conditions, observed a similar SMA reduction and consequent methanol accumulation. Thus, during methylothrophic methanogenic degradation of methanol, the decrease in sludge SMA with methanol as the substrate is the variable to follow for predicting possible methanol accumulation, further acetate accumulation and ultimately complete reactor acidification.

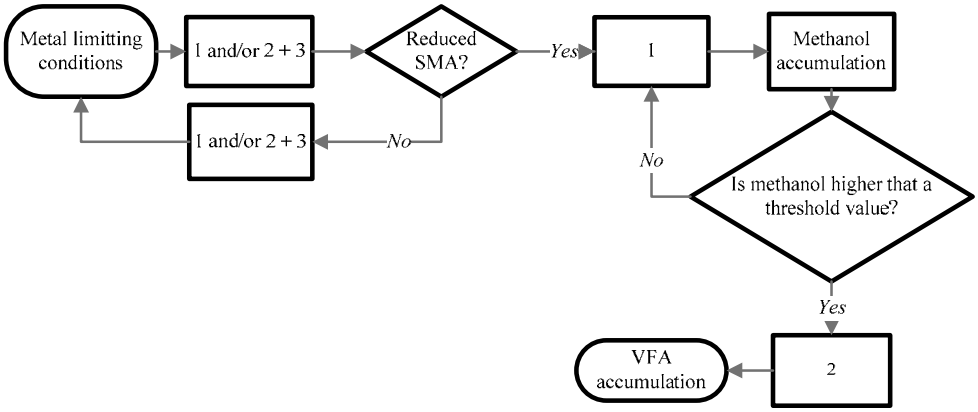

Figure 10.4. Proposed outline of the mechanism of induction of metal limitation in methanol-fed anaerobic reactors. Numbers indicate predominant process. (1) methylotrophic methanogenesis. (2) acetogenesis. (3) acetotrophic methanogenesis. SMA: specific methanogenic activity with methanol as the substrate.

The time period required to achieve nickel limitation (140 days of operation, Chapter 4), as also observed by Zandvoort et al. (2002b), was much longer compared to that required for cobalt, which is already achieved in 15 days (Chapter 3). Kida et al. (2001) observed that the methanogenic activity with acetate as the substrate (pH 7, 37 °C), as well as the F_{430} and corrinoid concentrations in methanogenic mesophilic biomass from a sewage treatment plant decreased with decreasing amounts of Ni^{2+} and Co^{2+} supplied. The omission of nickel might thus result in a reduced amount of coenzyme F_{430}, thereby decreasing the SMA of the sludge. The reduced SMA in the absence of nickel was not observed until day 129 (Chapter 4). The initial concentration of nickel in the seed sludge (43 μg·g TSS^{-1}) was probably enough to support the enzymatic activity and synthesis of coenzyme F_{430} for a considerably long time, even if the size of the *Methanosarcina* population increased (Chapter 4). Moreover, many organisms respond to lower metal bioavailability by an increased synthesis of metals transporters (Sunda and Huntsman, 1998). This type of adaptation to low nickel concentrations might also have contributed to the longer time-period before a decrease in methylotrophic activity is observed.

Only a few papers have so far reported on zinc deprivation during methanogenesis. Osuna et al. (2002) studied metal deprivation in volatile fatty acid (VFA) fed UASB reactors. The latter authors found that when adding solely zinc to the medium, the SMA with acetate as the substrate of metal deprived sludge increased by 36 %. Sauer and Thauer (1997) found in enzymatic studies that the transfer of the methyl group from CH_3-MT1 to coenzyme M by MT2 depends on the Zn^{2+} concentration. The K_M value of this transfer reaction amounted to 0.25 nM free Zn^{2+}. This K_M value is around 240 times lower than the dissolved zinc concentration in the medium of the reactor (60 nM) at the moment the limitation was developed in the present study. This difference between the Km value found by the latter authors (with enzymes) and the dissolved zinc concentration at the time of limitation in the present study (with sludge granules) can be explained by several interactions between the metal cation and the heterogeneous and complex environment inside a UASB reactor. Complexation by bicarbonates, phosphates, soluble sulfides or soluble extrapolymeric substance produced by the biomass reduce the free metal concentration (van Hullebusch et al., 2003).

Cobalt, nickel and zinc are components of different enzymes in the anaerobic methanol degradation (Chapter 3-5). This suggests that even other metals present in the enzyme system involved in methanol degradation could follow the same algorithm as well, e.g. iron which is present in heterodisulfide reductase (Deppenmeier et al., 1999) or copper which is present in Acetyl-CoA synthase (Seravalli et al., 2003).

10.4.2.2. Restoration of methanogenesis in metal limited sludge by metal dosing

The addition of nickel to the influent of a nickel limited UASB reactor after methanol and acetate accumulation occurred, almost instantaneously decreased the acidification and recovered the methanol removal efficiency (Chapter 4). The decreased VFA accumulation and complete methanol removal in the last days of operation of the reactor in Chapter 4, coupled to the lack of acetoclastic methanogenic activity, indicates that methylothrophic methanogens recovered their activity upon the nickel addition without boosting acetogenesis.

In contrast to the methanogenic reactor recovery upon nickel addition, cobalt (Chapter 3) and zinc (Chapter 5) addition in a cobalt-deprived and in a zinc-deprived reactor, respectively, actually enhanced the acetogenic activity of the biomass and methanogenesis did not recover. The different behavior upon nickel addition might be due to the lower zinc dependence of the enzymatic system of methanogenesis compared to nickel (Sauer and Thauer, 2000). In the case of cobalt, the high cobalt dependence (Bainotti and Nishio, 2000), but slight nickel dependence of the enzymatic system of acetogenesis (Diekert et al., 1981), most probably hampered the restoration of methanogenesis.

10.4.2.3. Oxyanions limitation

During propionate degradation to methane, molybdenum, tungsten and selenium are essential for formate dehydrogenase (FDH). FDH is needed for energy conservation both in propionate oxidizers and in methanogens. Methanogens make propionate oxidation energetically feasible by keeping the concentration of the products hydrogen and/or formate extremely low (Stams, 1994). During a propionate fed UASB reactor experiment (Chapter 6) no induction of molybdenum, tungsten and selenium limitation was observed in the SMA with propionate or with any of the intermediates (formate, H_2 and acetate) as the substrates. FDH-1 and FDH-2 gene transcription in *Syntrophobacter*, determined by Q-PCR, was found to decrease with no molybdenum, tungsten and selenium present in the influent (Chapter 6). The decrease in propionate oxidizing activity of the reactor in Chapter 6 along with the decrease in FDH-1 and FDH-2 gene transcription suggests that metal limitation affected the propionate oxidizers. The continuing activity decrease of the propionate oxidizers lowered the methanogenic activity in a further stage of the reactor experiment. The further decrease of FDH-1 and FDH-2 gene transcription, the increased hydrogen usage and decreased formate usage by methanogens at the end of the reactor experiment suggested that propionate degradation proceeded mainly via interspecies hydrogen transfer instead of formate transfer (Chapter 6).

The transcription of FDH-1 and FDH-2 are promising indicators for molybdenum, tungsten and selenium limitation in propionate fed anaerobic reactors. Q-PCR is a relatively new technique to follow gene expression levels that is more and more used in medical diagnostics as well as in other research fields (Noble and Weisberg, 2005; Okubara et al., 2005; Khan et al.,

2007). Q-PCR is an easy and fast technique and therefore a promising to detect oxyanions limitation in wastewater treatments.

10.4.3. Metal toxicity in granular sludge

Toxicity of cobalt cannot be evaluated solely based on the total cobalt concentration in the liquid phase, but requires also the determination of the amount of cobalt precipitated/sorbed in methanogenic granular sludge and the speciation of cobalt present in the bulk solution (Shuttleworth and Unz, 1991; Plette et al., 1999; Paquin et al., 2000). Moreover, the results of this thesis (Chapter 7) suggest that cobalt toxicity can be assessed based on the free cobalt concentration. Similar IC_{50} values, standard deviation of only 22%, for three different measurement sets were obtained when taking only the free cobalt species (Co^{2+}) concentration into account. The latter statement is only valid when the process conditions such as pH, concentration of competing metals (namely calcium or magnesium) etc. are kept constant.

Not only the free cobalt species can be transported into a microbial cell. Also compounds such as vitamin B_{12} (Ferguson and Deisenhofer, 2004), Cobalt-citrate (Krom, 2002) and siderophores (Worms et al., 2006) are taken up by the methanogenic cell. When equilibrium conditions are established, not only the free species, but any metal species can be used to predict metal toxicity (Worms et al., 2006).

There is only a small difference between the optimum and toxic free cobalt concentration (Fig. 10.1): the optimum concentration was shown to be approximately 7 $\mu mol \cdot L^{-1}$ with an apparent K_M value of 0.9 $\mu mol \cdot L^{-1}$, based on total cobalt addition (Zandvoort et al., 2002a), whereas a free cobalt concentration of only around 18 µmol/L is already significantly toxic, causing 50% inhibition. This means that in case only a small amount of complexing agents are present in the medium, a relatively low total cobalt concentration will cause operational problems with anaerobic reactors.

DMT was successfully used for measurement of the free cobalt (Co^{2+}) concentration in anaerobic granular sludge. Prior to the measurements on real samples, the suitability of this technique for cobalt was determined (Table 7.3). Similarly, Temminghoff et al. (2000) obtained a good agreement between calculated and DMT-measured free metal concentrations for copper and cadmium without and with inorganic and synthetic as well as natural organic complexation. The only problem is the long time required to obtain experimental data, i.e. 4 days for equilibrium establishment and 5 days for DMT measurement, which disables DMT for measurements of free metal concentrations under dynamic conditions of bioreactors.

10.5. EVALUATION OF THE CHOSEN SOLUTION: PULSE ADDITION

Repeated pulse addition of low amounts of cobalt is an efficient dosing strategy to maintain a stable methanogenic methanol fed UASB reactor (Fig. 8.1). Pulse addition of 5 μmoles cobalt per litre of reactor volume in the form of $CoCl_2$ creates a pool of cobalt into the granular sludge matrix due to the high cobalt retention, i.e. around 90 %. In contrast, a much smaller cobalt pool is formed when cobalt is dosed as Co-EDTA^{2-}; i.e. only 8 % of the supplied Co-EDTA^{2-} is retained (Fig. 8.3). Additionally, cobalt wash-out was much less compared to the pulse addition of high amounts of cobalt or the pre-loading cobalt strategies reported in previous studies with methanol fed UASB reactors (Table 8.3). The cobalt pool formed upon $CoCl_2$ dosing was enough to keep stable methanogenesis at an OLR of 8.5 g COD·$L^{-1}_{reactor}$·d^{-1} for more than 15 days between the pulses. Additionally, pulse addition of $CoCl_2$ presents several advantages compared to Co-EDTA^{2-}. First of all, cobalt added as chloride is much more retained in the granular sludge compared to cobalt bound to EDTA, indicating that the addition frequency would be much lower, i.e. once per 15 days for $CoCl_2$ versus once per 7 days for Co-EDTA^{2-}. The side-effects that EDTA causes to the granular sludge matrix and to the microbial cells (Chapter 8) make EDTA an unreliable ligand to use in full scale applications that require trace metal supplementation.

Calculation of the required amount of cobalt needed per day to support adequate methanogenesis is very important for practice. With the data provided in Chapter 8, the calculation of the mg of cobalt needed to be dosed per litre of reactor and per day was obtained empirically. In Chapter 8, considering a stable methanol removal period of 15 days after the $CoCl_2$ pulse addition and an OLR of 8.5 g COD·$L^{-1}_{reactor}$·d^{-1}, the required cobalt amounted to 0.36 μmoles·$L_{reactor}^{-1}$·d^{-1} of $CoCl_2$. In case of a Co-EDTA^{2-} pulse, considering a stable methanol removal period of 7 days upon the pulse, the required cobalt amounted to 0.77 μmoles·$L_{reactor}^{-1}$·d^{-1} of $CoCl_2$. In a methanol fed UASB reactor with an OLR of 2.6 g COD·$L^{-1}_{reactor}$·d^{-1}, (Zandvoort et al., 2002a) estimated that 0.15 μmoles·$L_{reactor}^{-1}$·d^{-1} would be required to maintain a stable methanol removal capacity when $CoCl_2$ was supplied continuously.

10.6. PRESENT THE SOLUTION: PREDICTION OF METHANOGENIC ACTIVITY BY METAL RETENTION

This thesis clearly shows how the retention of cobalt in the sludge during 24 hours following a cobalt pulse correlates with the decline during 16 and 35 days on the SMA with methanol as the substrate in the absence of cobalt of the sludge present in methanol fed UASB reactors (Fig. 9.4). This correlation shows that the type of ligand used does not solely determine the quantification of the reactor performance upon the pulse, but other conditions also affect the quantification of the reactor performance, e.g. the methanol or VFA effluent concentrations or concentration of cobalt in the sludge prior to the pulse addition. Nevertheless, the correlation

between metal retention and reactor performance does not indicate that metal retention is the cause of different reactor performances, thus, the predictions for reactor operation should not be overstretch, but it is the basis for further research on quantification of metal bioavailability.

Bioavailability is a characteristic of the extent that an active compound reaches a biological system and its availability at the site of action (Shargel and Yu, 1999). After the metal pulse, the retained metal is introduced in the biological system, however, it is not necessary bioavailable at the biocatalytic site. In order to become bioavailable, different mechanisms that involve many interrelationships between metal speciation and enzymatic reactions take place. For example, metal sulfide dissolution (Jansen et al., 2007), excretion of ligands to acquire metals from the media (Roth et al., 1996; Shaked et al., 2005), translocation of metals across the membrane in bacteria (Koster, 2001) or metal allocation in different enzymes (Mulrooney and Hausinger, 2003). The SMA increase after the pulse and its subsequent decline (Fig. 9.2) shows that a fraction of the total amount of retained cobalt is bioavailable cobalt just after the cobalt pulse addition, and most probably also the cobalt that is abiotically retained just after the cobalt pulse addition is taken up later by the cell in times of need or starvation.

The outcome of this thesis shows that the correlation between an initial stage in the reactor operation, following the cobalt pulse addition, and the reactor behavior in a long time period could be used to optimize the metal dosing strategies for UASB reactor operation (Chapter 9). This correlation could be used to minimize the cobalt supply to methanol fed UASB reactors, and at the same time maximize the organic removal efficiency of the bioprocess. Moreover, the correlation between the initial metal retention and further metal bioavailability could be a tool for metal dosing strategies for other bioprocess using biofilms (Nicolella et al., 2000; Skiadas et al., 2003).

10.7. FUTURE RECOMMENDATIONS

10.7.1. Mechanistic model of metal dynamics

A much more accurate quantification of the required amount of cobalt to be dosed can be obtained when a mechanistic model for the $CoCl_2$ dosing to methanol fed UASB reactors is developed. The mechanistic modelling of the cobalt uptake rate is, however, rather complicated because of the complexity of the system under study, where the relation between chemical speciation, biological uptake, biomass growth, hydrodynamic of the reactor and biofilm characteristics has to be taken into account (Jansen, 2004). Some of these aspects are described in the literature for biofilm systems other than granular sludge (Flemming, 1995; Arican et al., 2002; Flynn, 2003; Wang et al., 2003; Weng et al., 2003; Worms et al., 2006), but the aspects of greatest importance, i.e. chemical interactions between cobalt and the granular sludge matrix, as well as cobalt uptake and biomass growth kinetics are not yet quantified (van Hullebusch et al., 2003).

10.7.2. Metal dynamics in living cells

An important prerequisite for further understanding of metal dynamics in living cells is the ability to image the concentration of trace metals in living cells in real time. Fluorescence microscopy is ideally suited for this purpose, and the development of fluorescent probes that bind transition metal ions with high affinity and selectivity has recently become an area of active research (Van Dongen et al., 2006), and can contribute to depict the metal microorganism interaction at single cell level in the biofilm system.

Imaging of the element distribution in cells and biofilm sections is becoming possible with sub-micrometer spatial resolution and picogram-level sensitivity owing to advances in laser ablation MS (Mass Spectrometry), ion beam and synchrotron radiation X-ray fluorescence microprobes (Gordon et al., 2008). Progress in nanoflow chromatography and capillary electrophoresis coupled with element specific ICP MS (Saba et al., 2007; Schaumlöffel, 2007) and molecule-specific electrospray MS/MS and MALDI (Matrix-assisted laser desorption/ionization) (Tharamani et al., 2008) enables speciation of elements in microsamples in complex biological environment as the anaerobic granular sludge.

10.7.3. Metal dynamics and solid state speciation in anaerobic granular sludge

The increasing sensitivity of EXAFS (Extended X-ray Absorption Fine Structure) and XANES (X-ray Absorption Near Edge Structure), owing to the use of more intense synchrotron beams and efficient focusing optics, offers a unique non-destructive tool to identify and to determine in situ the relative amounts of functional sulfur species down to low concentrations providing information about the oxidation state, fingerprint speciation of metal sites and metal-site structures (Jalilehvand, 2006; Lobinski et al., 2006). The application of more elaborate sulfur extraction schemes for anaerobic granular sludge, yielding more specific fractions including elemental sulfurs and disulfides (Canheld et al., 1998; van der Veen, 2004), which have been successfully applied to freshwater and marine sediments, can also contribute to a better characterization of the sulfur pool in anaerobic granular sludge. Nuclear magnetic resonance (NMR) methods provide the ability to non-invasively monitor metal transport processes and biomass accumulation and distribution (Seymour et al., 2007). The further determination of cobalt taken up into the cell, e.g. by mapping radioactive metal isotopes previously supplied to the anaerobic granule, is another way to better characterization of this metal fraction (Lombi et al., 2001).

10.7.4. Process monitoring and control

The DMT principle, as studied in Chapter 7, can also be used in a "reversed" set-up to control the addition of trace metals to the reactor (Schöder, personal communication). Coupling the reactor via a DMT membrane with a nutrient solution, buffered by the addition of a strong

ligand, the DMT cell can be used as a nutrient source with a defined flux over the membrane surface, thus the free ion activity of the reactor solution can be controlled. The sensor and the dosing are then combined in one unit, which is a very elegant solution. Other kinds of cobalt dosing strategies that minimize the metal losses by manipulating the cobalt dissolution rate can be development in further research, e.g. including cobalt in slow release capsules (Im et al., 2005) or electrochemical release of cobalt (Cosnier et al., 1994).

Electrochemical sensors have been developed for trace metal analysis in water, using either microlithographically fabricated- or screen-printed microelectrode arrays (Xie et al., 2005; Noël et al., 2006). The sensors are chiefly based on the principles of stripping voltammetry (anodic, cathodic, and adsorptive voltammetry) and stripping potentiometry (PSA) (Ostapczuk, 1993). The combination of microelectrodes and computer-controlled miniaturized instrumentation is very suitable for the development of portable analytical instruments for in situ and on-site measurement of heavy metals in reactors (Xie et al., 2005). The inclusion of trace metal measurement on the reactor system as a controlled parameter will allow to implement metal dynamics in process control design. Different applicable control strategies include feedforward, supervisory, multivariable and adaptative control features as well as sophisticated digital logic control (Stephanopoulos, 1984). The implementation of metal pulse dosing as manipulated parameter in such process control algorithm can compensate process disturbances, such as variation in incoming flows, wastewater characteristics or temperature. Process control can thus alleviate fluctuations in metal loading rates and subsequently increase the conversion capacity of anaerobic wastewater treatment plants.

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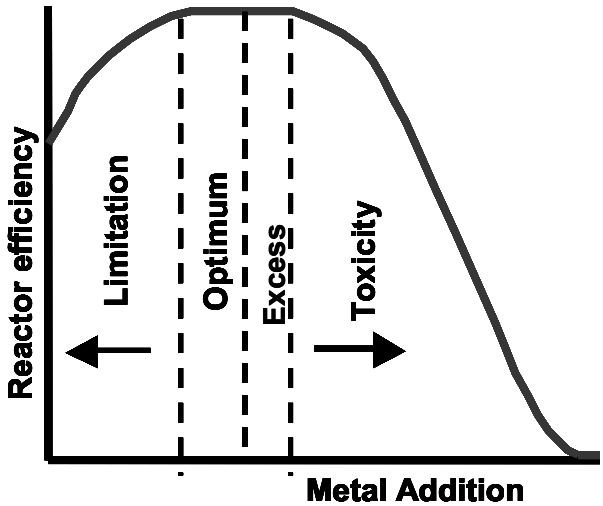
SAMENVATTING EN ALGEMENE DISCUSSIE

10.1. DOELSTELLING

De doelstelling van dit proefschrift is het optimaliseren van de dosering van sporen-metalen aan slib bed reactoren zoals de opstroom anaerobe slib bed (UASB) reactor en de geexpandeerde korrelslibbed bed (EGSB) reactor, die gebruikt worden voor anaerobe zuivering van afvalwater, waarbij methaan wordt geproduceerd uit organische verbindingen. De optimale dosering van sporen-metalen is een compromis tussen het bereiken van de maximale biologische activiteit, minimale kosten voor benodigde metalen en minimale uitspoeling van metalen naar het milieu. Enerzijds kan de biologische beschikbaarheid van de sporen-metalen te laag zijn (limitatie) en anderzijds kan de beschikbaarheid van de metalen te hoog zijn (toxiciteit). Alleen wanneer de metaal beschikbaarheid tussen deze grenswaarden ligt, is er optimale stabiele omzetting (Fig. 10.1). Beide grenswaarden moeten dus worden gekwantificeerd. De doseringsstrategie beïnvloedt de uitspoeling van metalen en de kosten voor de sporen-metalen. De optimalisatie van de metaaldosering is uitgevoerd vanuit een mileutechnologische benadering. Dit hoofdstuk vat de belangrijkste bevindingen samen en bevat de discussie hierover.



Figuur 10.1. Grenzen van metaaltoevoeging voor optimale bioconversie in slib bed reactoren.

10.2. MILIEU TECHNOLOGISCHE BENADERING VOOR PROCESOPTIMALISATIE

Het ontwerp en de optimalisatie van biotechnologische behandelingsprocessen van vervuilde grond-, lucht- en waterstromen is één van de taken waarvoor een milieu-ingenieur in onze technologie georiënteerde maatschappij verantwoordelijk is. Deze processen maken veelal

gebruik van micro-organismen en enzymen om een effectievere en schonere behandeling te realiseren, tegen veel lagere kosten dan mogelijk is met fysisch-chemische methoden. Het verschil met de meeste fysisch-chemische technieken is dat een biologisch proces vaak onstabiele componenten bevat, die speciale aandacht vereisen voor een optimale werking van de biokatalysator.

Vanwege de vele keuzes die gemaakt moeten worden tijdens het ontwerp van een biologisch proces en de grote persoonlijke invloed van de ontwerper hierop, zijn er meerdere uitkomsten mogelijk en is er geen manier beschikbaar om te bepalen wat de meest optimale procesconfiguratie is. Het vervaardigen van een ontwerp vergt inzicht en vaardigheid binnen de gebieden die relevant zijn voor bioprocestechnologieën zoals microbiologie, (bio-)chemie en fysica (Fig. 1.3).

Proces ontwerp is een open proces en er zijn meerdere manieren om naar een probleem te kijken. Er is niet slechts één “goede” oplossing en het bepalen van de “beste”oplossing is niet mogelijk (naar Sessink et al., 2007). Ontwerpen is een kwestie van keuzes maken. Wanneer men op een probleem stuit, dan zijn daar in theorie oneindig veel antwoorden op mogelijk en is het niet mogelijk te evalueren wat het enige beste antwoord is. Voor de meeste ontwerpprocessen is er een standaard set stappen die gebruikt kan worden als basis voor het ontwerpproces. De details van deze methodologische stappen zijn afhankelijk van het doel en de context van het ontwerp en kunnen in elke situatie verschillend zijn (naar Van Der Schaaf et al., 2003).

Voor het ontwerp van de in dit proefschrift behandelde onderzoeksvraag: “wat is de optimale strategie voor metaaldosering in anaerobe korrelslib bioreactoren?” is gebruik gemaakt van de set van methodische stappen zoals gegeven door Van Der Schaaf et al. (2003). Deze bestaan uit: analyseer het probleem, vorm een concept oplossing, kies een oplossing, werk de oplossing uit in detail, evalueer de oplossing, verander het ontwerp al naar gelang bevindingen en presenteer de oplossing (Fig. 10.2).

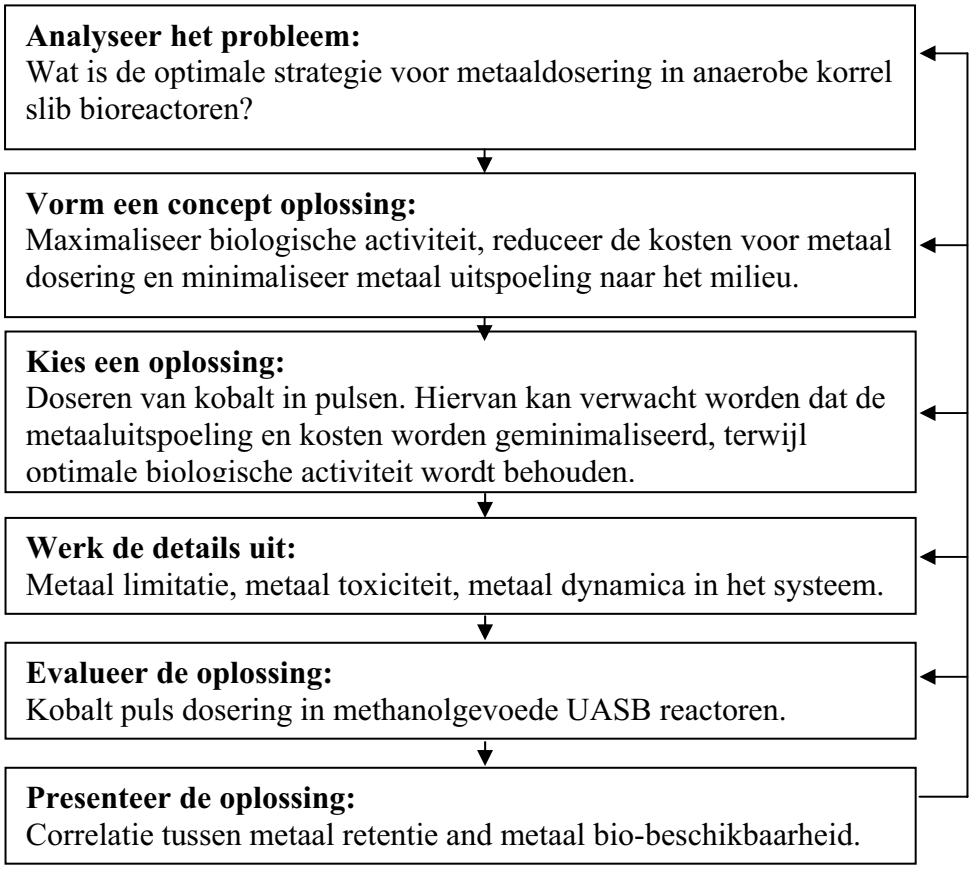
10.3. PROBLEEMANALYSE EN CONCEPTUELE OPLOSSING VOOR OPTIMALE METAAL DOSERING AAN UASB REACTOREN

10.3.1. Het milieutechnologisch probleem

Speece en medewerkers (1986) hebben laten zien dat de maximale potentiële omzettingssnelheid van acetaat en propionaat in 10 van de 30 bestudeerde anaerobe behandelingssystemen, verhoogd kon worden door de dosering van nikkel en ijzer. Metaal tekorten van anaerobe en aërobe behandelingssystemen zijn zeker geen zeldzaamheid, de dosering van een redelijke hoeveelheid sporen-metalen is noodzakelijk voor het bereiken van maximale omzettingssnelheden in afvalwaterzuiveringsinstallaties, zowel in aërobe (Clark en Stephenson, 1998; Burgess et al., 1999a; Burgess et al., 1999b; Philips et al., 2003) als in

anaerobe (Speece et al., 1983; Takashima en Speece, 1989; Patidar en Tare, 2004; Zitomer et al., 2008) installaties.

De dosering van micronutriënten aan een anaëroob korrelslib bioreactor moet onderzocht worden op celbiologie niveau, oftewel het effect van specifieke metalen op specifieke micro-organismen (bijvoorbeeld het effect op de expressie van genen en enzym activiteit). Ook op "full-scale" niveau moet de dosering worden onderzocht, waarbij de rol van bioreactorontwerp en de bedrijfsvoering van bioreactoren van belang zijn.



Figuur 10.2 Ontwerpstappen gemaakt in dit proefschrift ter beantwoording van de vraag: "Wat is de optimale strategie voor metaaldosering in anaerobe korrelslib bioreactoren?".

Kobalt is een essentieel sporen-element voor methanogenese in anaerobe reactoren gevoed met methanol (Florencio et al., 1993; Florencio et al., 1994; Gonzalez-Gil et al., 1999; Zandvoort et al., 2002a; Zandvoort et al., 2006a; Zandvoort et al., 2006b; Zandvoort et al.,

2006c, Hoofdstuk 3). Kobaltdosering aan continue methanogene reactoren is noodzakelijk om een hoge specifieke methanogene activiteit (SMA) te verkrijgen en/of te behouden en voor een stabiele omzetting in kobaltgelimiteerde methanol gevoede methanogene reactoren (Zandvoort et al., 2003).

Voor kobaltdosering aan methylotrofe methanogene UASB reactoren zijn verschillende strategieën toegepast (Tabel 8.3). Continue toevoeging van een kleine hoeveelheid $CoCl_2$ (0.62 µmol $L_{reactor}^{-1}$ d^{-1}) aan een methanol gevoede UASB reactor verhoogt de SMA van het slib naar 215 mg CZV-CH_4 g VSS^{-1} d^{-1} (CZV = chemische zuurstof vraag, VSS = vluchtige gesuspendeerde stoffen) (Zandvoort et al., 2002a). Dezelfde auteurs hebben de mogelijkheid van slib voorbehandeling onderzocht. Hierbij wordt het slib voor het enten van de reactor gedurende 24 uur geïncubeerd in een 1 mM $CoCl_2$ oplossing. Voorbehandeling voorkwam kobaltlimitatie in een methanolgevoede UASB reactor. Een nadeel was de uitspoeling van een aanzienlijk deel van het opgenomen kobalt (Zandvoort et al., 2004).

Pulsdosering, een doseerstrategie die een compromis biedt tussen voorbehandeling en continue dosering van kobalt, voorkomt acute kobaltlimitatie (Zandvoort et al., 2004). Herstel na kobalt limitatie was mogelijk door het gepulseerd doseren van grote hoeveelheden kobalt (77.5 µmol $_{kobalt}$ $L_{reactor}^{-1}$) (Zandvoort et al., 2004). De uitspoeling van kobalt bij pulsdosering was echter aanzienlijke hoger dan bij continue dosering: respectievelijk 3.5 µg g TSS^{-1} d^{-1} en 0.1 µg g TSS^{-1} d^{-1} (Tabel 8.3).

10.3.2. Conceptuele oplossing

Een alternatief voor de hierboven beschreven doseerstrategieën is het frequenter toedienen van kleinere hoeveelheden sporen-elementen aan het influent. Met deze strategie wordt de uitspoeling van metalen geminimaliseerd en kobaltlimitatie voorkomen. Om deze reden is deze strategie gekozen als conceptoplossing in dit proefschrift.

Fig. 10.3 toont de structuur van dit proefschrift, waarin de pulsdosering van kleine hoeveelheden kobalt aan UASB reactoren wordt onderzocht en geoptimaliseerd. Als eerste werden de interacties tussen metalen, biofilm en micro-organismen in korrelslib onderzocht (Metaal fractionering, **hoofdstuk 2**). Twee grensvoorwaarden voor stabiele reactor bedrijfsvoering werden gedefinieerd en onderzocht: metaal limitatie (**hoofdstuk 3 t/m 6**) en metaaltoxiciteit **(hoofdstuk 7**). Deze condities bepalen in feite wanneer de dosering van nutriënten aan een UASB systeem vereist is. Limitatie van anaerobe reactoren door kobalt, nikkel of zink is bestudeerd, waarbij deze metalen zijn beschouwd als tweewaardige metalen, die voornamelijk worden gereguleerd door de zwavelchemie. Limitatie van anaerobe reactoren door oxyanionen, die niet worden gecontroleerd door de zwavelchemie, werd tevens bestudeerd. De pulsdosering van kleine hoeveelheden kobalt aan methanolgevoede UASB reactoren wordt geëvalueerd in **hoofdstuk 8**. Tot slot wordt in **hoofdstuk 9** de relatie bestudeerd tussen

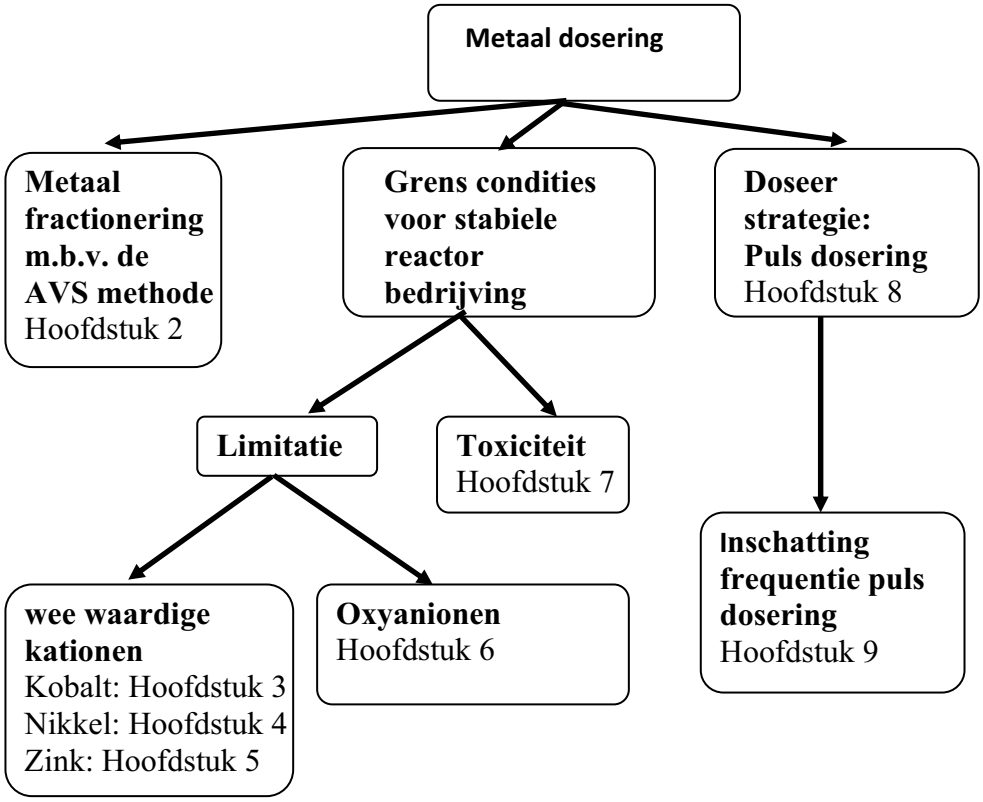
metaalretentie na pulsdosering aan het influent van een UASB reactor, en het functioneren van de reactor in de periode na pulsdosering van sporen-metalen.

10.4 METAAL TOEVOEGING AAN UASB REACTOREN

10.4.1. Metaal dynamiek in korrelslib

De overheersende rol van sulfiden bij het vastleggen van metalen in anaerobe systemen kan worden aangetoond door het hoge gehalte aan metalen en sulfiden dat vrijkomt bij het aanzuren van de oxideerbare fractie, die zowel sulfiden als organisch gebonden verbindingen bevat. Zo wordt de biobeschikbaarheid en mobiliteit van essentiële sporen-elementen in UASB reactoren voornamelijk bepaald door de zwavelchemie. Omdat metaalsulfiden een laag oplosbaarheidproduct hebben, zou verwacht kunnen worden dat deze metalen niet biobeschikbaar zijn voor methanogene consortia. Jansen et al. (2007) laat echter zien dat in de meeste gevallen de methanogene activiteit in anaëroob slib niet wordt beperkt door de oplossnelheden van Co en Ni. Een kanttekening hierbij is dat dit gebaseerd is op ladingsgewijze testen met opgehoopte methanogene cultures en chemisch bereide metaalsulfide precipitaten. Veroudering van sulfide precipitaten, zoals dat bij het slib van een werkende reactor plaatsvindt, zal de oplossnelheden verlagen en kan zodoende de biobeschikbaarheid verminderen. (Gonzalez-Gil et al., 2003).

De potentiële biobeschikbaarheid van metalen in het slib kan worden bepaald uit de verdeling van de metalen over verschillende fracties bij sequentiële extractie, ook al wordt de indeling van verbindingen in fracties bepaald door oplossing, vervluchtiging of retentie in de vaste fase, na een bepaalde fysisch/chemische behandeling die niet verbindingsspecifiek is. De fracties hebben een afnemende oplosbaarheid/reactiviteit van de eerste tot de laatste behandelingsstap. Deze afname in oplosbaarheid kan worden gebruikt als maat voor de potentiële biobeschikbaarheid (Jong en Parry, 2004), de eerste fractie (geadsorbeerd) is het meest beschikbaar en de laatste fractie (residueel) het minst beschikbaar. Uit eerdere studies is gebleken dat sulfiden beschikbaar zijn voor metanogene bacteriën, zelfs onder anoxische condities (Gonzalez-Gil et al., 2003). Hierdoor kunnen de eerste drie fracties worden opgeteld en worden beschouwd als de totale beschikbare fractie. In slib uit een UASB reactor gebruikt voor de behandeling van afvalwater uit een papierfabriek, is mangaan het meest beschikbaar (Mn > Co > Ni >> Fe). In slib uit een UASB reactor die het afvalwater van een alcohol distillatie fabriek behandelt, zijn kobalt en nikkel het best beschikbaar (Co = Ni > Mn >> Fe). Het effect van ijzer is klein, aangezien ijzer vooral in de residu fractie aanwezig is (Fig. 2.4.).



Figuur 10.3. Structuur van dit proefschrift. (AVS = Acid Volatile Sulfides: bij lage pH vluchtige sulfides).

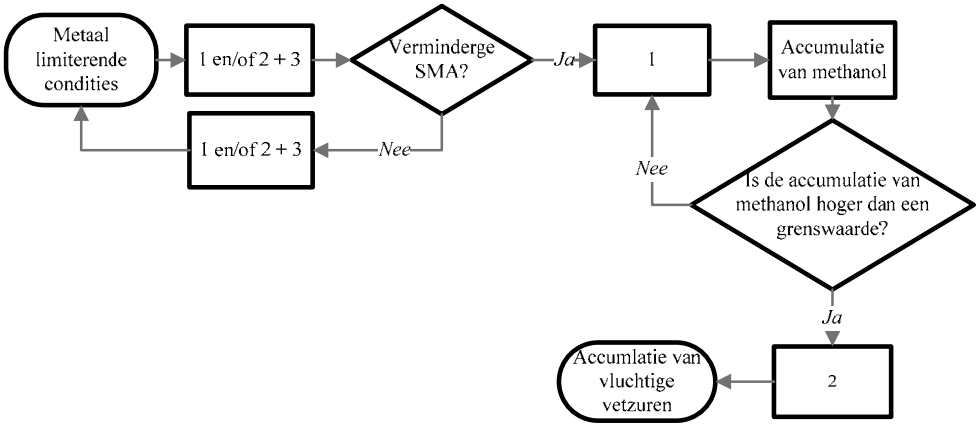
10.4.2. Metaal limitatie in korrelslib

10.4.2.1. Limitatie van methanogenese door divalente kationen

Het weglaten van tweewaardige metaalionen (kobalt, nikkel of zink) uit de voeding van methanolgevoede korrelslib reactoren leidde tot een verminderde specifieke methanogene activiteit (SMA) voor methanol en daardoor tot methanolophoping in het effluent, gevolgd door verhoogde acetaat vorming (zie Fig. 10.4. Zandvoort en medewerkers (2006) zagen onder kobaltlimiterende condities een zelfde vermindering in SMA en methanol ophoping. De vermindering van de SMA tijdens methylotrofe methanogene afbraak van methanol is dus een geschikte indicator om mogelijke methanol ophoping, acetaataccumulatie en reactorverzuring te voorspellen.

De benodigde tijd voor nikkellimitatie (140 dagen reactor bedrijf, hoofdstuk 4), zoals waargenomen door Zandvoort et al. (2002b), was veel langer dan de tijd benodigd voor kobaltlimitatie. Kobaltlimitatie wordt al na 15 dagen bereikt (Hoofdstuk 3). Kida et al. (2001)

vonden in methanogene mesofiele biomassa van een afvalwaterzuivering dat zowel de methanogene activiteit met acetaat als substraat (pH 7, 37 °C), als de coënzym F_{430} en corrinoiden concentratie afnam, met afnemende hoeveelheden gedoseerd Ni^{2+} en Co^{2+}. Het ontbreken van nikkel kan een verminderde hoeveelheid coënzym F_{430} tot gevolg hebben en zo de SMA van het slib reduceren. De verminderde SMA in afwezigheid van nikkel werd niet gevonden tot dag 129 (hoofdstuk 4). De initiële nikkelconcentratie in het uitgangsslib (43 μg·g TSS^{-1}) was waarschijnlijk voldoende om de enzymactiviteit en synthese van coenzym F_{430} een aanzienlijke tijd te behouden. De populatie *Methanosarcina* nam zelfs toe. Bovendien reageren veel organismen op een lage biobeschikbaarheid van metalen met een toename van de synthese van metaal transportsystemen (Sunda and Huntsman, 1998). Deze aanpassing aan lage nikkel concentraties kan ook hebben bijgedragen aan de lange periode voordat een afname in methylotrophe activiteit werd waargenomen.



Figuur 10.4. Voorgesteld mechanisme voor de inductie van metaallimitatie in methanol gevoede anaerobe reactoren. De nummers geven het belangrijkste proces aan: (1) methylotrofe methanogenese. (2) acetogenese. (3) acetotrofe methanogenese. SMA: specifieke methanogene activiteit met methanol als substraat.

Slechts enkele publicaties maken melding van zinktekorten gedurende methanogenese. Osuna et al. (2002) bestudeerden metaaltekorten in UASB reactoren gevoed met vluchtige vetzuren. Deze auteurs vonden dat toevoeging van alleen zink aan het medium, de SMA met acetaat als substraat van het verarmde slib toenam met 36%. Sauer and Thauer (1997) vonden in enzymatische studies dat de overdracht van de methylgroep van CH_3-MT1 naar coënzym M door MT2 afhangt van de Zn^{2+} concentratie. De waarde van de affiniteitsconstante (K_M) van de overdrachtreactie bedroeg 0.25 nM vrij Zn^{2+}. Deze K_M waarde is ongeveer 240 keer lager dan de concentratie opgelost zink in het reactormedium (60 nM) op het moment dat limitatie ontstond. Het verschil tussen de K_M waarde gevonden door genoemde auteurs (met enzymen) en de

opgeloste zinkconcentratie op het moment van limitatie, gevonden in de huidige studie (met korrelslib), kan worden verklaard door verschillende interacties tussen het metaalion en de heterogene en complexe omgeving in een UASB reactor. Complexen gevormd met bicarbonaten, fosfaten, opgeloste sulfiden, of opgeloste extrapolymere stoffen geproduceerd door de biomassa, reduceren de vrije metalen concentratie (van Hullebusch et al., 2003).

In verschillende enzymen die een rol spelen in de anaerobe afbraak van methanol, is kobalt, nikkel of zink ingebouwd (Hoofdstuk 3-5). Dit suggereert dat bij andere metalen die aanwezig zijn in het enzymsysteem en een rol spelen in de afbraak van methanol, dezelfde processen een rol zouden kunnen spelen. Voorbeelden van zulke metalen zijn ijzer, dat aanwezig is in heterodisulfide reductase (Deppenmeier et al., 1999), en koper, dat deel uitmaakt van Acetyl-CoA synthase (Seravalli et al., 2003).

10.4.2.2. Herstel van methanogenese door metaal dosering in metaal gelimiteerd slib

Toevoegen van nikkel aan het influent na accumulatie van methanol en acetaat, verminderde de mate van verzuring en herstelde vrijwel onmiddellijk de verwijdering van methanol (hoofdstuk 4). De verminderde VVZ ophoping, de complete methanol verwijdering en de afwezigheid van acetoclastische methanogene activiteit tijdens de laatste dagen van het reactorexperiment (beschreven in hoofdstuk 4), zijn een indicatie dat methylotrofe methanogenen herstelden na de toevoeging van nikkel.

In tegenstelling tot bovenstaande, nam bij toevoeging van kobalt en zink in respectievelijk kobalt en zink verarmde reactoren, steeg de acetogenese activiteit van de biomassa terwijl de methanogene activiteit niet herstelde. Het verschillend gedrag in vergelijking met nikkeltoevoeging is mogelijk het gevolg van een lagere gevoeligheid van het enzymatisch systeem van methanogenese voor zink dan voor nikkel, (Sauer and Thauer, 2000). In het geval van kobalt, beperkte een hoge afhankelijkheid van het acetogenese systeem van kobalt (Bainotti and Nishio, 2000), maar geringe afhankelijkheid van nikkel (Diekert et al., 1981) waarschijnlijk het herstel van de methanogenese.

10.4.2.3. Oxyanionen limitatie

Bij afbraak van propionaat naar methaan zijn molybdeen, wolfraam en selenium noodzakelijk voor formiaat dehydrogenase (FDH). FDH is nodig voor energie opslag zowel bij propionaat oxideerders als voor methanogenen. Methanogenen maken propionaatoxidatie energetisch mogelijk doordat zij de concentratie H_2 en/of formiaat extreem laag houden (Stams, 1994). In een propionaat gevoede UASB reactor werd molybdeen, wolfraam en selenium limitatie niet geobserveerd in SMA testen met propionaat of met een van de tussenproducten (formiaat, H_2 en acetaat) als substraat. FDH-1 en FDH-2 gen transcriptie in *Syntrophobacter*, bepaald met Q-PCR, nam af wanneer er geen molybdeen, wolfraam en selenium in het influënt

aanwezig was (hoofdstuk 6). De afname van propionaatoxidatie activiteit in hoofdstuk 6, in combinatie met de afname van FDH-1 en FDH-2 gen transcriptie, suggereert dat metaallimitatie de propionaat oxideerders beïnvloedt. De steeds verdere afname in activiteit van de propionaat oxideerders zorgde voor een verlaging van de methanogene activiteit in latere stadia van dit experiment. De verdere afname van FDH-1 en FDH-2 gen transcriptie, het toegenomen verbruik van waterstof en het afgenomen verbruik van formiaat door methanogenen tegen het einde van de reactor bedrijfsvoering, suggereren dat propionaatafbraak voornamelijk verloopt via H_2 overdracht in plaats van door formiaatoverdracht (hoofdstuk 6).

De transcriptie van FDH-1 en FDH-2 is een veelbelovende indicator voor limitatie van molybdeen, wolfraam en selenium in propionaatgevoede anaerobe reactoren. Q-PCR is een relatief nieuwe techniek om genexpressie niveaus te volgen. Deze methode wordt gebruikt in medische diagnostiek, maar ook in andere onderzoeksgebieden (Noble and Weisberg, 2005; Okubara et al., 2005; Khan et al., 2007). Q-PCR is een eenvoudige en snelle methode en derhalve veelbelovend bij het bepalen van oxyanionlimitatie in afvalwaterbehandeling.

10.4.3. Metaal toxiciteit in korrelslib

Toxiciteit van kobalt kan niet worden bepaald aan de hand van slechts de totale kobaltconcentratie in de vloeistof fase. Ook de hoeveelheid kobalt die geprecipiteerd of geabsorbeerd is in methanogeen korrelslib en de speciatie van kobalt in de bulk oplossing dienen bepaald te worden (Shuttleworth and Unz, 1991; Plette et al., 1999; Paquin et al., 2000). Verder suggereren de resultaten van dit proefschrift (hoofdstuk 7), dat kobalttoxiciteit vastgesteld kan worden aan de hand van de concentratie vrij kobalt. Met drie verschillende experimenten werden vergelijkbare IC_{50} waarden verkregen, met een standaarddeviatie van slechts 22%, wanneer alleen de vrije kobalt (Co^{2-}) concentratie werd meegenomen. Dit laatste geldt alleen indien de procescondities, zoals pH, concentratie van concurrerende metalen (bijvoorbeeld calcium of magnesium), enz., constant gehouden worden.

Niet alleen vrij kobalt kan door een celwand van een bacterie worden getransporteerd. Ook stoffen zoals bijvoorbeeld vitamine B_{12} (Ferguson and Deisenhofer, 2004), kobalt-citraat (Krom, 2002) en sideroforen (Worms et al., 2006) kunnen worden opgenomen door methanogenen. Wanneer evenwichtscondities zijn bereikt, kan niet alleen het vrije ion, maar elke metaalvorm worden gebruikt voor het voorspellen van metaaltoxiciteit (Worms et al., 2006).

Er is slechts een klein verschil tussen de optimale en de toxische concentratie vrij kobalt (Fig. 10.1). De optimale concentratie bleek ongeveer 7 µmol L^{-1} te zijn met een schijnbare K_M waarde van 0.9 µmol L^{-1} (gebaseerd op totale kobalt toevoeging) (Zandvoort et al., 2002a), terwijl een concentratie vrij kobalt van slechts ongeveer 18 µmol L^{-1} al significant toxisch is (50% inhibitie). Dit betekent dat bij aanwezigheid van slechts een geringe hoeveelheid

complexvormers in het medium, een relatief lage totale kobaltconcentratie operationele problemen zal veroorzaken in anaerobe reactoren.

De Donnan Membrane Techniek (DMT) werd met succes toegepast om de concentratie vrij kobalt (Co^{2+}) in anaëroob korrelslib te meten. Voordat metingen met monsters werden uitgevoerd, werd de geschiktheid van de techniek bepaald (Tabel 7.3). Op dezelfde wijze verkregen resultaten (Temminghoff et al., 2000) gaven een goede overeenstemming tussen berekende en gemeten waarden (met DMT) voor de concentraties vrije metalen van koper en cadmium, zonder en met anorganische- en organische (synthetische en natuurlijke) complexatie. De enige moeilijkheid is de lange tijd die benodigd is voor het verkrijgen van experimentele data (vier dagen voor instellen van evenwicht, vijf dagen voor de DMT procedure). Dit maakt DMT metingen ongeschikt voor het bepalen van concentraties vrije metalen onder de dynamische condities in bioreactoren.

10.5. EVALUATIE VAN DE GEKOZEN OPLOSSING: PULS DOSERING

Herhaaldelijke puls dosering van kleine metaalhoeveelheden is een effectieve doseerstrategie om een stabiele omzetting in methanolgevoede UASB reactoren te behouden (Fig, 8.1). Pulsdosering van kobalt (5 $\mu mol_{kobalt} \cdot L_{reactor}^{-1}$) in de vorm van kobaltchloride ($CoCl_2$) creëert een kobaltvoorraad in de matrix van korrelslib, 90% van de kobalt wordt daarin geïmmobiliseerd. Wanneer kobalt daarentegen als Co-$EDTA^{2-}$ word gedoseerd, wordt slechts 8% van de toegevoegde kobalt vastgehouden (Fig. 6.3). Hierdoor hoeft kobalt dosering, om limitatie te voorkomen, minder frequent plaats te vinden bij dosering van $CoCl_2$ dan wanneer kobalt als Co-$EDTA^{2-}$ wordt toegevoegd, namelijk een keer per 15 dagen en een keer per 7 dagen voor respectievelijk $CoCl_2$ en Co-$EDTA^{2-}$. Een ander nadeel van het gebruik van EDTA zijn de neveneffecten van EDTA op het korrelslib en de micro-organismen (hoofdstuk 8). EDTA is daarom een ongeschikte ligand voor sporen-element dosering aan full-scale reactoren.

De berekening van de hoeveelheid kobalt die moet worden gedoseerd om een adequate substraatomzetting te behouden is van groot belang voor de bedrijfsvoering van full-scale reactoren. In hoofdstuk 9 wordt deze berekening empirisch verkregen met behulp van data uit hoofdstuk 8. De hoeveelheid $CoCl_2$ die 15 dagen na de laatste $CoCl_2$ dosering moet worden toegevoegd, bij stabiele methanol verwijdering en een organische belasting van 8.5 g $CZV \cdot L^{-1}_{reactor} \cdot d^{-1}$, is 0.36 $\mu mol_{Co} \cdot L_{reactor}^{-1} \cdot d^{-1}$. In het geval van Co-$EDTA^{2-}$ pulsdosering, is de benodigde hoeveelheid kobalt, na een periode van 7 dagen stabiele methanolverwijdering na de laatste pulsdosering, 0.77 $\mu mol_{kobalt} \cdot L_{reactor}^{-1} \cdot d^{-1}$. Wanneer $CoCl_2$ continu wordt toegevoegd aan een methanolgevoede UASB reactor met een organische belasting van 2.6 g $CZV \cdot L^{-1}_{reactor} \cdot d^{-1}$, is een geschatte dosering van 0.15 $\mu mol_{kobalt} \cdot L_{reactor}^{-1} \cdot d^{-1}$ nodig om een stabiele methanolverwijdering te behouden (Zandvoort et al., 2002a).

10.6 PRESENTATIE VAN DE OPLOSSING: VOORSPELLING VAN DE METHANOGENE ACTIVITEIT DOOR MIDDEL VAN METAAL RETENTIE

Dit proefschrift toont duidelijk hoe de retentie van kobalt in het slib gedurende 24 uur na een kobaltpuls correleert met de SMA, wanneer methanol wordt gebruikt als substraat voor slib uit methanolgevoede UASB reactoren (gedurende 16 en 35 dagen afwezigheid van kobalt, Fig. 9.4). Deze correlatie toont aan dat niet alleen het gebruikte type ligand de omzetting in de reactor na de puls beïnvloedt, maar dat ook andere parameters dit beïnvloeden, bijvoorbeeld de concentraties van methanol en VVZ in het effluent en de kobalt concentratie in het slib voor de pulsdosering.

De correlatie tussen de metaalretentie en de reactorefficiëntie betekent echter niet dat de metaalretentie de oorzaak is van de verschillen in omzettingsefficiëntie. De inschattingen wat betreft efficiëntie moeten dus niet worden overschat, het vormt een basis voor verder onderzoek naar de kwantificering van de biobeschikbaarheid van metalen.

Biobeschikbaarheid is een kenmerk van de mate waarin een actieve verbinding een biologisch systeem bereikt en beschikbaar is op de plek waar de reactie plaatsvindt (Shargel and Yu, 1999). Na de metaalpuls wordt het ingevangen metaal geïntroduceerd in het biologisch systeem, maar het is daarmee niet per definitie biobeschikbaar. Om biobeschikbaar te worden, vinden veel verschillende mechanismen plaats die samenhangen met de vele relaties tussen metaalspeciatie en enzymatische reacties, zoals het oplossen van metaalsulfiden (Jansen et al., 2007), de excretie van liganden (Roth et al., 1996; Shaked et al., 2005), de metaaltranslocatie door de membranen (Koster, 2001), of de metaalallocatie in verschillende enzymen (Mulrooney and Hausinger, 2003). De verhoging van de SMA na de puls en de daaropvolgende afname hiervan (Fig. 9.2) laat zien dat een fractie van de totale hoeveelheid kobalt biobeschikbaar is. Gedurende een periode van kobaltlimitatie, neemt de cel waarschijnlijk het abiotisch gebonden kobalt op.

De uitkomsten van dit proefschrift laten zien dat de correlatie tussen de beginfase van de reactor na de toevoeging van de kobaltpuls en het gedrag van de reactor over een langere tijdsperiode, gebruikt kunnen worden voor optimalisatie van de strategie voor metaaldosering om de werking van een UASB reactor te optimaliseren (hoofdstuk 9). Deze correlatie kan worden gebruikt om de kobaltdosering in methanolgevoede UASB reactoren te minimaliseren en tegelijkertijd de efficiëntie voor verwijdering van organische stof van het bioproces te maximaliseren. Bovendien kan de correlatie tussen de initiële metaalretentie en de metaal biobeschikbaarheid een hulpmiddel zijn bij de strategie-ontwikkeling van de metaaldosering voor andere bioprocessen (Nicolella et al., 2000; Skiadas et al., 2003).

10.7. AANBEVELINGEN VOOR VERDER ONDERZOEK

10.7.1. Mechanistisch model voor metaal dynamica

De ontwikkeling van een mechanistisch model voor de dosering van kobalt kan ervoor zorgen dat er een veel preciezere kwantificering gemaakt kan worden van de benodigde hoeveelheid $CoCl_2$ van methanol gevoede UASB reactoren. Het mechanistisch modelleren van de kobalt opnamesnelheid is echter vrij gecompliceerd vanwege de complexiteit van het bestudeerde systeem waarin rekening gehouden moet worden met de relatie tussen chemische speciatie, biologische opname en groei, de hydrodynamica van de reactor en de biofilmeigenschappen (Jansen, 2004). Sommige van deze aspecten zijn reeds beschreven in de literatuur over biofilmsystemen (Flemming, 1995; Arican et al., 2002; Flynn, 2003; Wang et al., 2003; Weng et al., 2003; Worms et al., 2006), maar de belangrijkste aspecten, zoals de chemische interactie tussen kobalt en de slibmatrix, de kobaltopname en de groeikinetiek van de biomassa in korrelslib zijn nog niet gekwantificeerd (van Hullebusch et al., 2003).

10.7.2. Metaal dynamica in levende cellen

De mogelijkheid om 'real time' de concentratie van sporen-metalen in levende cellen te meten, is een belangrijke vereiste voor een beter begrip van metaaldynamica in levende cellen. Fluorescentiemicroscopie is hiervoor uitermate geschikt, de ontwikkeling van fluorescente probes die transitiemetalen met een hoge selectiviteit en affiniteit binden is een nieuw onderzoeksgebied (Van Dongen et al., 2006) en kan bijdragen aan het begrijpen van de interacties tussen metaal en micro-organisme op het niveau van intacte cellen in een biofilm systeem.

Het visualiseren van de verdeling van elementen binnen een cel of biofilm is mogelijk met resoluties beneden de micrometer en gevoeligheden tot op picogram niveau, dankzij vooruitgangen in "laser ablation massaspectroscopie" (MS), ion-beam en synchrotron radiatie X-ray fluorescentie microprobes (Gordon et al., 2008). Vooruitgang in de nano chromatografie en capillaire electroforese gecombineerd met element specifieke ICP MS (Saba et al., 2007; Schaumlöffel, 2007) en molecuul specifieke electrospray MS/MS en MALDI (Matrix-assisted laser desorption/ionization) (Tharamani et al., 2008), maakt het mogelijk de verdeling van elementen te meten in complexe biologische systemen zoals anaëroob korrelslib.

10.7.3. Metaal dynamica en vaste stof verdeling in anaëroob korrelslib

De dankzij het gebruik van intensere synchrotron stralen en efficiëntere optica steeds grotere gevoeligheid van EXAFS (Extended X-ray Absorption Fine Structure) en XANES (X-ray Absorption Near Edge Structure), biedt een unieke niet-destructieve mogelijkheid om in-situ relatieve hoeveelheden van functionele zwavel soorten te meten en te identificeren. Het geeft informatie over de oxidatiestaat en de structuur van metaalbindende locaties (Jalilehvand, 2006;

Lobinski et al., 2006). Het toepassen van meer uitgebreide zwavel sequentiële extractie methoden voor anaeroob korrelslib, die meer specifieke fracties opleveren, inclusief elementaire zwavel soorten en di-sulfiden (Canheld et al., 1998, van der Veen, 2004), die al succesvol zijn toegepast op zoetwater en mariene sedimenten, kunnen ook een bijdrage leveren aan het beter karakteriseren van de zwavelverdeling in anaëroob korrelslib. Nuclear magnetic resonance (NMR) methoden geven de mogelijkheid om transportprocessen, en de metaaldistributie te meten (Seymour et al., 2007). Ook metingen aan door cellen opgenomen kobalt, door bijvoorbeeld de toepassing van radioactieve metaal isotopen, kunnen bijdragen aan het karakteriseren van deze metaalfractie (Lombi et al., 2007).

10.7.4. Proces controle

Het DMT principe, zoals beschreven in hoofdstuk 7, kan ook "andersom" gebruikt worden om de toevoeging van sporen-metalen aan de reactor te sturen (Schöder, persoonlijke communicatie). Met het via een DMT membraan koppelen van de reactor aan een nutriënten oplossing, gebufferd door de toevoeging van een sterke ligand, kan de DMT cel worden gebruikt als een nutriënten bron met een gedefinieerde flux over het membraan oppervlak, waardoor de vrije ion activiteit van de reactor vloeistof kan worden gecontroleerd. De sensor en de dosering zijn dan gecombineerd in een enkele unit. In verder onderzoek kunnen andere manieren om kobalt te doseren ontwikkeld worden, met een minimaal verlies aan metalen door sturing van de kobalt oplossnelheid. Hierbij valt de denken aan "slow release capsules" (Im et al., 2005) of elektrochemische dosering van kobalt (Cosnier et al., 1994).

Er zijn elektrochemische sensoren ontwikkeld voor de analyse van sporen-metalen in water. Deze maken gebruik van microlithografisch geproduceerde of 'screen-printed' microelectrode arrays (Xie et al., 2005; Noël et al., 2006). De sensoren zijn vooral gebaseerd op stripping voltometrie (anodisch, cathodisch en adsorptieve voltametrie) en stripping potentiometrie (PSA) (Ostapczuk, 1993). De combinatie van micro-elektroden en computer gestuurde geminiaturiseerde instrumenten is erg geschikt voor de ontwikkeling van draagbare analytische instrumenten voor in-situ metingen van zware metalen in reactoren (Xie et al., 2005). Het meten van de sporen-metalen in een reactor systeem als een gecontroleerde parameter maakt het mogelijk om metaaldynamica op te nemen in het ontwerp van de processturing. Verschillende toepasbare processturingsstrategieën zijn voedingsgestuurd, supervisory, multivariabel en adaptieve controle, naast verfijnde digitale logische controle mechanismen (Stephanopoulos, 1984). Het toepassen van pulsdosering van metalen als een controle parameter in een dergelijk algoritme kan compenseren voor proces verstoringen zoals concentratie veranderingen in het influent, de afvalwatereigenschappen of temperatuurvariaties. Processturing kan dus de invloed van fluctuaties in de metaaldosering verminderen en zo de efficiëntie van afvalwater zuivering verhogen.

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LIST OF ABBREVIATIONS

A0: Retained cobalt during 24 hours upon a cobalt pulse minus B0
Area16: Area below the SMA-time profile 16 days upon a cobalt pulse
Area35: Area below the SMA-time profile 32 days upon a cobalt pulse
ASS: Acid soluble sulfur
AVS: Acid volatile sulfides
B0: Retained cobalt instantaneously upon a cobalt pulse
BLM: Biotic ligand model
COD: Chemical oxygen demand
CSTR: Complete stirred tank reactor
DGGE: Denaturing gradient gel electrophoresis
DGT: Diffusive gradient in thin films
DMT: Donnam membrane technique
EDTA: Ethylene diamine tetraacetic acid
EGSB: Expanded granular sludge bed
EPS: Extrapolimeric substance
FDH: Formate dehydrogenase
FIAM: Free ion activity model
FISH: Fluorescence *in situ* hybridization
HRT: Hydraulic retention time
I: Inhibition of specific methanogenic activity
IC_{50}: Concentration of metal to provoke 50 % inhibition
ICP-OES: Inductively coupled plasma optical emission spectrometry
K_M: Apparent affinity constant for metals
K_r: Retention constant, it s a sum of all involved metal retention mechanism
K_s: Affinity constant for substrate
MDI: Morril dispersion index
OLR: Organic loading rate
Q-PCR: Quantitative polymerase chain reaction
SEM: Simultaneously extracted metals
SMA: Specific methanogenic activity
S_{res}: Residual sulfur
TSS: Total suspended solids
UASB: Upflow anaerobic sludge bed
VFA: Volatile fatty acids
VSS: Volatile suspended solids

LIST OF PUBLICACTIONS IN SCIENTIFIC JOURNALS

Zandvoort, M.M., Van Hullebusch, E.D., Fermoso, F.G. and Lens, P. (2006) Trace metals in anaerobic granular sludge reactors: Bioavailability and dosing strategies. Engineering in Life Sciences 6 (3), 293-301.

Van der Veen, A., Fermoso, F.G. and Lens, P. (2007) Bonding form analysis of metals and sulfur fractionation in methanol-grown anaerobic granular sludge. Engineering in Life Science 7 (5), 480-489. A slightly modified version is presented in **Chapter 2**.

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Fermoso, F.G., Collins, G., Bartacek, J., O'Flaherty, V. and Lens, P. (2008) Role of nickel in high rate methanol degradation in anaerobic granular sludge bioreactors. Biodegradation DOI : 10.1007/s10532-008-9177-3. A slightly modified version is presented in **Chapter 4**.

Fermoso, F.G., Collins, G., Bartacek, J. and Lens, P.N.L. (2008) Zinc deprivation of methanol fed anaerobic granular sludge bioreactors. Journal of Industrial Microbiology & Biotechnology DOI: 10.1007/s10295-008-0315-z. A slightly modified version is presented in **Chapter 5**.

Fermoso, F.G., Bartacek, J., Chung, L.C. and Lens, P.N.L. (2008) Effect of chelation on cobalt dosing strategy in methanol fed anaerobic granular sludge bioreactors Biochemical Engineering journal. In press. A slightly modified version is presented in **Chapter 8**.

Bartacek, J., Fermoso, F.G., Baldó-Urrutia, A.M., van Hullebusch, E.D. and Lens, P.N.L. Cobalt Toxicity in Anaerobic Granular Sludge: Influence of Chemical Speciation. Submitted for publication. A slightly modified version is presented in **Chapter 7**.

Word, P.; Fermoso, F. G.; Plugge, C.M.; Lens, P.N.L. Transcription of formate dehydrogenase encoding genes in *Syntrophobacter* spp. And *Methanospirillum hungatei* in a UASB reactor lacking tungsten, molybdenum and selenium. In preparation. A modified version is presented in **Chapter 6**.

Fermoso, F. G.; Bartacek, J.; Manzano, R.; Lens, P.N.L. Cobalt dosing to anaerobic granular sludge bioreactors: Quantification of metal retention to predict methanogenic activity. In preparation. A slightly modified version is presented in **Chapter 9**.

Fermoso, F. G.; Bartacek, J.; Lens, P.N.L. Enhancement of the performance of anaerobic granular sludge bioreactors by pulse addition of vitamin B_{12} as cobalt source: Comparison with cobalt chloride pulse addition. In preparation.

Fermoso, F. G.; Bartacek, J.; Jansen, S.; Lens, P.N.L. Metal supplementation to UASB reactors: from cell-metal interactions to full-scale application. In preparation.

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Fernando G. Fermoso
Wageningen, The Netherlands, May 2008.

Netherlands Research School for the
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- **SENSE Symposium Sensible Water Technology, 12-13 April 2007, Leeuwarden, The Netherlands**

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