Trace metal dynamics in methanol fed anaerobic granular sludge bed reactors

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ABSTRACT

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Trace metals are essential for anaerobic microorganisms, because they are present as cofactor in many of their enzymes. Therefore anaerobic wastewater treatment systems using these microorganisms to perform biological conversions are dependent on these metals for their (optimal) performance. In practice these metals are supplied to the influent when they are absent or insufficiently present in the wastewater. This supply is generally not very rational. However, such a rational metal supply is desired in order to reduce the costs, to minimize the introduction into the environment and to maximize the biological activity. To achieve this insight, in the trace metal dynamics in anaerobic granular sludge bed reactors is required. This thesis therefore focuses on the retention, accumulation and release of trace metals in anaerobic granular sludge and the factors affecting these processes. Further the impact of metal presence/absence and dosing on the anaerobic conversion of methanol was addressed.

This investigation showed that trace metal deficiencies are in some cases already present in granular sludge from full-scale reactors, although mainly for the substrate methanol and for the metal cobalt, as evidenced by a significant increases of the specific methanogenic activity (SMA) of the sludge with methanol upon cobalt addition. If not already present limitations for cobalt, nickel or iron can be easily induced in Nedalco granular sludge (e.g. within ± 70 days), present in an upflow anaerobic sludge bed (UASB) reactor (pH 7; 30 °C) fed with methanol at an OLR of ± 5 g COD. l reactor^{-1}.d^{-1} under metal deprived conditions. The response of the systems to metal deprivation differed depending on the metal for which it was deprived. Cobalt deprivation leads to a low methanol removal capacity without any volatile fatty acid (VFA) accumulation, nickel deprivation resulted in methanol accumulation with a slowly increasing moderate VFA accumulation, while under iron deprived conditions an instant, fast and significant methanol and VFA accumulation occurred.

Different cobalt supply strategies (continuous, pulse and pre-loading of the sludge) to overcome and prevent limitations in the sludge were studied with respect to their effectiveness in metal retention and improvement of the methanogenic methanol conversion. Continuous cobalt dosing at low concentrations was found to be favorable with respect to the amount of cobalt required and the minimal losses with the effluent, although the impact on the SMA was relatively limited. After termination of the cobalt supply, the activity and methanol removal capacity could be maintained for more than 100 days. Pulse dosing comprises an intermediate dosing strategy between continuous dosing and pre-loading of the sludge, the strategy is very effective in overcoming almost immediately acute cobalt limitations. However, the losses of cobalt were considerably higher compared to the continuous dosing strategy. Pre-loading of the sludge, although also effective in overcoming cobalt limitations and resulting in high a SMA's, was ineffective reducing cobalt losses, especially immediately after reactor start-up.

The reactor conditions can not only influence the metal retention by the sludge they can also influence dynamics of the metals within the sludge. This research showed that the fractionation of the sludge metal content over operationally defined fractions by sequential extraction is a good method to create insight in these internal metal dynamics. Under 'normal' operational conditions cobalt leached from pre-loaded Nedalco granular sludge at an initial fast rate of ± 22 μg. g TSS^{-1}, the cobalt was lost from the more loosely bound exchangeable and carbonate fraction. After depletion of the latter fractions cobalt, was lost mainly from the more strongly bound organic/sulfide fraction of the sludge at a slower rate of ± 9 μg. g TSS^{-1}. The pH and sulfur source determine metal solubility and therefore are important operational parameters that can influence metal retention. For instance the presence of a sulfur source was required for the onset of iron and molybdenum accumulation, and the sulfur source (sulfate or cysteine) determined the preferred fraction for zinc accumulation in the sludge. Further short term pH shocks (pH 5; 30 h) were found to strongly affect the metal speciation in the granular sludge pre-loaded with cobalt, nickel and Iron.

Stellingen

1 Het is thans mogelijk de noodzaak van metaal dosering aan praktijkreactoren door uitvoering van eenvoudige laboratoriumtesten adequaat vast te stellen, derhalve gedurende het bedrijf van de reactoren te blijven volgen.
(dit proefschrift)

2 Het verdwijnen van methanogene activiteit op acetaat als substraat bij langdurig eenzijdig voeden van een anaërobe reactor op methanol draagt bij aan de instabiliteit van anaërobe bioreactoren welke methanol houdende afvalwaters moeten behandelen.
(dit proefschrift)

3 Yu en medewerkers (2000) spannen duidelijk het paard achter de wagen met het toedienen van zeer hoge ijzer (Fe^{2+}) concentraties (tot 800 $mg.l^{-1}$) aan de voeding van hun anaërobe reactor t.b.v. de anaërobe korrelslibvorming, want de ijzersulfide korrels die ze krijgen bezitten inferieure biologische eigenschappen.
Yu HQ, Fang HHP, Tay JH (2000). Effect of Fe^{2+} on sludge granulation in upflow anaerobic sludge blanket reactors, Wat Sci Tech, 41, 199-205

4 Hoewel de toekomst van wetenschappelijk onderzoek in sterke mate gebaat is bij het realiseren van goed functionerende multidisciplinaire samenwerkingsverbanden, moeten beleidsmakers er goed bewust van zijn dat de moeilijkheidsgraad van het van de grond krijgen hiervan veel gelijkenis vertoont met het inburgeringproces van allochtone medeburgers, er gaat aan tijd minstens een generatie mee gemoeid, in dit geval dus minstens een generatie promotie onderzoekers.

5 Een expert is iemand die op een heel klein gebied alle mogelijke fouten heeft gemaakt.
Niels Bohr, Deens Natuurkundige (1885-1962)

6 Het huidige "normen en waarden" debat munt uit in een dusdanige vrijblijvendheid t.a.v. de verantwoordelijkheid hierin van alle mogelijke (semi-)overheidsinstellingen, dat de gemiddelde Nederlander weinig vertrouwen kan opbrengen in de zin en geloofwaardigheid van de aangezwengelde discussies.

7 Ondersteun de bacteriën, het is de enige "cultuur" die sommige mensen hebben.
Steven Wright Amerikaans komiek en acteur

Stellingen behorende bij het proefschrift "**Trace metal dynamics in methanol fed anaerobic granular sludge bed reactors**" door Marcel Zandvoort, Wageningen 23 maart 2005

Contents

Chapter 1

Introduction

Biological effects of metals

All live forms are dependent on nutrients for their preservation and growth. Besides the macronutrients such as e.g. carbon, nitrogen. phosphorus and sulfur; also trace elements are essential for life. Trace elements can be defined as "any various chemical elements that occur in very small amounts in organisms, but are essential for many physiological and biochemical processes" [http://www.epa.gov/trs]. Many of these essential trace elements are metals. In general, the role of metals in biology has received a lot of attention and publications on the subject are numerous. Research towards the role of trace elements in humans, animal and plants mainly focuses on their mode of action in health and disease and nutritional importance.

Human

The effects of metals on human health are not only plenteous, they are also very complex, it is for instance well known that iron is an essential constituent of hemoglobin and thus regulates the uptake of oxygen in blood. The availability of nutritional non-heme iron is limited and deficiencies can occur even when it is present in the nutrition at apparently sufficiently high concentrations. The availability of iron can, however, be enhanced by for instance cysteine [Glahn and van Campen, 1997] or vitamin C [Halberg, 1995]. Although, iron supplementation can be positive in preventing anemia, it could at the same time promote the growth of the malaria parasite [Verhoef et al, 2002]. Another example of an important metal for humans is zinc, as it is a cofactor in over 300 enzymes [Coleman, 1992; Vallee and Falchuk, 1993]. Zinc was found to be important for the human immune system, e.g. supplementation of zinc is even effective in reducing the duration of the common cold [Hulisz, 2004]. Several trace elements can also influence the cognitive functions: Se, Cr, Co and Fe have a positive effect, while Cu and Al negatively influence the cognitive function, aluminum for example contributes to the pathogenesis of Alzheimer dementia [Smorgon et al., 2004].

Animal

The requirement for trace metals for animals is evident as well and they are generally supplemented with commercial animal feeds to improve animal health and growth. The bioavailabity of these trace elements is essential. The processes determining whether a metal is bioavailable for animals can be complex and unanticipated. For instance, cattle grazing in molybdenum rich pastures can develop secondary copper (and sulfur) deficiency due to the antagonistic effect of molybdenum on copper uptake [Farmer et al., 1982]. The requirement of metals varies significantly between animals species, e.g. sheep are very sensitive to copper

toxicity [Ishmael et al., 1972], while copper is supplemented (max. conc. 170 $mg.kg^{-1}$) to the feed of piglets to promote their growth [Janssens, 2004]. The manure of piglets contains such high concentrations (up to 914 $mg.kg^{-1}$ dry matter) of copper [Jordeville et al., 2003] that sheep are not allowed to graze on pastures fertilized with pig manure. New legislation of the European Union aims at the reduction of the metal output to the environment, the maximum concentrations of trace metals allowed in animal feeds has therefore been reduced (Directive 70/524/EEC, maximum levels of trace metals, SANCO/367 rev. 2/2000). In order to have the same impact on animal health and growth, the limited amount of metal supplied with the feed should be (more) bioavailable. As a result a new source of trace metals, metals chelated to amino acids and organic acids, has become more important [Janssens, 2004]. In theory, these metal have a higher bioavailability, because of the different uptake mechanism and lower competition with other complex forming compounds in the feed and intestines [Apines et al., 2003].

Microorganism

Similar to higher life forms, microorganisms are dependent on trace elements as well. The effects of metals on humans and animals described in the above section can be more or less directly translated to microorganisms viz. the requirement, essentiality and effects of a trace metal may vary with the species and the metal bioavailability for microorganisms is a very intricate subject as well. The essential metals are often present in the enzyme system as part of a cofactor or they are of vital importance for the enzyme system. An overview of some of the metallo-enzymes present in different microorganisms and the metals that are essential for these enzymes is presented in Table 1. On the non-enzymatic level some metals can be involved in microbial processes with the electron transfer in redox reactions. For example, this is the case for Fe(III)- and Mn(IV)-reducing bacteria [Lovley, 1993; van der Maas, 2005]. On the other hand, all metals are potential toxicants. "Metal toxicity" is the generic term for the total of possible ways in which metals may inhibit microbial activity.

Biological effects of metals in bioreactors

The essential requirement of trace metals for optimal functioning of microorganisms and the thus the conversions that they perform is clear (Table 1). Many biotechnological processes use microorganisms, these processes are therefore highly dependent on the presence of metals for their optimal performance. For example, in anaerobic wastewater treatment systems such as upflow anaerobic sludge bed (UASB) reactors a consortium of microorganisms is present immobilised in sludge granules. The microorganisms present in these granules degrade organic compounds to methane (CH_4) and carbon dioxide (CO_2) via a

complex metabolic network (Fig. 1). Each subsequent conversion is carried out by a different microbial group, which has different metal requirements.

Table 1. The key metals in enzymes of microbial conversions

Enzyme	Organism(s)	Metal	Reference
Methyltransferase	Methanogens and acetogens	Co (B12)	Beveridge and Doyle, 1989
B12-enzymes	Many organisms	Co (B12)	Beveridge and Doyle, 1989
CO-dehydrogenase	Methanogens/Acetogens	Co, Ni, Fe	Ferry,1999
Acetyl-CoA synthase	*Moorella thermoacetica*	Fe, Ni, Cu	Seravalli et al., 2003
Tetrachloroethene reductive dehalogenase	*Dehalospirillum multivirans*	Co, Fe	Neuman et al., 1996
Methyl-CoM-reductase	Methanogens	Ni	Hausinger, 1987
Uerase	Several organisms	Ni	Hausinger, 1987
Hydrogenase	*Desulfovibrio*	Fe	
		Ni, Fe	
		Ni, Fe, Se	Fauque et al., 1988 ; Albracht, 1994
	E. coli	Ni, Fe	Sawers, 1994
	Facultative anaerobes	Cu, Zn	Takashima and Speece, 1990; Patel et al.,1993
MMO (free)[1]	*M. trichosporium*	Fe	Lipscomb, 1994
NO-reductase	*P. denitrificans*	Fe (haem)	Ferguson, 1994
Nitrite reductase	*P. stutzeri*	Cu, Fe (haem)	Ferguson, 1994
Ammoniummonooxygenase	*N. europaea*	Cu	Ensign et al., 1993
SOD aerobes, anaërobes[2]		Fe, Cu, Zn, Mn	Hughes and Poole, 1991
Formiate dehydrogenase	*Methylobacterium*	Mo or W	Girio et al., 1992
	E. coli	Mo-Se	Sawers, 1994
Formylmethanofuran-dehydrogenase	*M. thermoautotrophicum*	Mo or W[3]	Bertram et al., 1994
Aldehyde-oxydoreductase[4]	*Clostridium*	Mo or W	White and Simon, 1992
Nitrate reductase	*P. denitrificans*	Mo, Fe, Fe (haem)	Ferguson, 1994
Nitrogenase		Mo, Fe	Schindelin et al., 1997
	M. Barkeri	Mo or V, Fe	Chien et al., 2000
Chloroperoxydase	*C. inaequalis*	V	Schijndel et al.,1993
Bromineperoxydase	*A. nodosum*	V	Schijndel et al., 1993
Glycin reductase	*E.coli*	Se	Heider and Bock, 1993

[1] Methane monooxygenase; [2] Superoxide dismutase; [3] With W in the growth medium an iso-enzyme was synthesized.

Anaerobic degradation of complex organic compounds

The following conversions and trophic groups of microorganisms are involved in anaerobic degradation of complex organic compounds (Fig 1.). The primary fermentative bacteria first hydrolyse polymers such as proteins, carbohydrates and lipids to monomers, they then further ferment these monomers to e.g. acetate, carbon dioxide (CO_2), hydrogen, alcohols lactate and fatty acids. Secondary fermentative bacteria or obligate hydrogen producing bacteria are required for the degradation of fatty acids longer than two C-atoms and alcohols longer than one C-atom.

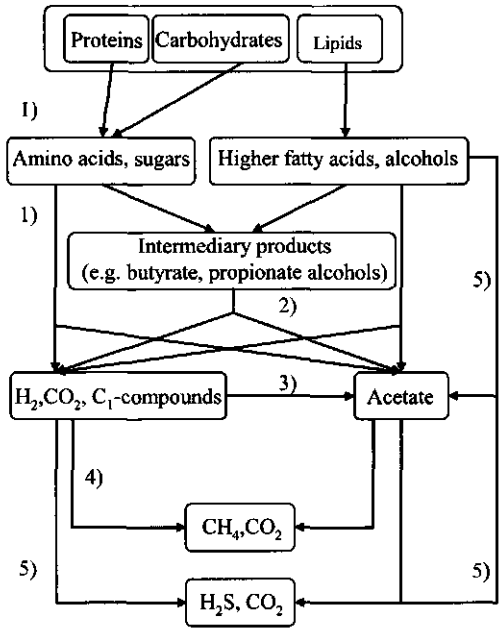


Figure 1 Anaerobic conversion of complex organic substrates; 1. Hydrolyses by primary fermentive bacteria, 2. Secondary fermentive bacteria, H_2 producing (syntrophic) bacteria, 3. Homo acetogenic bacteria, 4. Methanogenesis by acetotrophic-, methylotrophic- and hydrogenotrophic methanogens, 5. Sulfate reduction.

The final step in the anaerobic degradation process is performed by methanogenic archae. These methanogenic archae are highly specialized and they can only use H_2/CO_2, acetate, formate and other C_1-compounds such as methanol, methylamines and methylthiols as substrates, which they disproportionate to CH_4 and CO_2 [Thauer et al., 1998]. Another important process in anaerobic treatment systems is sulfate reduction (Fig. 1), were sulfate reducing bacteria convert sulfate into hydrogen sulfide. Sulfate reducing bacteria are able to use several intermediates of the anaerobic mineralization process as substrates, not only the

direct methanogenic substrates but also e.g. the propionate, butyrate, higher branched fatty acids, lactate, ethanol, higher alcohols [Colleran et al., 1995]. Hence, different groups of microorganisms present in the bioreactor will compete for the same substrate.

Metal requirement of methanogens

As described in the above section, the final step of the anaerobic conversion is performed by methanogens. Methanogens can be stimulated by various metals (Table 2), but all methanogens were found to require cobalt, nickel and iron [Whitman, 1985]. Table 2 gives an indication of the medium concentrations that were stimulating for pure cultures of methanogens and illustrates that their needs for metals can vary strongly [for review see; Takeshima and speece, 1990; Jarrel and Kalmokoff, 1988]. The cell metal content of 10 methanogens has been determined by Scherer et al. [1983], this study showed that the metal content varies considerably between the different species of methanogens even when they are from the same genus and converting the same substrate.

Table 2. Metal stimulation of pure cultures of methanogens.

Pure culture	Conversion	Stimulating conc. (µM):	Reference
Methanosarcina barkeri	Methanol (methanogenic)	Fe(II) (35)	Lin et al., 1990
Methanosarcina barkeri	Methanol (methanogenic)	Co (1), Ni (1), Se (1), Mo (1)	Scherer and Sahm, 1981
Methanothrix soehngenii VNBF	Acetate (methanogenic)	Fe (20-100), Co (2), Ni (2), Mo (2)	Fathepure, 1987
Methanobacterium thermoautotrophicum	H_2/CO_2 (methanogenic)	Se (1), W (10)	Gerhard et al., 1993
Methanobacterium thermoautotrophicum	H_2/CO_2 (methanogenic)	Fe (>5), Co (>0,01), Ni (>0,1), Mo (>0,01)	Schönheit et al., 1979
Methanosarcina barkeri	Nitrogen fixation (methanol C: source)	Mo (5) or V (2)	Scherer, 1989
Methanococcus ofnielli	growth on formiate	Se (1), W (100)	Jones and Stadtman, 1977
Methanospirillum hungatei GP1	H_2/CO_2 (methanogenic)	Mn (50)	Pankhania and Robinson, 1984

Metals in the metabolic pathways of methanogenesis

The enzymology of methanogenesis and acidogenesis has been studied extensively, which resulted in a detailed description of the hypothetical metabolic pathways of methanogenesis from methanol, H_2/CO_2 and acetate and the formation of acetate from methanol [Ferry, 1999; Thauer, 1998; Shima et al., 2002]. Figure 2 presents the different metabolic pathways and the enzymes involved. Several of the enzymes in these pathways

contain metals such as cobalt, nickel, iron, zinc, molybdenum and/or tungsten in the form coenzymes and cofactors (Fig. 2), which explains the high dependence of methanogens on the presence and availability of these metals.

All methanogenic pathways converge to the enzymatic reduction of methyl coenzyme M to methane (Fig 2). This reduction is catalyzed by the Methyl-coenzyme M reductase complex, which contains a nickel containing cofactor called F_{430} [Friedman et al.,1990]. Another metallo-enzyme that is present in the methanogenic pathways is the cobalt/corrinoid containing methyl-H_4MPT:Coenzyme M methyltransferase complex [Thauer, 1998]. The first step of methanogenesis from methanol is also catalyzed by a specific cobalt dependent methyltransferase, Methanol:Coenzyme M, next to cobalt this enzyme contains also zinc [Sauer and Thauer, 2000].

The key enzyme complex in the methanogenesis from acetate is carbon monoxide dehydrogenase (CODH). CODH cleaves the C-C and C-S bonds in the acetyl moiety of acetyl-CoA, oxidizes the carbonyl group to CO_2 and transfers the methyl group to Coenzyme M. The CODH complex is composed of two enzyme components: a nickel/iron-sulfur component and a corrinoid/iron-sulfur component [Ferry, 1999]. This enzyme complex is also involved in the formation of acetate by acetogens from e.g. H_2/CO_2 and methanol [Bainoti and Nishio, 2000].

Formylmethanofuran dehydrogenase is a molybdenum containing enzyme that catalyzes the terminal step in the oxidation of methanol to CO_2 (Fig. 2A) and the first step in CO_2 reduction to CH_4 in autothrophic CO_2 fixation (Fig. 1B) [Bertram and Thauer, 1994; Wasserfallen, 1994]. *Methanobacterium thermoautotrophicum* contains two formylmethanofuran dehydrogenase iso-enzymes, a tungsten form and a molybdenum containing form. The molybdenum enzyme is synthesized only when molybdenum is available in the growth medium. The tungsten enzyme is synthesized when either tungsten or molybdenum is present. If the growth medium contains molybdenum, the tungsten enzyme will contain molybdenum instead of tungsten [Hochheimer et al, 1995].

Hydrogenases play a key role in the metabolism of methanogenic Archaea during autotrophic growth on H_2/CO_2, uptake hydrogenases consume hydrogen to provide electrons for the reduction of CO_2 to CH_4 (Fig 2B) [Hausinger 1994]. Two of the four reductive steps require reduced F_{420} (Fig. 2B), this reduction is catalyzed by F_{420}-reducing hydrogenase. This enzyme of e.g. *Methanosarcina barkeri* contains nickel and iron [Michel et al, 1995], while the hydrogenase of *Methanoccus voltae* grown in the presence selenenium, contains this metal as well. In the absence of selenium, a selenium free hydrogenase is produced [Berghöfer et al., 1994].

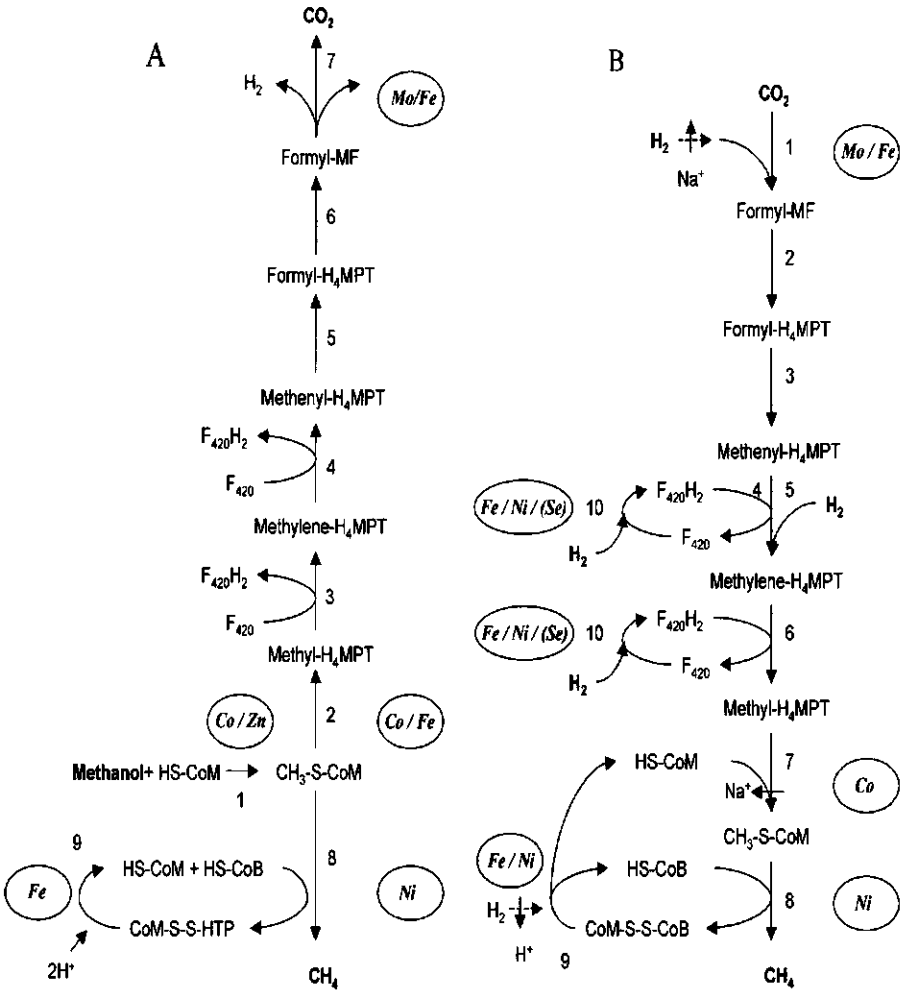
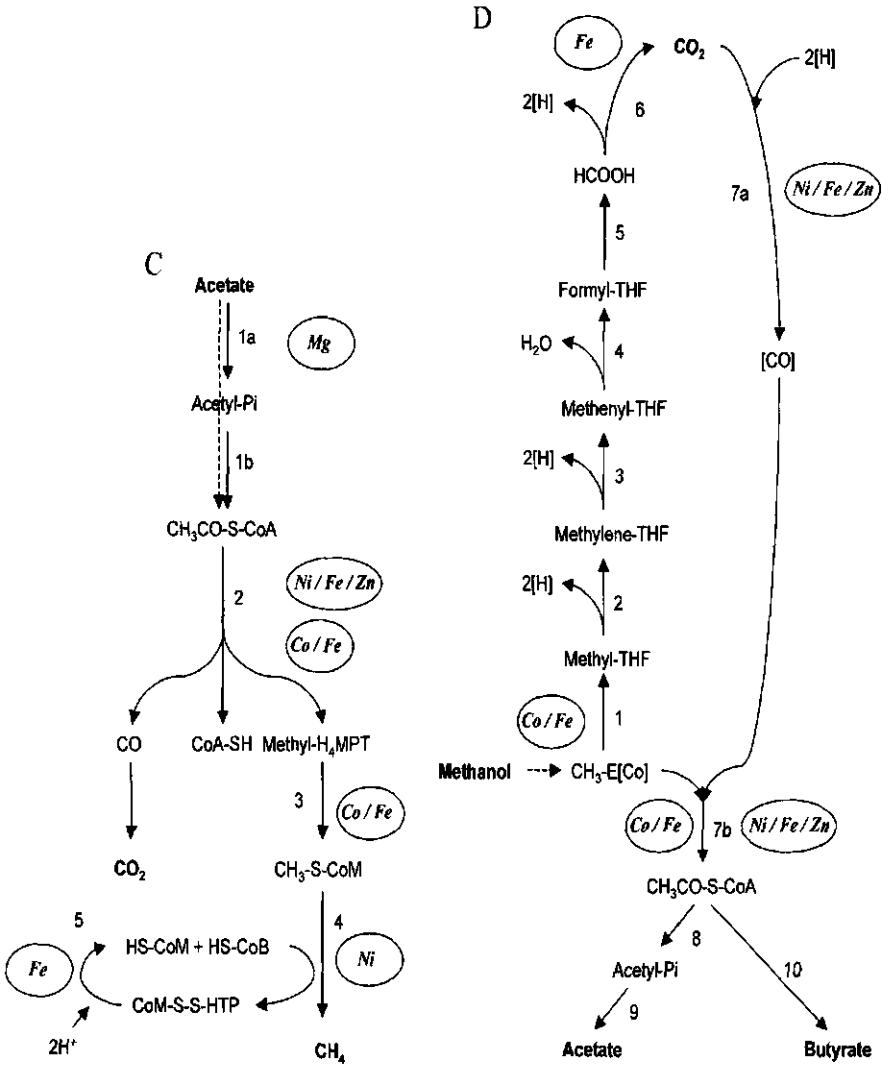


Figure 2 Metabolic pathways. MF, methanofuran; H_4MPT, tetrahydromethanopterin; HS-CoM, Coenzyme M, HS-CoB, coenzyme B; CoM-S-S-CoB, heterodisulfide of HS-CoM and HS-CoB; CH_3CO-S-CoA Aceteyl coenzyme A; Acetyl-Pi, Acetyl phosphate; THF, tetrahydrofolate; CH_3-E[Co], corrinoid bound methyl.

(A) Enzymes involved in methanogensis from methanol (1) Methanol:Coenzyme M methyltransferase; (2) methyl-H_4MPT:Coenzyme M methyltransferase; (3) Methylene-H_4MPT reductase; (4) methylene-H_4MPT dehydrogenase; (5) Methenyl-H_4MPT cyclohydrolase; (6) Formylmethanofuran: H_4MPT transferase; (8) Methyl-coenzyme M reductase; (9) Heterodisulfide reductase.

(B) Enzymes involved in methanogensis from H_2/CO_2, (1) Formylmethanofuran dehydrogenase; (2) Formylmethanofuran: H_4MPT formyltransferase (3) Methenyl-H_4MPT cyclohydrolase; (4) F_{420} dependent methylene-H_4MPT dehydrogenase ; (5) H_2-forming methylene-H_4MPT dehydrogenase; (6) Methylene-H_4MPT reductase; (7) Methyl-H_4MPT:Coenzyme M methyltransferase; (8) methyl-coenzyme M reductase; (9) Heterodisulfide reductase; (10) F_{420}-reducing hydrogenase.



(**C**) Enzymes involved in methanogensis from acetate. (1a) Acetate kinase and (1b) Phosphotransacetylase in *Methanosarcina* and catalyzed directly by Acetate thiokinase in *Methanosaeta* (dotted arrow); (2) CO dehydrogenase; (3) Methyl-H_4MPT:Coenzyme M transferase (4); Methyl-CoM reductase; (5) Heterodisulfide reductase.

(**D**) Enzymes involved in acetate (and or butyrate) formation from methanol by acidogenic bacteria (1) methyltransferase; (2) methylene-THF reductase; (3) methylene-THF dehydrogenase; (4) methenyl-THF cyclohydrolase; (5) Formyl-THF synthase; (6) formate dehydrogenase; (7a) CO dehydrogenase; (7b) acetyl-CoA synthase; (7a and 7b are catalyzed by the same enzyme); (8) phosphateacetyltransferase; (9) acetate kinase

Dosing metals to bioreactors

Metal dynamics in bioreactors

For reactor operation practice a better understanding of the effect and effectiveness metal supply is required, in order to reduce the costs of metal supply, to minimize the introduction of metals into the environment (with the effluent and sludge) and to maximise the effect on the biological activity. The practical questions that should be answered to achieve an optimised metal supply are e.g., which metals are essential for the anaerobic treatment of different wastewater streams, how should the metals be supplied to the bioreactor, when should they be dosed, how long can the reactors operate stably without metal supplementation, can the bioavailability of the supplied metals be increased and can the sludge provide in its own metal needs from the "stock" present within the granule. In order to be able to answer these questions, a multidisciplinary approach which integrates the microbiological, physical-chemical and environmental technological aspects of metal supply is mandatory (Fig. 3). Fundamental knowledge concerning the effects of metals on microorganisms (trophic groups), the impact of media composition and the impact sludge matrix on the metal bioavailability has to be incorporated in strategies for metal supply in practice.

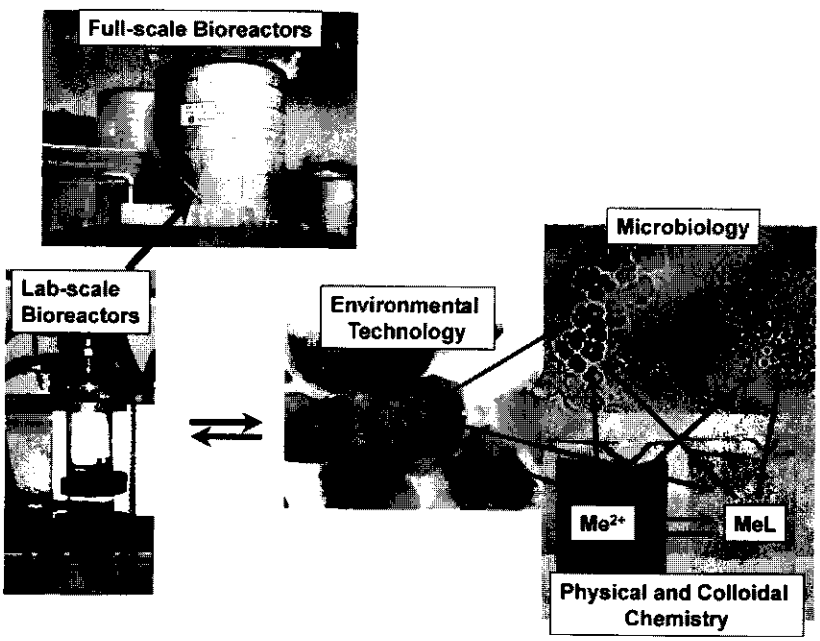


Figure 3 Multidisciplinary approach required for determining the interactions of metals with the solid phase, liquid phase and the microorganisms present in anaerobic bioreactors, which will lead to a more rational and efficient metal supply to bioreactors.

Metal speciation in solution

The fate (bioavailability and retention) of metals in the bioreactors depends on the complex interactions between the liquid phase and the solid phase present in the bioreactor (Fig. 4). The uptake of metals by microorganisms is assumed to proceed mainly via the transport of free metal ions over the cell membrane. However, before the essential metals actually reach the biomass present in the sludge granule, they are subjected to complex (bio)chemical processes in the reactor liquid such as precipitation (e.g. as sulfides), inorganic- and organic complex formation. These processes can reduce the free metal concentration in solution to extremely low values. A lot of research has been published on the metal speciation and the effect of metals on organisms in natural systems. However, little knowledge is available on the relation between metal speciation and metal bioavailability in anaerobic bioreactors.

In general anaerobic wastewaters have a high capacity to form metal complexes. Relatively high concentrations of inorganic ligands such as S^{2-}, PO_4^{3-} and CO_3^{2-} are present in the wastewaters. The importance of PO_4^{3-} and CO_3^{2-} complexes was already shown for anaerobic media [Callendar and Barford, 1983], but also dissolved sulfide complexes are very important in the metal speciation [Jansen, 2004]. Next to inorganic ligands, bioreactors often contain a high concentration of soluble microbial products (SMP) as well [Barker and Stuckey, 1999]. These SMP can have a metal binding capacity as was demonstrated for nickel [Kuo and Parkin, 1996]. In some cases, microorganisms were even found to actively excrete organic ligands to overcome metal limitation, e.g. in case of iron [Neilands, 1995] and cobalt [Saito, 2002], but so far such an active involvement in acquiring essential metals has not been demonstrated for anaerobic microorganisms. In an extensive study towards the effect of metal speciation on the bioavailability in anaerobic wastewater treatment, Jansen [2004] concluded that in cases were the free metal ion concentration is controlled by precipitation equilibria, strong complexation acts as a dissolved metal buffer, preventing the system from dissolution rate limitation.

Metal dynamics in granular biomass

The metals supplied to the bioreactor have to be retained by the sludge in order to prevent losses with the effluent and to be available for the biomass present in the granules (Fig. 4). The main mechanisms involved in the accumulation of metals within biofilms are complex formation, chelation of metals, ion exchange, adsorption, inorganic micro-precipitation and translocation of metals into the cells [Veiglio and Beolchini, 1997; van Hullebusch et al., 2003]. Precipitation of metals, e.g. as carbonates and especially as sulfides, is important for the accumulation of metals in the sludge. Sulfide is ubiquitously present in anaerobic bioreactors because of the occurrence of sulfate reduction or organic matter

mineralization. This property of sulfides is even used for the removal of metals from wastewaters [Kaksonen et al., 2003; Jong and Perry, 2003]. Although, the metals are better retained in the sludge due to sulfide precipitation, it may also influence their bioavailability, e.g. making them no longer 'directly' bioavailable [Gonzalez-Gil et al., 2003]. This may be especially the case when the sulfide precipitates age with time from amorphous to more crystalline forms [Jansen et al., 2004]. Moreover, metal precipitates (sulfides and carbonates) may actually take part in the sludge granulation process in anaerobic bioreactors [Oleskiewicz and Romanek, 1989; Shen et al., 1993b; Yu et al., 2000; Sharma and Sing, 2001].

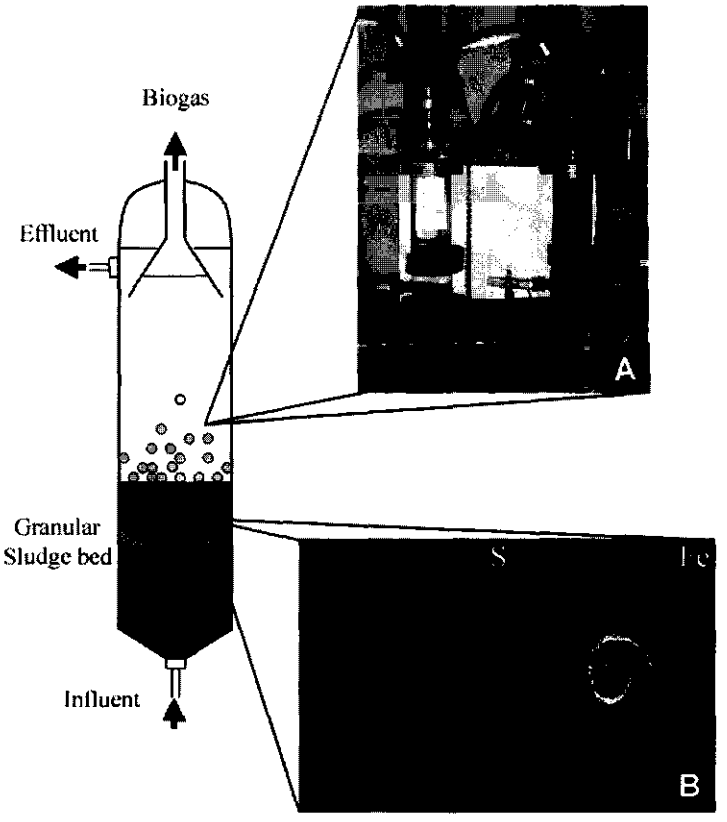


Figure 4 Metal speciation in solution and biomass of bioreactors (A) UASB reactors used in Chapter 6 fed without (left) and with (right) a sulfur source. The black liquor (right reactor) indicates the formation of metal sulfide precipitates and/or dissolved metal sulfide complexes. (B) Energy dispersed 'X' ray analyses (EDXA) photographs showing the sulfur and iron distribution (light zones) in a sludge granule.

Van Hullebusch et al., [2004, 2005a] studied the kinetics and capacity of cobalt and nickel sorption onto granular sludge. The sorption capacity, expressed in terms of q_m (Langmuir saturation constant), was generally lower when compared with other sorbents (Table 3), however, from a microbial point of view such metal contents are still substantial. Considering the complexity of the granular matrix, the total metal content of the sludge is a poor indicator of metal bioavailability and mobility.

Table 3. Comparison of the Langmuir constant (q_m) for nickel and cobalt for different sorbents.

Metal	Sorbent	q_m ($mg.g^{-1}$)	T(°C)	pH	Reference
Nickel	Anaerobic granules	7.9	30	6	van Hullebusch et al.,
		9.4	30	7	2005a
		11.5	30	8	
Nickel	Anaerobic digested sludge	25.2		7.2	Artola et al., 2000
Nickel	Sphagnum moss peat	9.7	4.5		Ho et al., 1996
Nickel	Anaerobic dead biomass	227			Haytoglu et al., 2001
	Dried *Chlorella vulgaris*	54.8			Aksu, 2002
Nickel	Biomass *Pseudomonas aeruginosa*	62.6	35	7.2	
Nickel	Free celss	145	30	8	Lopez et al., 2000
	Immobilized cells	37	30	8	
Cobalt	Anaerobic granules	8.4	30	6	van Hullebusch et al,
		8.9	30	7	2005a
		9.5	30	8	
Cobalt	Ion exchange resin	60.0	25	5.3	Rengaraj et al., 2002
		75.6	25	5.3	
Cobalt	Carbon sorbent	17.3	25	6	El-Shafey., 2002
Cobalt	*Oscillatoria anguitissima*	131.5	25	5	Ahuja et al., 1999
	Biomass	150.0	25	6	

Little is known about how the prevailing reactor conditions can influence the distribution of the trace metals over different (chemical) fractions present within the granular sludge. Only a few attempts to fractionate the metal content present in anaerobic sludge granules have been reported in literature, e.g. Shen et al. [1993a] studied the effect of heavy metals on the extra cellular polymeric substance (EPS) and Espinosa et al. [1995] used a sequential extraction scheme to define the metal fractionation in anaerobic sludge granules during different operational periods. Sequential extraction schemes are widely used for the evaluation of availability and mobility of trace elements in solid matrices [Filgueiras et al., 2002]. In these schemes, extractants are applied in order of increasing reactivity so that the successive fractions obtained correspond to metals associated in a form with lower mobility [van Hullebusch et al., 2005b]. Despite its intrinsic disadvantages (operationally defined, e.g. extraction method determined), sequential extractions are a useful tool to gain insight in the metal dynamics within granular sludge as a function of operation time and reactor conditions.

Stimulating anaerobic treatment processes by metals

Anaerobic bioreactors

Although trace metals are essential for anaerobic treatment processes, the supply of metals to bioreactors has received less attention than the inhibiting effects of metals on the microbial activity due to toxicity. There are, however, some examples in literature that do report the stimulating effect of metal supply to full- and lab-scale UASB reactors (Table 4) and other types of anaerobic reactor (Table 5). Metal deficiencies of anaerobic treatment systems are therefore certainly not a rare phenomenon and the importance of metal supply is still largely underestimated. For instance Speece et al. [1986] showed that the removal capacity of 10 out of 30 anaerobic treatment systems could be improved by the addition of cobalt, nickel and iron. The stimulating effects of metals can occur even when high total concentrations of these metals are present in the reactor system, this shows that the metals can be present in a non-bio available form. For instance Ni (10 μM) stimulated the biogas production of a chicken manure digester, while nickel was present in the effluent (253 μM) before 'extra' Ni was added [William *et al.*, 1986]. In a reactor treating a high sulfide cane stillage, iron bioavailability decreased due the formation of sulfide precipitates, iron had to be supplied continuously at a concentration as high as 600 $mg.l^{-1}$ in order to improve the acetic acid conversion [Callendar and Barford, 1983]. Some industrial wastewaters e.g. condensates may be devoid of one or more essential trace metals, which makes additional metal supply to systems treating such wastewaters essential.

Table 4. Stimulation of biological conversions by metal supply in UASB reactors.

System	Conditions	Experiment	Parameters	Results	Ref.
UASB, fatty acids C2:C3:C4 = 4:1:1	Granular sludge. buffer HCO_3^-; pH = 6.8; T = 35°C; COD-load= 6.5 g COD.l^{-1}; HRT 0.6 d.	5 different combinations of Fe, Ni and Co. I: 200 d. infl. + yeast extract (100 mg.l^{-1}); II: 60 d. infl. - yeast extract; III: 30 d liquid recirc., no feed. trace-metals with or without Fe (1100 µg.l^{-1}), Ni (50 µg.l^{-1}), Co (75 µg.l^{-1})	COD-removal Metal content granules; Metal content extra cellular polymers	After period II: - Fe/Ni/Co COD-rem. % = 81.1% - Fe/Ni/Co COD-rem. % = 98.7% with Co/Fe COD-rem. % = 99.5% with Ni/Fe COD-rem. % = 98.5% with Ni/Co COD-rem. % = 52.3% 22% increase Fe (1100 µg.l^{-1})	Shen et al., 1993a
UASB, influent fatty acids C2:C3:C4 = 4:1:1	Granular sludge. buffer HCO_3^-; pH = 6.8 ± 0.2; Organic loading rate (OLR): stepwise to 39.6 g COD.l^{-1}.d^{-1}.	8 combinations of Fe, Co and yeast extract. trace-elements with or without Fe (1000 µg.l^{-1}), Co (100 µg.l^{-1}), yeast extract	COD-removal; Metal composition granules; Settleability granules.	After 56 days reactor operation: - Fe/Co/yeast COD-rem.= 57.8% + Fe/Co COD-rem. = 93.0% + Fe COD-rem. = 93.9% + Co COD-rem. % = 75.1% 62% increase by Fe (1000 µg/l); 30% increase by Co (100 µg/l);	Shen et al.. 1993b
UASB; Substrate; Cane molasses stillage	OLR 21.5 kg COD. m^{-3}.d^{-1}; pH_{inf} = 4.7, $pH_{eff.}$ = 7.8; T = 35 °C	Metals (in mg.l^{-1}) Fe (100), Ni (15), Co (10) and Mo (0.2).	Methanogenic conversion of vinasse	Influent metal supply reduced the effluent VFA conc. by 94%, the COD rem. and gas production increased by 32 and 38 %, respectively. The SMA on propionate increased from 0.085 to 0.32 g CH_4-COD.gVSS^{-1}.d^{-1}, and on acetate from 0.23 to 0.32 g CH4-COD.gVSS-1.d-1	Espinosa et al., 1995
Lab scale methanol fed UASB-reactors, receiving metal cocktail with or without Co	Granular sludge from UASB treating distillery wastewater. T = 30°C, pH_{batch}= 6.8	Effect of cobalt on the methanol conversion.	Methanol, VFA, biogas composition, Metal content of the sludge	UASB with cobalt Supply complete COD removal. UASB without Co supply COD-removal low on average ± 40%.	Florencio et al., 1993
Lab-scale UASB reactors fed with food industry wastewater	Sludge from pilot reactor with the same influent; pH: 6.9-7.3	effect of de addition of (in mg.l^{-1}); Fe^{3+} (20-40), Ni (0.5), Co (0.5)	VSS, methanogenesis	Faster sludge growth during start-up when Fe/Ni/Co are supplied; Higher COD-rem, better sludge retention and granule formation when Fe/Ni/Co are supplied.	Oleszkiewicz and Romanek, 1989
Fed- Batch (granular UASB sludge); distillery wastewater	OLR 5.9 kg COD.m^{-3}.d^{-1}; pH 6.7-7.5; T = 35 °C	(in mg.l^{-1}) $FeSO_4.7H_2O$ (10 and 50) $NiSO_4.6H_2O$ (0.1 and 1) $CoCl_2.6H_2O$ (0.2 and 2)	Methanogenesis	Metals improved both SVI and methanogenic activity of the sludges	Sharma et al., 2001

Table 5. Stimulation of biological conversions by metal supply in continuous and batch anaerobic systems.

System	Conditions	Experiment	Parameters	Results	Ref.
Anaerobic downflow fixed film reactors; substrate: Bean wastewater	I: pH = 6.5 – 7.2; acetate = 17to 62 mM II: COD load resulted in $VFA_{eff.}$ 200 to 400 $mg.l^{-1}$; III: COD load resulted in VFA_{eff} ± 300 $mg.l^{-1}$	Effect of 100 nM, Ni, 50 nM Co and 50 nM Mo on the specific methanogenic activity (SMA) on acetate: I effluent II performance reactor, III performance newly started reactors.	Biogas formation; gas composition; VFA	I: SMA stimulated by Ni en Co; Mo stimulates only in combination with Ni en Co; II: 42% higher OLR after Ni, Co and Mo were supplied with the influent III: 2 times higher OLR possible when Ni, Co and Mo is supplied reactor.	Murray & van den Berg, 1981
AFEB reactor (Anaerobic Film Expanded Bed); Whey 10 $g.l^{-1}$	buffer HCO_3^{-1}, 5 $g.l^{-1}$; T: 20, 25, 30 en 35 °C; pH=6.9	Operation at different temperatures; without trace metals and with trace metals	Methane formation; fatty acid concentration	T=35°C: COD-rem.79.6% +metal/ 60.0% -metal. T=30°C: COD-rem 75.5% +metal/ 47.5% -metal. T=25°C: COD-rem 64.7% +metal./ 37.2% -metal.	Kelly & Switzen-baum, 1984
Upflow flocculent sludge reactor; substrate: sugar cane fermentation waste; no trace-metal supply	after 120 days sudden reactor performance deterioration	Effect of iron addition (batch-activity : 140 $mg.l^{-1}$ FeCl2; reactor 600 $mg.l^{-1}$ FeCl2)	VFA, VSS, methane formation	Batch: 20 times increased acetate conversion ate; 0.7 to 5.0 $kgCOD.m^{-3}.d^{-1}$ VFAeffluent:>600 to 100 $mg.l^{-1}$	Callander & Barford, 1983
CSTR (Continuously Stirred Tank Reactor); Substrate: Molasses wastewater	1.5-2 kg COD m-3 d-1; T = 35 °C	Co (0.0 and 0.12$mg{\cdot}l^{-1}$) Fe (1.12 $mmol{\cdot}l^{-1}$)	Influence of the addition of trace metals on methane production	Iron supply was required to precipitate the sulfide before cobalt addition. Without this, cobalt was probably precipitated by sulfide and not available for the microorganisms.	Percheron et al., 1997
Countinous mixed culture with acetate	T = 35°C acetate = 4 $mmol.l^{-1}d^{-1}$ dilution rate = 0.02 d^{-1},	Effect of Fe-dosing (0.08 mM) on SMA on acetate	Methane formation; acetate; Fe in solution.	Acetate conversion rate 10 times higher when Fe is supplied.	Hoban & van den Berg, 1979
pH-stat; substrate: acetate; conc. 2 to 3 $g.l^{-1}$ trace-metals	digester sludge (domestic), during 5 years enriched with acetate	Effect of combinations of Ni (4.5 $m.l^{-1}$), Fe (70.2 $mg.l^{-1}$), Co (4,5 $mg.l^{-1}$), yeast extract (200 $mg.d^{-1}$) and phosphate (40 $mg.d^{-1}$) on the acetate conversion	Acetate conversion rate, VSS	- Ni: 2 – 4.5 g acetate g $VSS^{-1}.d^{-1}$ + Ni: 10 g acetate g $VSS^{-1}.d^{-1}$ + Ni + yeast extract: 12 to 15 g acetate g $VSS^{-1}.d^{-1}$	Speece et al., 1983

Table 5. Continued.

System	Conditions	Experiment	Parameters	Results	ref.
Thermophilic digestion of energy crops 1:1 sorghum/cellulose dry weight (30%).	T = 55°C	I: addition (in mg.g VS^{-1}) 0.11 Ni, 0.05 Co, 0.02 Mo. II: addition (in mg. g VS^{-1}) 0.19 Ni, 0.13 Co, 0,16 Mo, 0.025 W, 0.0002 Cu, 0.0003 V, 0.025 Zn, 0.0007 B, 0.0011 Mn and 0.39 Fe.	VS, methane formation, VFA, total-ammonia-N	I: max. OLR from 8 to 24 $gVS.l^{-1}.d^{-1}$ and the methane production increased from 2 to 6 1 $.kg^{-1}.d^{-1}$ when Ni/Co/Mo are supplied II: max.OLR from 4.5 to 12 $gVS.l^{-1}.d^{-1}$ and the methane production increased from1.3 to 3.1 $kg^{-1}.d^{-1}$ when trace metals are supplied.	Jewell et al.,1992
Thermophilic digestion of chicken manure; batch reactors	T= 50°C, HRT = 5 d; pH=7.5	Biogas formation increase as result of Ni doses	Biogas formation; Ni-concentration	1 µM Ni 2% extra biogas (not sign.) 10 µM Ni 5% extra biogas (sign.) 100 µM Ni 8% extra biogas (sign.) already 253 µM Ni present in the medium, however low extra Ni doses stimulated biogas formation.	Williams et al., 1986
Batch; substrate VFA	Sludge from sludge digester	Effect of metal supply on acetogenesis and methanogenesis	VFA, methane production	Low doses of Ni stimulated de acetogenesis	Lin, 1992
Batch; anaerobic digestion		Effect of the addition of pyrite (1-3 $mg.l^{-1}$) on the methanogenesis	Methane produced Metal composition biomass	3 times higher methane formation; Fe, Ni en Co were taken up from the pyrite by the biomass.	Sanchez-Hernandez, 1994

Methanol conversion as a model system for metal studies

Bioreactor

Methanol can be utilized by both methanogens and acetogens (Fig. 2). In the anaerobic treatment of methanol containing wastewater, the utilization of methanol by acetogens is undesired, because the volatile fatty acid formation can lead to low COD removal and reactor instability. From the first studies towards the feasibility of anaerobic conversion of methanol using UASB reactors, the importance of trace metals on the methanol conversion pathway already became evident [Lettinga et al., 1979]. Following this research, detailed investigations towards the factors determining the fate of methanol in anaerobic bioreactors was performed [Florencio, 1994]. The cobalt concentration was found to be one of the factors determining whether methanol will be used by methanogens or acetogens, e.g. low cobalt concentrations favor the direct formation of methane from methanol. Cobalt and to a lesser extent nickel was also found to greatly enhance methanogenesis from methanol [Florencio et al., 1993]. This property makes methanol an excellent substrate to study the impact of metal supply and the dosing strategy of supply on their bioavailability for anaerobic sludge, as was demonstrated in batch activity tests by Gonzalez-Gil et al. [1999].

Methanosarcina

On the microbial level the genus *Methanosarcina*, the methanogens responsible for the direct conversion of methanol to methane, has been studied extensively. *Methanosarcina species* are the most versatile methanogen capable of using H_2/CO_2, acetate, formate and C_1-compounds such as methanol, methylthiols and methylamines [Thauer et al., 1998]. They were found to contain a high cobalt concentration in the form of corrinoids (part of the methyltranferase enzyme, Fig. 2), especially when grown on methanol [Krzycki and Zeikus, 1980]. Several authors have investigated the cobalt and nickel requirement of *Methanosarcina sp.* [Diekert et al., 1981; Scherer and Sahm, 1981; Scherer et al., 1983; Mazumder et al., 1987; Lin et al., 1989; Silveira et al., 1991a,b; Nishio et al., 1992]. From these studies the order of magnitude of nickel and cobalt content in the cells and effects on the methanol conversion are well known. The high requirement and impact of cobalt and nickel on the methanogenic activity of *Methanosarcina sp.*, makes it an ideal organism for metal uptake and availability studies in controlled batch media. For instance Gonzalez-Gil et al. [2003] used an enrichment of *Methanosarcina sp.* to study the effect of nickel and cobalt complexation by yeast extract on their bioavailabilty in sulfidic media. Nickel and cobalt form relatively strong organic complexes with yeast extract. The bioavailability of these metals was dramatically increased by the addition of yeast extract. This is due to the formation of dissolved bioavailable complexes, which favor the dissolution of metals from their sulfides. Uptake of cobalt and nickel by a pure culture of *Methanosarcina barkeri* was studied and modeled by Jansen [2004], in a defined medium which contained the ligand EDTA to prevent sulfide

precipitation. This enabled direct study into the relationship between the speciation and uptake of cobalt and nickel and the influence on the methanogenic activity.

Scope of the thesis

The main objective of this thesis was to elucidate the trace metal dynamics in methanol fed anaerobic granular sludge bed reactors. The information available about the retention of essential metals during anaerobic bioreactor operation, and the factors that influence this retention, is limited. The effects of the metals (presence, absence and mode of supply) on the micro-biological conversion of methanol were taken in to consideration as well.

Chapter 2 describes the trace metal content of granular sludges from full-scale anaerobic treatment systems. It was investigated whether the metal contents of these sludges were limiting for the conversion of different methanogenic substrates by assessment of the specific methanogenic activity on methanol, acetate and H_2/CO_2 in the presence and absence of a metal cocktail in the medium. In **Chapter 3 and 4** the effect of cobalt, nickel and iron deprived (or limited) operation on the methanol conversion in lab-scale UASB reactors is studied. **Chapter 5** investigates the impact of two different cobalt dosing strategies, viz. pre-loading and in situ loading on the performance and cobalt accumulation/retention in methanol fed bioreactors inoculated with initially cobalt limited granular sludge. **Chapter 6** deals with the effects of different sulfur sources (cysteine and sulfate) on the performance and metal retention of methanol fed UASB reactors. Metals were sequentially extracted from the sludge in order to study the metal dynamics within the sludge granules. **Chapter 7** describes the effect of imposed pH shocks on the metal retention of granular sludge, which was pre-loaded with cobalt, nickel and iron. Two sludges that originated from different full scale bioreactors were used in order to determine the effect of the sludge source on the metal retention.

REFERENCES

Ahuja P, Gupta R, Saxena RK (1999). Sorption and desorption of cobalt by *Oscillatoria anguitissima*, *Curr Microbiol*, **39**, 49-52

Aksu Z (2002). Determination of the equilibrium, kinetics and thermodynamic parameters of the batch sorption of nickel (II) ions onto *Chlorella vulgaris*. *Process Biochem*, **38**, 89-99

Albracht SPJ (1994). Nickel hydrogenases: In search of the active site. *Biochim. et Biophys. Acta*, **1188**, 167-204

Apines MJS, Satoh S, Kiron V, Watnabe T, Aoki T (2003). Availability of supplemental amino acid-chelated trace elements in diets containing tricalcium phosphate and phytate to rainbow trout *Oncorhynchus mykiss*. *Aquacult*, **225**, 431-444

Artola A, Martin M, Balaguer DM, Rigola M (2000). Isotherm model analyses for the adsorption of Cd (II), Cu(II), Ni (II) and Zn (II) on anaerobically digested sludge. *J Colloid Inter Sci*, **232**, 64-70

Bainoitti AE, Nishio N (2000). Growth kinetics of Acetobacterium sp. on methanol-formate in continuous culture. *J Appl Microbiol*, **88**, 191-201

Barker DJ, Stuckey DC (1999). A review of soluble microbial product (SMP) in wastewater treatment systems, *Wat Res*, **33**, 3063-3082

Betram PA, Thauer RK (1994). Thermodynamics of the formylmethanofuran dehydrogenase reaction in Methanobacterium thermoautotrophicum. *Eur J Biochem*, **226**, 811-818

Berghofer Y, Agha-Amiri K, Klein A (1994). Selenium is involved in the negative regulation of the expression of selenium-free [NiFe] hydrogenases in *Methanococcus voltae*. *Mol Gen Genet*, **242**, 369-73

BeveridgeTJ, Doyle RJ (1989). Metal ions and bacteria. Wiley-Interscience, USA

Callander IJ, Barford JP (1983). Precipitation, chelation and the availability of metals as nutrients in anaerobic digestion. II: *Biotechnol Bioeng*, **25**, 1959-1972

Chien YT, Auerbuch V, Drabban AD, Zinder SH (2000) Analyses of genes encoding for an alternative nitrogenase in the archaeon *Methanosarcina barkeri* 227. *J Bacteriol*, **182**, 3247-3253

Coleman JE (1992). Zinc proteins: enzymes, storage proteins, transcription factors and replication proteins. *Annu Rev Biochem*, **16**, 897-946

Colleran E, Finnegan S, Lens P (1995). Anaerobic treatment of sulphate containing wastewater. *Antonie van Leeuwenhoek*. **67**, 29-46

Diekert, G, Konheiser U, Piechull K, Thauer RK (1981). Nickel requirement and factor F430 content of methanogenic bacteria. *J Bacteriol*, **148**, 459-464

Ensign SA, Hyman MR, Arp DJ (1993). In vitro activation of ammonia monooxygenase from *Nitrosomonas europaea* by copper. *J Bacteriol*, **175**, 1971-1980

Espinosa A, Rosas L, Ilangovan K, Noyala A (1995). Effect of trace metals on the anaerobic degradation of volatile fatty acids in molasses stillage. *Wat Sci Tech*, **32**, 121-129

El-Shafey E, Cox M, Pichugin AA, Appleton Q (2002). Application of a carbon sorbent for the removal of cadmium and other heavy metal ions from aqeous solution. *J Chem Technol Biotechnol*, **77**, 429-436

Farmer PE, Adams, TE, Humphries WR (1982). Copper supplementation of drinking-water for cattle grazing molybdenum-rich pastures. *Vet Rec*, **111**, 193-195

Fathepure BZ (1987). Factors affecting the methanogenic activity of *Methanothrix soehngenni VNBF*. *Appl Env Microbiol*, **53**, 2978-2982

Filgueiras AV, Lavilla I, Bendicho C (2002). Chemical sequential extraction for metal partitioning in environmental solid samples. *J Env Monit*, **4**, 823-857

Fauque G, Peck HD, Moura JJD, Huynh BH, Berlier Y (1988). The three classes of hydrogenases from sulfate-reducing bacteria of the genus *Desulvovibrio*. *FEMS Microbiol Rev*, **54**, 299-344

Ferguson SJ (1994). Denitrification and its control. *Antonie van Leeuwenhoek Int J. of Gen. and Mol Microbiol*, **66**, 89-110

Ferry JG (1999). Enzymology of one-carbon metabolism in methanogenic pathways. *FEMS Microbial Rev*, **23**, 13-38

Friedman HC, Klein A, Thauer RK (1990). Structure and function of the nickel porphinoid, coenzyme F_{430}, and its enzyme, methyl coenzyme M reductase, *FEMS Microbiol Rev*, **87**, 339-348

Florencio L, Jenicek P, Field JA, Lettinga G (1993). Effect of cobalt on the anaerobic degradation of methanol. *J Ferment Bioeng*, **75**, 368-374

Florencio L (1994). The fate of methanol in anaerobic bioreactors. PhD Thesis, Wageningen University Wageningen, The Netherlands

Gerhard E, Butsch BM, Marison IW, Stockar von U (1993). Improved growth and methane production conditions for *Methanobacterium thermoautotrophicum*. *Appl Microbiol Biotechnol*, **40**, 432-437

Girio FM, Marcos JC, Amaral CMT (1992). Transition metal requirement to express high level NAD-dependent formate dehydrogenase from a serine-type methylotrophic bacterium. *FEMS*, **97**, 161-166

Glahn RP, van Campen DR (1997). Iron uptake is enhanced in in Caco-2 Cell monolayers by Cysteine and reduced cystenyl glycine, *J Nutr*, **127**, 643-647

Gonzalez-Gil G, Kleerebezem R and Lettinga G (1999). Effects of nickel and cobalt on kinetics of methanol conversion by methanogenic sludge as assessed by on-line CH_4 monitoring. *Appl Environ Microbiol*, **65**, 1789-1793

Gonzalez–Gil G, Jansen S, Zandvoort MH, van Leeuwen HP (2003). Effect of yeast extract on speciation and bioavailability in nickel and cobalt in anaerobic bioreactors. *Biotech Bioeng*, **82**, 134-142

Halberg L, (1995). Iron and vitamins, *Bibl Nutr Dieta*, **52**, 9-20

Hausinger RP (1994). Nickel enzymes in microbes. *Sci tot Environ*, **148**, 157-166

Haytoglu B, Demerir GN, Yetis U (2001). Effectiveness of anaerobic biomass in adsorbing heavy metals. *Wat Sci Technol*, **44**, 245-252

Heider J, Bock A (1993) Selenium metabolism in micro-organisms. *Adv Microbial Phys*, **35**, 71-107

Hensgens CMH, Nienhuis-Kuiper ME, Hansen TA (1994). Effects on the growth of *Desulfovibrio gigas* NCIMB 9332 and other sulfate-reducing bacteria with ethanol as substrate. *Arch Microbiol*, **1662**, 143-147

Ho YS, Wase DAJ, Forster CF (1996). Kinetic studies of competitive heavy metal sorption by sphagnum moss peat. *Environ Technol*, **17**, 71-76

Hoban DJ, Van den Berg L (1979). Effect of iron on conversion of acetic acid to methane during methanogenic fermentations. *J Appl Bacteriol*, **47**, 153-159

Hochheimer A, Schmitz RA, Thauer RK, Hedderich R (1995). The tungsten Formylmethanofuran dehydrogenase from Methanobacterium thermoautothrophicum contains sequence motifs characteristic for enzymes containing molybdopterin dinucleotide. *Eur J Biochem*, **234**, 910-920

Hulisz D (2004). efficacy of zinc against common cold viruses: an overview. *J Am Pharm Assoc*, **44**, 594-603

Hullebusch van ED, Zandvoort MH, Lens PNL (2003). Metal immobilisation in biofilms: mechanisms and analytical tools. *Re/view Envion Sci Bio/Technol*, **2**, 9-33

Hullebusch van E, Zandvoort MH, Lens PNL (2004). Nickel and cobalt sorption on anaerobic granular sludges: kinetic and equilibrium studies. *J Chem Technol Biotechnol*, **79**, 1219-1227

Hullebusch van ED, Peerbolte A, Zandvoort MH, Lens PNL (2005a). Sorption of cobalt and nickel on anaerobic granular sludges: isotherms and sequential extraction. *Chemosphere*, **58**, 493-505

Hullebusch van ED, Utomo S, Zandvoort MH, Lens PNL (2005b). Comparison of three extraction procedures to describe metal fractionation in anaerobic granular sludges. *Talanta*, **65**, 549-558

Ishmael J, Gopinath C, Howell JM (1972). Experimental chronic copper toxicity in sheep – biochemical and haematological studies during development of lesions in liver. *Res Vet Sci*, **13**, 22-30

Jansen S (2004). speciation and bioavailability of cobalt and nickel in anaerobic wastewater treatment. Ph. D Thesis, Department of physical and colloidal chemistry, Wageningen Univerisity, The Netherlands, ISBN 90-8504-114-7

Janssens G (2004). Sporenelementen en zwavel: essentieel voor de ontwikkeling van plant, mens en dier, Technologisch Instituut, Antwerpen, in Dutch

Jarrell KF, Kalmokoff MI (1988). Nutritional requirements of the methanogenic archaebacteria. *Can J Microbiol*, **4**, 557-576

Jones JB, Stadtman TC (1977). *Methanococcus vannielii*: effects of selenium and tungsten on growth. *J Bacteriol*, **130**, 1404-1406

Jong T, Perry L (2003). Removal of sulfate and heavy metals by sulfate reducing bacteria in short-term bench scale upflow anaerobic packed bed reactors. *Wat Res*, **37**, 3379-3389

Jondreville C, Revy PS, Dourmand JY (2003). Dietery means of better control of the environmental impact of copper and zinc by pigs from weaning to slaughter. *Livestock Prod Sci*, **84**, 147-156

Jewell WJ, Cummings RJ, Richards BK (1992). Energy production from biomass and wastes: present limitations and potential improvements. Proceedings of the 3rd workshop of the working group on biogas production technologies CNREE Network on biomass production and conversion for energy. Braunschweig, Germany, 5-7 May 1992. Ed.: Baader, W. FAO

Kaksonen AH, Riekkola-Vanhanen ML, Puhakka JA (2003). Optimization of metal sulfide precipitation in fluidized-bed treatment of acidic wastewater. *Wat Res*, **37**, 255-266

Kelly CR, Switzenbaum MS (1984). Anaerobic treatment: temperature and nutrient effects. *Agric Wastes*, **10**, 135-154

Kuo WC and Parking GF (1996). Characterization of soluble microbial products from anaerobic treatment by molecular weight distribution and nickel-chelating properties. *Wat Res*, **30**, 915-922

Krzycki J and Zeikus JG (1980). Quantification of corrinoids in methanogenic bacteria. *Curr Microbiol*, **71**, 243-245

Lipscomb JD (1994). Biochemistry of the soluble methane monooxygenase. *Ann Rev Microbiol*, **48**, 371-399

Lin CY (1992). Effect of heavy metals on volatile fatty acid degradation in anaerobic digestion. *Wat Res*, **26**, 177-183

Lin D, Kakinozo T, Nishio N, Nagai S. (1990). Enhanced cytochrome formation and stimulated methanogenesis rate by the increased ferrous concentration in *Methanosarcina barkeri* culture. *FEMS Microbiol Lett*, **68**, 89-92

Lin DG, Nishio N, Mazumder TK, Nagai S (1989). Influence of Co^{2+}, Ni^{2+} and Fe^{2+} on the production of tetrapyrroles by *Methanosarcina barkeri*. *Appl Microbial Bioetechnol*, **30**, 196-200

Lettinga G, van der Geest AT, Hobma S, van der Laan J (1979). Anaerobic treatment of methanolic wastes. *Wat Res*, **13**, 725-737

Lovley, D.R. (1993). Anaerobes into Heavy Metal - Dissimilatory Metal Reduction in Anoxic Environments. *Trends in Ecology & Evolution*, **8**, 213-217

Lopez A, Lazaro N, Priego JM, Marques AM (2000). effect of pH on the biosorption of nickel and other metals by *Pseodomonas fluorescens* 4F39. *J Ind Microbiol Bioetechnol*, **24**, 146-151

Maas van der P, Peng S, Klapwijk B, Lens PNL (2005). Enzymatic versus nonenzymatic conversions during the reduction of EDTA chelated Fe(III) in BioDeNOx reactors. *Environ Sci Tech (in press)*

Mazumder TK, Nishio N, Fukazaki S, Nagai S (1987). Production of extracellular vitamin B_{12} compounds from methanol by *Methanosarcina barkeri*. *Appl Microbiol Biotechol*, **26**, 511-516.

Murray WD, Van den Berg L (1981). Effects of nickel, cobalt, and molybdenum on performance of methanogenic fixed-film reactors. *Appl Environ Microbiol*, **42**, 502-505

Michel R, Massanz C, Kostka S, Richer M, Fiebig K (1995). Biochemical characterization of the 8-hydroxy-5-deazaflavin hydrogenase from Methanosarcina barkeri. *Eur J Biochem*, **233**, 727-735

Nishio N, Kakizono T, Silveira RG, Takemoto S, Nagai S (1992). Nutrient control by the gas evolution in methanogenesis of methanol by Methanosarcina barkeri. *J Ferment Bioeng*, **73**, 481-485

Neuman A, Wolfarth G, Diekert G (1996). Purification and characterization of tetrachloroethene reductive dehalogenase from *Dehalospirillum multivorans*, *J Biol Chem*, **271**, 6515-6519

Neilands JB, (1995). Siderophores structure and function of microbial iron transport compounds. *J Biol Chem*, **270**, 26723-26726

Oleszkiewicz JA, Romanek A. (1989). Granulation in anaerobic sludge bed reactors treating food industry wastes. *Biological Wastes*, **27**, 217-235

Pankhania IP, Robinson JP (1984). Heavy Metal inhibition of methanogenesis by *Methanospirillum hungatei* GP1. *FEMS Microbiol Lett*, **22**, 277-281

Patel VB, Patel AR, Patel MC, Madamwar DB (1993). Effect of Metals on Anaerobic Digestion of Water Hyacinth Cattle Dung. *Appl Biochem Biotech*, **43**, 45-50

Percheron G, Bernett N, Moletta R (1997). Start up of anaerobic digestion of sulfate wastewater. *Biores Technol*, **61**, 21-27

Rengaraj S, Moon SH (2002). Kinetics of adsorption of Co (II) removal from water and and wastewater by ion exchange resin. *Water Res*, **36**, 1783-1793

Saito MA, Moffet JW, Chisholm SW, Waterbury JB (2002). Cobalt limitation and uptake in Prochlorococcus. *Limnol Oceanogr*, **47**, 1629-1636

Sanchez Hernandez EP (1994). Effect of pyrite addition on anaerobic digestion. *Biores Technol*, **47**, 189-190

Sauer K & Thauer R (2000). Methyl-coenzyme M formation in methanogenic archaea; involvement of zinc in coenzyme M activation. *Eur J Biochem*, **267**, 2498-2500

Sawers G (1994). The hydrogenases and formate dehydrogenases of *Escherichia coli*. *Anthonie.Van Leeuwenhoek Int J of Gen and Mol Microbiol*, **66**, 57-88

Scherer P (1989) Vanadium and molybdenum requirement for the fixation of molecular nitrogen by two *Methanosarcina* strains. *Arch Microbiol*, **151**, 44-48

Scherer P, Lippert H, Wolff G (1983). Composition of the major elements and trace elements of 10 methanogenic bacteria determined by inductive coupled plasma emission spectroscopy. *Biol Trace Elem Res*, **5**, 49-163

Scherer P, Sahm H (1981). Effect of trace elements and vitamins on the growth of *Methanosarcina barkeri, Acta Biotechnol*, **1**, 57-65

Schijndel JWPMV, Vollenbroek EGM, Wever R (1993). The chloroperoxidase from the fungus *Curvularia inaequalis*: A novel vanadium enzyme. *Biochim Et Biophys Acta*, **1161**, 249-256

Schindelin H, Kisker C, Schlessman JL, Howard JB, Rees DC (1997). Structure of ADP-AlF_4^- stabilized nitrogenase complex and its implications for signal transduction. *Nature*, **387**, 370-376

Schönheit P, Moll J, Thauer RK (1979). Nickel, Cobalt, and Molybdenum requirement for growth of *Methanobacterium thermoautotrophicum. Arch Microbiol*, **123**, 105-107

Seravalli J, Gu W, Tam A, Strauss E, Tadhg P, Begley P, Cramer SP, Ragsdale SW (2003). Functional copper at the acetyl-CoA synthase active site. *Chemistry*, **100**, 3689-3694

Shima S, Warkentin E, Thauer RK, Ermler U (2002). Structure and function of enzymes involved in the methanogenic pathway utilizing carbon dioxide and molecular hydrogen. *J Biosci Bioeng*, **6**, 519-530

Sharma J, Singh R (2001.) Effect of nutrient supplementation on anaerobic sludge development and activity for treating distillery effluent. *Biores Technol*, **79**, 203-206

Shen CF, Kosaric N, Blaszczyk R (1993a).The effect of selected heavy metals (Ni, Co and Fe) on anaerobic granules and their extracelllular polymeric substance (EPS). *Water Research Oxford*, **27**, 25-33

Shen CF, Kosarik N, Blaszcyk R (1993b). Properties of anaerobic granular sludge as affected by yeast extract, cobalt and iron supplements. *Appl Microbiol Biotechnol*, **39**,132-137

Silveira RG, Nishio N, Nagai S (1991a). Growth characteristics and corrinoid production with *M. Barkeri* on methanol-acetate medium. *J Ferment Bioeng*, **71**, 28-34

Silveira RG, Kakizono T, Takemoto S, Nishio N, Nagai S (1991b). Medium optimization by orthogonal array design for the growth Methanosarcina barkeri, *J Ferment Bioeng*, **72**, 20-25

Singh RP, Surendra K, Ojha CSP (1999). Nutrient requirement for UASB process: a review. *Biochem Eng J*, **3**, 35-54

Smorgon C, Mari E, Atti AR, Dalla Nora E, Zamboni PF, Calzoni F, Passaro A, Fellin R (2004). Trace elements and cognitive impairment: an elderly cohort study. *Arch Gerontol Geriatr Suppl*, **9**, 393-402

Speece RE, Parkin GF, Gallagher D (1983). Nickel stimulation of anaerobic digestion. *Wat Res*, **17**, 677-683

Speece, RE, Parkin GF, Bhattacharya S, Takashima M (1986). Trace nutrient requirements of anaerobic digestion Proc. EWPCA Conf. on Anaerobic Waste Water Treatment, 15-19 Sept. 1986, Amsterdam, The Netherlands, 175-187

Takashima M, Speece RE (1990). Mineral requirements for methane fermentation *Crit Rev Environ Contr*, **19**, 465-479

Thauer RK (1998). Biochemistry of methanogenis: a tribute to Majory Stephenson. *Microbiol*, **144**, 2377-2406

Vallee BL, Falchuk KH (1993). the biological basis of zinc physiology. *Physiol Rev*, **73**, 79-118

Veiglio F, Beolchini F (1997). Removal of metals by biosorption: a review. *Hydrometal*, **44**, 301-316

Verhoef H, West CE, Nzyuko SM, Vogel de S, Valk van der R, Wanga MA, Kuijsten A, Veenemans J, Kok FJ (2002). Intermittent administration of iron and sulfadoxine-pyrimethamine to control anaemia in Kenyan children: a randomised controlled trial, *Lancet*, **360**, 908-914

Wasserfallen A (1994). Formylmethanofuran synthesis by formylmethanofuran dehyrogenase from *Methanobacterium thermoautohrophic* Marburg. *Biochem Biophys Res Commun,* **199**, 1256-1261

White H, Simon H (1992). The role of tungstate and/or molybdate in the formation of aldehyde oxidoreductase in *Clostridium thermoaceticum* and other acetogens: Immunological distances of such enzymes. *Arch Microbiol*, **158**, 81-84

Whitman WB (1985). Methanogenic bacteria, The bacteria vol. 8, Academic Press, New York, 3-84

Williams CM, Shih JCH, Spears JW (1986). Effect of nickel on biological methane generation from a laboratory poultry waste digester. *Biotechnol Bioeng*, **28**, 1608-1610

Yu HQ, Fang HHP, Tay JH (2000). Effect of Fe^{2+} on sludge granulation in upflow anaerobic sludge blanket reactors, *Wat Sci Tech*, **41**, 199-205

Chapter 2

Granular sludge in full-scale anaerobic bioreactors: trace element content and deficiencies

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INTRODUCTION

The trace element requirement of anaerobic microorganisms is a rather specific phenomenon, because many enzymes involved in the biochemistry of fermentation and methane (CH_4) production contain trace metals like e.g. cobalt, nickel, zinc and iron. Besides present in enzyme systems of the microorganisms, part of the metals in methanogenic granular sludge is present extracellularly, i.e. associated to the extracellular polymeric substances (EPS) or as inorganic precipitates. These extracellular metals can be considered as a stock of metals, which can be either non-bioavailable or (partly) bioavailable for the biomass. The relative contribution to the metal retention of these fractions depends on both the wastewater composition and bioreactor operational parameters.

In order to prevent trace metal limitations of the methanogens, essential trace elements are usually supplied to the media in the cultivation of these microorganisms or in the media of specific activity assays. But also in practical applications of anaerobic microorganisms such as in Anaerobic Upflow Sludge Bed (UASB) or Expanded Granular Sludge Bed (EGSB) reactors, the supply of a balanced trace element cocktail is essential to maintain good reactor performance. Trace element limitations result in lower sludge activities and consequently in a sub-optimal reactor operation. The effect of a sub-optimal dosing of trace metals in laboratory-scale UASB-reactors has been shown to induce limitations for cobalt [Florencio et al., 1993, Zandvoort et al., 2002a], nickel [Zandvoort et al., 2002b] and iron [Zandvoort et al, 2003]. In some full-scale UASB applications trace metals are not supplied because they are already ubiquitously present in the wastewater influent streams. But in these cases, some of the trace metals still might be present in sub-optimal concentrations and therefore the performance of these reactors then in principle could be improved substantially by a rational addition of trace metals.

Little is known so far about the relation between the amount of metals present in the anaerobic granular sludges and their effect on the biological activity of the concerning sludge. Moreover, very little attention has been given so far to the existence of trace element limitations in full-scale bioreactors and how and when they manifest, e.g. when any supply of trace metal is omitted, or when they are dosed at sub-optimal concentrations or when they are induced by some change in an environmental factor, e.g. a pH shock. In order to elucidate whether limitations for trace elements prevail in full-scale bioreactors, anaerobic granular sludge's from four full-scale bioreactors were screened for their response to trace metals, using three methanogenic substrates, viz. methanol, acetate and H_2/CO_2. The response on the supply of a trace metal cocktail and on supply of individual metals was assessed and it was attempted to relate the metal content of the sludges with the assessed specific methanogenic activity responses.

MATERIAL AND METHODS

Source of biomass

Four types of granular sludge, which originated from four different full-scale anaerobic bioreactors were used in this study. Nedalco granular sludge originates from a UASB reactor treating alcohol distillery wastewater, consisting of ethanol and volatile fatty acids (Nedalco, Bergen op Zoom, the Netherlands). Eerbeek granular sludge was obtained from a UASB treating paper mill wastewater (Industriewater, Eerbeek, The Netherlands). Hoogeveen granular sludge was obtained from a UASB treating groundwater contaminated with perchloroethene (Hoogeveen, The Netherlands). Heineken granular sludge originated from an EGSB reactor treating brewery wastewater (Heineken, Zoeterwoude, The Netherlands).

Specific maximum methanogenic activity tests

Maximum specific methanogenic activity (SMA) of the sludge was determined in duplicate at 30 (±2)°C using on-line gas production measurements as described in chapter 3. Tests compared the methane evolution upon the addition of either a full trace metal cocktail, a single trace metal or the full cocktail from which cobalt was omitted. The full metal cocktail contained iron at a concentration of 50 μM and cobalt, nickel, copper, zinc, manganese, molybdenum and selenium at a concentration of 5 μM. Approximately 1g (wet weight) of granular sludge was transferred to 120 ml (245 ml for SMA on H_2/CO_2) serum bottles containing 50 ml of basal medium with the same composition as described in chapter 3, supplemented with either methanol (4 g COD.l^{-1}) or acetate (2 g COD.l^{-1}) as the substrate. The pH was maintained at ±7 by addition of 2.52 g.l^{-1} $NaHCO_3$. For the SMA with H_2/CO_2 as the substrate, bottles were flushed with 0.8 bar of nitrogen gas, a further 1.8 bar of H_2/CO_2 (80%/20%) equivalent to 3.4 g COD.l^{-1} was added to the bottles resulting in a total pressure of 2.6 bar. In the H_2/CO_2 test, the pH was maintained at approximately 7 by the addition of 3.7 g.l^{-1} $NaHCO_3$. For the SMA at pH 6 (Hoogeveen sludge), 6.81 g of KH_2PO_4 and 4 ml 1.4 M NaOH was added per liter of medium. The SMA with H_2/CO_2 as the substrate was calculated from the rate of the gas pressure decrease in the bottles (except for the Hoogeveen sludge which was determined by the methane evolution in the headspace of the bottles) and the methane concentration in the headspace after depletion of the substrate.

Metal analyses

The total metal concentration in the sludge was determined by ICP-OES, after microwave destruction of the sample, as described by van Hullebusch et al. [2004] The sequential extraction procedure of the cobalt from the sludge was performed at the beginning

and the termination of the reactor run using an extraction scheme as described by Osuna *et al.* [2004]. This scheme consists of four extraction steps, which become more stringent with each subsequent step. The following nomenclature is applied for the subsequent extraction steps, viz. the exchangeable fraction (1M NH_4CH_3COO), the carbonate fraction (1M CH_3COOH), the organic/sulfide fraction (30% H_2O_2) and the residual fraction (3:1 HCl/HNO_3)

RESULTS

Metal content of sludge from full scale UASB reactors

The results of the analyses of the four sludge's for VSS/TSS ratio and their metal content are sumarised in Table 1. The VSS/TSS ratio of the sludges varied between 0.75 for the Hoogeveen sludge and 0.92 for the Nedalco sludge, differences which largely can be explained by the differences in the iron and sulfur contents between these sludge's, e.g. the combined iron and sulfur content of the Hoogeveen sludge is 145.5 mg.g TSS^{-1} while it is only 43.5 mg.g TSS^{-1} for the Nedalco sludge. The high iron content of the Hoogeveen sludge is due to the high influent iron concentration (10.1 $mg.l^{-1}$). The molar ratio Fe:S of the sludges was 1:1.9, 1:2.0, 1:1.6 and 1:1.1 for Nedalco, Eerbeek, Hoogeveen and Heineken sludge, respectively.

In general, the trace metal contents of the Nedalco and Eerbeek sludge are lower compared to that of the Hoogeveen and Heineken sludge. The total cobalt content of all the sludges is low compared to those of the other trace elements, i.e. the concentration ranged from 27 μg.g TSS^{-1} for the Nedalco sludge to 51 μg.g TSS^{-1} for the Heineken sludge. The results of the fractionation of the cobalt by the sequential extraction procedure (Table 2, Fig. 1) show that cobalt is mainly present in the more strongly bound fractions: the organic/sulfide- and the residual fraction, in the Eerbeek sludge cobalt is rather equally distributed over these two fractions, while in the other sludges cobalt predominates in the organic/sulfide fraction.

Cobalt, nickel, manganese, selenium, zinc and copper show remarkable similarities in distribution over the different fractions of the sludges, except for the Eerbeek sludge (Fig. 1). The largest variation was observed for the zinc content, i.e. being 2267 μg. g TSS^{-1} for the Hoogeveen sludge and only 104 μg. g TSS^{-1} for the Eerbeek sludge. The content of nickel, copper and zinc is the lowest for the Eerbeek sludge and selenium is hardly present. In the Eerbeek sludge the metals predominantly accumulated in the residual fraction, most remarkably also for molybdenum and nickel, while in the other sludges these metals were mainly present in the organic/sulfide and/or carbonate fraction.

Table 1. Initial chemical composition (μg. g TSS^{-1}) of the sludge granules

	Nedalco	**Eerbeek**	**Hoogeveen**	**Heineken**
VSS (g VSS.g wet $weight^{-1}$)	0.076	0.172	0.076	0.079
TSS (g TSS.g wet $weight^{-1}$)	0.083	0.210	0.101	0.098
VSS/TSS	0.92	0.82	0.75	0.81
Cobalt[a]	27	31	33	51
Nickel	130	30	105	230
Copper	690	50	142	300
Manganese	55	102	122	117
Zinc	760	104	2267	1524
Selenium	n.d.	1.9	49	124
Molybdenum	n.d.	49	52	36
Magnesium	n.d.	737	718	1180
Iron[b]	20.8	25.9	75.7	36.0
Calcium[b]	4.7	20.3	8.9	n.a.
Phosphorous[b]	6.6	6.6	8.4	12.5
Sulfur	22.7	29.3	69.8	23.2

n.a. not analysed; [a] sum of the 4 sequential extracted fractions; [b] mg.g TSS^{-1}

Table 2. Fractionation of the cobalt in the granular sludges

	Nedalco		**Eerbeek**		**Hoogeveen**		**Heineken**	
	μg.g TSS^{-1}	%	μg.g TSS^{-1}	%	μg.g TSS^{-1}	%	μg.g TSS^{-1}	%
Exchangeable	3	10	5	17	1	4	4	7
Carbonates	5	18	6	20	4	13	13	25
Organic / sulfides	15	58	9	29	25	75	29	56
Residual	5	17	10	33	3	2	6	12

Specific methanogenic activity measurements

Methanol as the substrate

The results are presented graphically in Fig. 2 and summarized in Table 3. Remarkable differences were observed for the four sludges. It is clear that the SMA of the Nedalco sludge responded positively to the supply of cobalt, i.e. the SMA increase was very pronounced, viz. a maximum value of only 306 mg CH_4-COD.g $VSS^{-1}.d^{-1}$ in the absence of metals, and 535 mg CH_4-COD.g $VSS^{-1}.d^{-1}$ in the presence of 5 μM cobalt (Table 3). Even in absence of trace element supply, clearly a peak – though rather 'flat' in the SMA appears. In the period of 'constant' methanogenesis, preceding the peak, an increased concentration of volatile fatty acid (VFA) in the medium was not observed (data not shown). In the presence of all metals (including cobalt), even a 36% higher SMA was found (730 mg CH_4-COD. g $VSS^{-1}.d^{-1}$) compared to the assay with 5 μM of cobalt alone. When all the metals (including cobalt) were supplied to the assay medium, except molybdenum, selenium and tungsten, the methane production rate curve and the SMA found (data not shown) appeared to be similar to those found in presence of merely 5 μM cobalt. Apparently, except of cobalt, the content (or availability) of one of these elements represents a limiting factor in the Nedalco sludge.

The SMA of the Eerbeek sludge clearly remained unaffected by the addition of 5 μM of cobalt, very similar curves were found with merely cobalt addition and in absence of metal addition, and in both cases the SMA slowly improved to a value of 101 mg CH_4-COD. g $VSS^{-1}.d^{-1}$. Surprisingly, contrary to the other sludges a lag-phase was almost absent. The effect of the supply of the complete metal cocktail was not tested for the Eerbeek sludge.

The SMA of the Hoogeveen sludge clearly improved with the addition of 5 μM cobalt alone (Table 3): the SMA increased 115% compared to the activity in the absence of metals. The SMA found with the addition of 5 μM cobalt was very similar to that found with the complete trace element cocktail + 5μM cobalt, indicating that the SMA could only be improved by the additions of cobalt and not by any other metal. A second feed of methanol increased the SMA in the "no metals" assay to 325 mg CH_4-COD. g $VSS^{-1}.d^{-1}$ (compared to 155 mg CH_4-COD. g $VSS^{-1}.d^{-1}$ in the first feed), i.e. similar to that in the first feed with the "all metals except cobalt" assay (344 mg CH_4-COD. g $VSS^{-1}.d^{-1}$), but also the SMA in the presence of the cobalt-addition assay increased substantially in the second feed, i.e. to 514 mg CH_4-COD. g $VSS^{-1}.d^{-1}$.

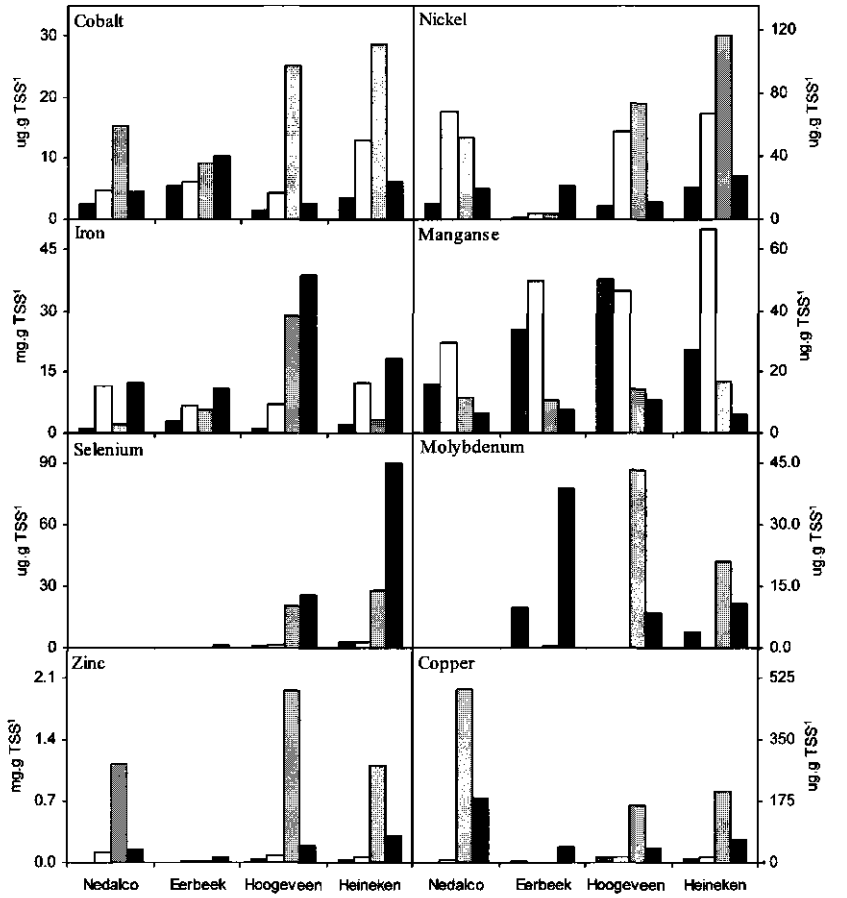

Figure 1 Metal fractionation in the sludges determined by sequential extraction. Exchangeable (dark grey), carbonate (white), Organic/sulfide fraction (light grey) and Residual fraction (Black)

The response of the Heineken sludge to the supply of the metal cocktail without cobalt on the SMA clearly is the highest for all four sludges with a maximum value of 756 mg CH_4-COD. g $VSS^{-1}.d^{-1}$, while here any clear add-on effect of cobalt supply was present (Fig. 2, Table 3). Moreover, also remarkable is the shape of the methane formation rate curve found for the assay conducted with 'no metal' addition (Fig 2), because here, following the lag phase, the SMA attained a peak value in the beginning, but it then after approximately 300 h dropped to a much lower values.

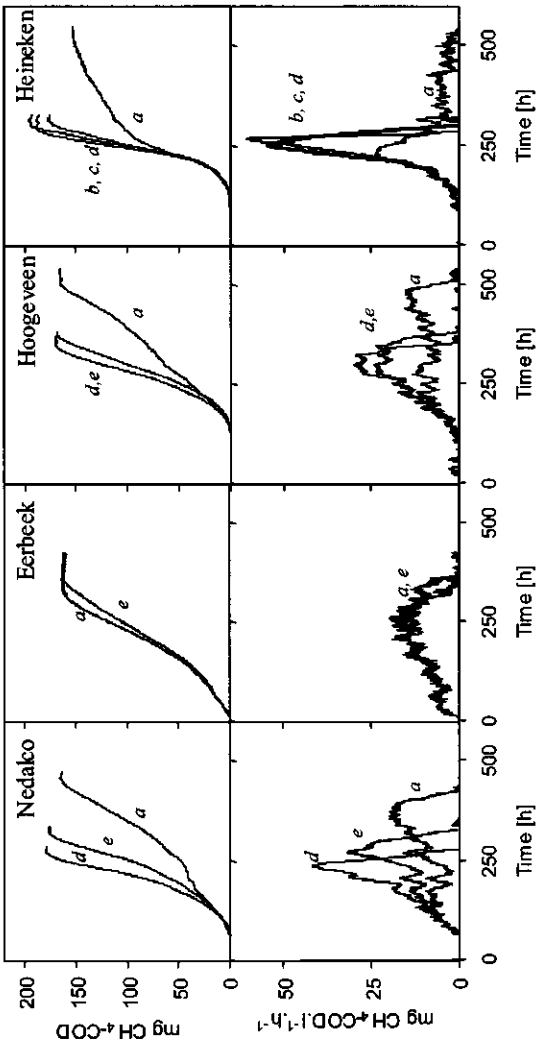

Figure 2 Cumulative methane production and methane (mg CH_4-COD) production rate curve (mg CH_4-COD.l^{-1}.h^{-1}) with methanol as the substrate. (a) no metals; (b) all metals, no cobalt; (c) all metals+1μM cobalt; (d) all metals+5μM; (e) no metal+5μM cobalt.

Table 3. Average specific methanogenic activity (mg CH_4. g $VSS^{-1}.d^{-1}$) with methanol as the substrate, the observed lag-phase (h) and the time (h) after start of the experiment after which maximum activity was observed.

	Nedalco			**Eerbeek**			**Hoogeveen**			**Heineken**		
	Act	Lag	Max	Act	Lag	Max	Act	Lag	Max	Act	Lag	Max
No Metals	306	70	350	101	10	248	155	142	480	390	93	242
All metals	344	70	350	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	756	93	248
All +1μM Co	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	747	93	236
All +5μM Co	730	70	231	n.d.	n.d.	n.d.	285	142	269	670	93	233
No +5μM Co	535	70	262	106	10	251	334	142	269	n.d.	n.d.	n.d.

n.d. not determined

Acetate as the substrate

The results are shown graphically in Figure 3 and summarized in Table 4. Regarding the curves assessed with acetate as substrate it is clear that the maximum values of SMA of Nedalco, Eerbeek and Hoogeveen sludge are not affected by the addition of cobalt or the trace elements cocktail (including cobalt) (Table 4). Only in case of the Heineken sludge a slight increase in SMA upon the addition of 5 μM of cobalt manifested, i.e. 184 mg CH_4-COD.g $VSS^{-1}.d^{-1}$ in presence of 5 μM of cobalt compared to 115 mg CH_4-COD.g $VSS^{-1}.d^{-1}$ in the assay conducted with the trace element cocktail without cobalt (Table 4). The SMA on acetate found with the Nedalco sludge was clearly the highest (639 mg CH_4-COD.g $VSS^{-1}.d^{-1}$).

A pronounced shortening of the lag-phase was observed for the Heineken sludge (Fig. 3), merely in presence of 5 μM of cobalt, the lag-phase decreased from 560 h to 234 h (Table 4, Fig. 3). The prevalence of a long lag-phase in case of the Heineken sludge indicates that the absence of an active acetotrophic population in this sludge, contrary to the other sludge tested

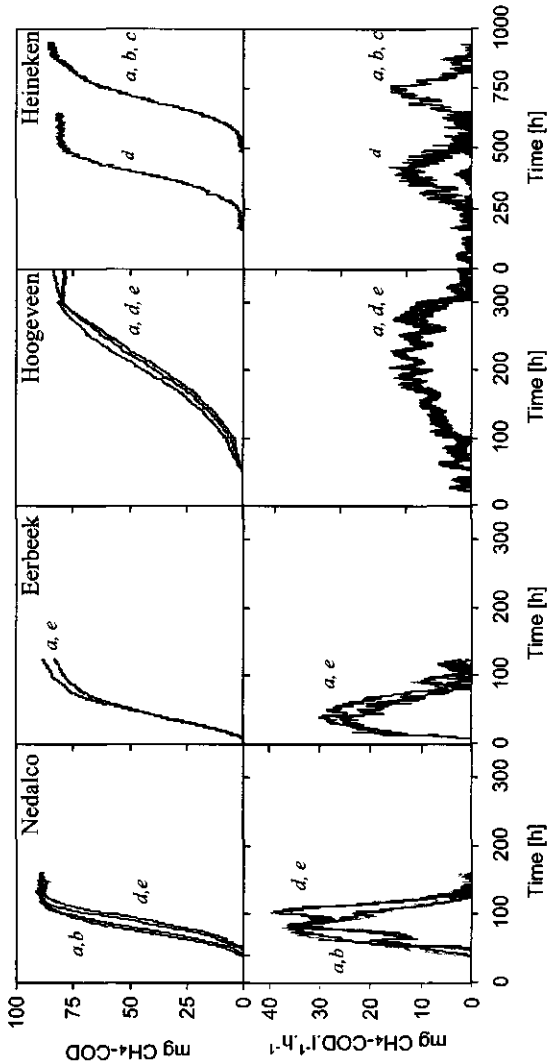

Figure 3 Cumulative methane production and methane (mg CH_4-COD) production rate curve (mg CH_4-COD.l^{-1}.h^{-1}) with acetate as the substrate. (a) no metals; (b) all metals, no cobalt; (c) all metals+1μM cobalt; (d) all metals+5μM; (e) no metal+5μM cobalt.

H_2/CO_2 as the substrate

The results of these assays, summarized in table 5 and 6, clearly do not reveal an effect of either the full metal cocktail nor of the mere cobalt addition on the maximum SMA and duration of the lag phase. The Heineken and Eerbeek sludge converted H_2/CO_2 without any clear lag phase, but with the Nedalco and Hoogeveen sludge a lag phase of approximately 13 and 50h was found, respectively. The highest values for the SMA were found for the Hoogeveen and Nedalco sludge (Table 5). The SMA of the Hoogeveen sludge assessed in a second feed did not show any response to metal addition (at pH 7) and only slightly higher values for the SMA were found for the second feed, while then no lag-phase was present (Table 6).

Table 4. Average specific methanogenic activity (mg CH_4. g $VSS^{-1}.d^{-1}$) with acetate as the substrate, the observed lag-phase (h) and the time (h) after start of the experiment after which maximum activity was observed.

	Nedalco			**Eerbeek**			**Hoogeveen**			**Heineken**		
	Act	Lag	Max	Act	Lag	Max	Act	Lag	Max	Act	Lag	Max
No metals	639	52	96	180	10	29	113	60	219	118	560	775
All metals	655	52	97	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	115	580	775
All +1μM Co	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	150	560	729
All +5μM Co	641	40	75	n.d.	n.d.	n.d.	109	60	204	184	234	409
No +5μM Co	617[a]	40	74	195	10	30	106	60	219	n.d.	n.d.	n.d.

n.d. not determined; Heineken all metals+1μM Cobalt average of 109 and 191 mg g $VSS^{-1}.d^{-1}$; [a] result of one measurement.

The SMA was also determined at pH 6, the operational pH of the Hoogeveen bioreactor, and a lower value was found compared to pH 7, viz. 296, 294 and 264 mg CH_4-COD.g $VSS^{-1}.d^{-1}$ for the no metal, cobalt (5 μM) and 'all metal + 5 μM cobalt' batches, respectively, but apparently there was no response to the different conditions applied (Table 6). The methane formation rate with H_2/CO_2 was initially similar for all conditions, but decreased from 48 h onwards in the assays conducted with the complete trace element cocktail but not in the assays conducted in absence trace metal element addition and cobalt-addition, which likely can be attributed to formation of acetate. The total methane formation was 1318 mg CH_4-COD after 212 h in the assay incubated with the complete metal cocktail, compared to 1807 and 1709 mg CH_4-COD respectively in the assays without metal addition

and merely cobalt addition. The pH assessed at termination of the experiment in the "all metal batches" was 5.95 and 6.2 for the other assays.

Table 5. Average specific methanogenic activity (mg CH_4. g $VSS^{-1}.d^{-1}$) with H_2/CO_2 as the substrate, the observed lag-phase (h) and the time (h) after start of the experiment after which maximum activity was observed.

	Nedalco			**Eerbeek**			**Hoogeveen**			**Heineken**		
	Act	Lag	Max	Act	Lag	Max	Act	Lag	Max	Act	Lag	Max
No Metals	419	-	12.5	121	-	91	444	>50	n.d.	315	-	13
All metals	452	-	13	134	-	81	n.d.	n.d.	n.d.	305	-	13
All +1μM Co	n.d	n.d	n.d	146	-	85	n.d.	n.d.	n.d.	311	-	14
All +5μM Co	417	-	13	n.d.	n.d.	n.d.	438	>50	n.d.	306	-	14
No +5μM Co	432	-	13	n.d.	n.d.	n.d.	413	>50	n.d.	n.d.	n.d.	n.d.

n.d. not determined

Table 6. Average specific methanogenic activity (mg CH_4. g $VSS^{-1}.d^{-1}$) with H_2/CO_2 as the substrate for the Hoogeveen sludge at pH 6 and 7 after the first and second feed, the observed lag-phase (h) and methane formation (mg CH_4-COD.l^{-1}).

	Hoogeveen (pH 7)						**Hoogeveen (pH 6)**					
	First Feed			Second feed			First Feed			Second feed		
	Act	Lag	CH_4	Act	Lag	CH_4	Act	Lag	CH_4	Act	Lag	CH_4
No Metals	444	>50	1814	528	-	1807	296	>50	2012	367	-	2009
All +5μM Co	438	>50	1870	468	-	1317	264	>50	2009	357	-	1352
No +5μM Co	413	>50	1700	534	-	1710	294	>50	1988	412	-	1848

n.d. not determined; Heineken all metals+1μM cobalt average of 109 and 191 mg g $VSS^{-1}.d^{-1}$

DISCUSSION

Granule metal content and SMA response

This study showed a significant response to the supply of metals although merely in case the sludges were fed with methanol (Table 3): particularly the SMA of the Nedalco and

Hoogeveen sludge increased significantly when cobalt was supplied as trace element (Fig. 2). While the SMA of the Heineken sludge increased by the presence of another trace element.

The response to cobalt clearly cannot be related to the initial cobalt contents of the sludges (Fig. 1, Table 2), which was approximately the same for three of the sludges tested, except for the Heineken sludge, where it was slightly higher. Interestingly, also the cobalt fractionation for the Nedalco, Hoogeveen and Heineken sludge shows a similar pattern (Fig. 1, Table 2). The strong positive response to cobalt addition most probably has a biological reason, i.e. cobalt is present in corrinoid structures, which play key role in enzymes involved in methanogenesis. The strong response of the granular sludge to cobalt addition with methanol and that cobalt limitations can easily be induced in the sludge in lab-scale reactors [Chapter 3]. It makes this feed very suitable as a model system to elucidate some of the mechanisms and processes involved in e.g. the trace metal uptake, its bioavailability and its impact on the SMA of methanogenic sludges. This particularly is true with respect to the mode these 'trace-metals' should be dosed, i.e. chemical form, concentration, dosing frequency.

The finding that Eerbeek sludge in the experiments with methanol did not respond to the supply of merely cobalt may be due to an underlying dominating deficiency of another essential trace metal, unfortunately the response of this sludge to the addition of the complete metal cocktail has not been investigated. Sludge freshly harvested from the Eerbeek reactor (data not shown) responded to the supply of nickel at a content of 38 $\mu g.g\ TSS^{-1}$, which is slightly higher than the nickel content of the Eerbeek sludge tested in the present. Further 70% of the (already low) nickel content of the Eerbeek sludge is present in the residual fraction (Fig. 1), while in the three other sludges significantly higher amounts of nickel were present in the less strongly bound carbonate and organic/sulfide fractions. It also should be noted here that the methanol conversion by the Eerbeek sludge starts without any clear lag-phase, which indicates that a methanol degrading population was already present in this sludge.

The Heineken sludge did not show any clear response to the addition of cobalt, while the supply of the trace metal cocktail, resulted in a distinct improvement of the SMA of the Heineken sludge. All trace metals are present in this sludge at similar or higher content compared to the other sludges, with the exception of molybdenum (Table 1), further research is needed to elucidate whether molybdenum or another element is limiting in this sludge.

Total cobalt versus biologically active cobalt - Methanol

The 5 μM cobalt supplied in the assays is expected to become completely sorbed in/to the sludge with the highest affinity of cobalt for the organic/sulfide fraction, where it either precipitates as sulfide, becomes incorporated in the pyrite matrix or sorbed to the cell membrane and/or EPS [van Hullbusch et al., 2004]. Initially sulfide was almost completely

absent in the medium but it gradually appears as a result of the reduction of the sulfate, present in the system, and this then even may change the chemical form of the cobalt during the course of the experiment. According to Jansen (personal communication) the dissolution rate of fresh/non-aged metal precipitates (cobalt, nickel and iron) proceeds rapidly enough to prevent a limitation in the metal uptake in batch experiments conducted with a *Methanosarcina sp.* enrichment cultivated on methanol.

The observed response to the cobalt addition with methanol can be related to the relatively high corrinoid content of methanol grown methanogens (and acetogens), i.e. which is generally substantially higher than when grown on other substrates. Indeed, e.g. *Methanosarcina barkeri* contains 4.1, 2.5 and 1.6 μmoles corrinoids.g dry cell^{-1} weight when grown on methanol, acetate and H_2/CO_2, respectively [Kryzycki and Zeikus; 1980]. The high corrinoid content under methanol fed conditions is likely related to the pathway of the direct methanogenesis from methanol, which contains a methanol specific corrinoid containing methyl-transferase [Deppenmeier et al., 1999], responsible for the introduction of methanol into the methanogenic pathway. The Nedalco sludge contained 0.03 μmoles corrinoids.g VSS^{-1}, viz. only 7% of the cobalt was present as corrinoid structures (data not shown). Differences in corrinoid content will manifest in the (granular) sludge dependending on the substrate used, i.e. methanol, VFA and sucrose grown granular sludges contained respectively, 0.71, 0.32 and 0.44 μmoles corrinoids.g VSS^{-1} after more than 6 months of operation [Eekert et al., 1998]. Regarding the above, organisms fed with and growing on methanol, obviously require more cobalt than those growing on other substrates.

Total versus biologically active cobalt – Acetate and H_2/CO_2

Cobalt is also present in carbon monoxide dehydrogenase (CODH) complex, which is responsible for the cleavage of acetate [Ferry, 1999] and in methylcobalamin:coenzyme M methyltransferase [Sauer and Thauer, 2000] which is a key intermediate in methanogenesis of all methanogenic substrates. The observation that the supply (presence) of cobalt (5 μM) only has a slight positive response on the methanogenic activity on acetate (accompanied by a significant lag-phase reduction) for the Heineken sludge may be related to the long incubation time needed in that case, during this period an acetotrophic population, initially almost absent in the sludge, could develop. Apparently cobalt was important for the growth and onset of growth of this population in the Heineken sludge. Similar to methanogenesis on methanol, the first step in the enzymatic acetogenic conversion of methanol by the acetogen *Eubacterium limosum* is catalyzed by a methyl-transferase containing corrinoid [van der Meijden et al., 1984]. Consequently, methanol grown acetogens also contain a high corrinoid concentration, e.g. 8.2 μmoles corrinoids.g^{-1} cell dry weight for *Acetobacterium sp* [Inoue et al., 1992].

In order to elucidate the influence of nickel and cobalt addition, Kida et al. [2001] cultivated a mixed culture of mesophilic methanogenic organisms on acetate as the sole substrate in a continuously stirred tank reactor (CSTR). The cobalt content of the sludge assessed at the moment of the maximum gas production rate (135 ml. g $VSS^{-1}.h^{-1}$) amounted to 39.2 μg.g VSS^{-1}, which was found to be present mainly in corrinoids (38 μg.g VSS^{-1}). The cobalt initially present in the granular sludge (see Table 1) therefore could suffice or almost suffice for attaining the optimal activity on acetate for all four the sludges tested. However, apparently this 'bound' cobalt is not completely bioavailable, and therefore a clear response to the addition of cobalt can be expected, especially when new bacterial growth is required for the conversion of the substrate. The addition of 5 μM cobalt would theoretically suffice, in case all cobalt supplied to the system was taken up by the 1 g of wet weight of granular sludge present in the activity vial. The cobalt content of the sludge then would increase from 31 to 116 μg.g VSS^{-1} for the Eerbeek sludge from 27 to 220 μg.g VSS^{-1} for the Nedalco sludge, i.e. a content greatly exceeding the theoretical optimum values needed for the conversion of acetate.

The duration of the SMA assays with H_2/CO_2 was relatively short and lag-phases were short or absent. This indicates the presence of active hydrogenotrophic methanogenic populations in all the sludges tested. The absence of a response of the SMA to cobalt is probably related to the lower cobalt requirement for hydrogenotrophic methanogens compared to methylotrophic methanogens. Indeed, an acclimated mixed culture of hydrogen utilising methanogens contained only 12 μg.g dry $cell^{-1}$ [Zhang et al., 2003].

SMA versus growth

The maximum SMA-values assessed in this research can likely be mainly attributed to new bacterial growth, because the maximum activities were always found near the termination of the experiment, in some cases after more than 10 days such as for instance in the case with methanol as the substrate (Table 3). This new growth obviously requires the presence and bioavailability of metals such as cobalt to enable its incorporation in the newly formed biomass.

The rate curves of the Nedalco and Hoogeveen sludge on methanol in the absence of metals show a remarkable similarity (Fig. 2) to those found in previous investigations Gonzalez-Gil et al. [1999] with cobalt and nickel limited sludge. Three clear phases could be distinguished, namely a first exponential phase, followed by a temporary decrease in the rate and finally an arithmetic increase of the gas production rate. Gonzalez-Gil et al. [1999] suggested to atribute this arithmetic phase to the dissolution of metal sulfides present in the system. If correct this would imply that, during the current experiments with Hoogeveen sludge, but especially for the Nedalco sludge, that the activity increase at least 'partly' would

be cobalt dissolution driven (either from sulfides or biomass decay) towards the termination of the experiment (after ±300h for Hoogeveen and 250h for the Nedalco sludge). The same reasoning may hold for the immediate arithmetic increase in the methane production rate found for the Eerbeek sludge with methanol (Fig. 2), i.e. also metal dissolution driven, although in this case not of cobalt but of some other metal, for instance nickel. However, in case of the Hoogeveen sludge, although similar rate curves for acetate were found as those for the Eerbeek sludge with methanol a constant arithmetic increase in the methane production rate manifested, indicating non-exponential growth. In the presence of the complete metal cocktail+5μM cobalt and merely 5 μM cobalt the methane production rate curves were similar to those found in the 'no metal batches', this indicates that an other factor than addition of metals prevented exponential growth/SMA increase. In contrast, the initial activity on acetate for the Heineken sludge appeared to be extremely low, here apparently (uninhibited) an exponential growth gradually developed.

The on-line SMA measurement procedure applied in this study allows a detailed analysis of the change in SMA and growth dynamics during the experiment and it therefore enables to distinguish subtle differences, which are impossible to distinguish in the conventional SMA-assessment techniques via e.g. the measurement of methane formation in the headspace. For instance the remarkable drop in methane production in the Heineken 'no metal' assay with methanol would not have manifest in such a test. An explanation for this drop could be the occurrence of a partial conversion of the methanol into acetate. The drop in methane production rate can than attributed to the depletion of the methanol, after which the remaining acetate was converted to CH_4. Nevertheless, this does not seem a very likely explanation, because the activity on acetate only developed after a long lag-phase of 560 h in the absence of metals. It only would be possible when the *Methanosarcina* population, responsible for the direct conversion of methanol to methane, had been already activated/enriched during the previous 300 h with the capacity to convert the acetate. Methanol grown *Methanosarcina* were shown to be unable to degrade acetate in the presence of methanol, they merely can convert acetate after the depletion of the methanol [Smith and Mah, 1978]. This would then explain the observed residual methane formation, although sometimes methanol grown *Methanosarcina* are unable to degrade acetate at all, even in the absence of methanol [Smith and Mah, 1978].

Reactor operation implications for practice

Considering the importance of cobalt for different trophic groups and the fact that cobalt is not present at high concentrations or even absent in most of the wastewater streams treated in full-scale reactors, it is clear that anaerobic bioreactors will be vulnerable to limitations for this element. This particularly is true for operating a reactor with methanol,

because the requirement for cobalt then is unambiguous. Even when a limitation initially does not manifest in a SMA assay, it can easily develop during reactor operation [Chapter 3]. Cobalt contents in the sludge matrix in the range as observed in the tested anaerobic granular sludge samples (27 to 51 μg.g TSS^{-1}) likely will result in limitations. Freshly harvested Eerbeek sludge (59 μg cobalt .g TSS^{-1}) was found to become clearly cobalt limited after 55 days of operation in the absence of Cobalt supply [Zandvoort et al., in prep.]. The SMA of this sludge 1687 mg CH_4-COD. g VSS^{-1}, was relatively high, but by addition of 5μM cobalt it still could be increased with 49% to 2515 mg CH_4-COD. g $VSS^{-1}.d^{-1}$ [Zandvoort et al., in prep.]. Even larger responses were observed with Nedalco sludge (19 μg cobalt. g TSS^{-1}) when operated for a period of 48 days under conditions without any trace metals supply. The SMA of this sludge could be increased by 379% from 402 to 1927 mg CH_4-COD. g $VSS^{-1}.d^{-1}$ upon the addition of 5μM cobalt [Chapter 8; Table 1]. Similarly limitations for other metals can be induced e.g. for nickel, methanol grown Nedalco sludge (initial nickel-content 61 μg.g TSS^{-1}) became clearly nickel limited after 89 days of reactor operation [Chapter 4] with a methanol feed lacking any nickel. Eerbeek sludge (nickel-content 38 μg.g TSS^{-1}) even appeared to be immediate nickel limited [Zandvoort et al., in prep.]. Apparently nickel contents below approximately 100 μg.g TSS^{-1} should be avoided in methanol fed reactors. Although the sludge samples tested in this research did not show an immediate response of the SMA on metal addition when fed with acetate, Osuna et al. [2003] showed that such limitations can be induced upon prolonged reactor operation [Chapter 8; Table 1].

Metal addition not only can result in an improved reactor performance, also the pathway of substrate conversion (competition for the substrate) can be influenced such as the direct methane formation or acetate formation from methanol by supplying cobalt [Florencio et al., 1994]. This impact of the supply of trace metal(s) on the substrate conversion pathway/competition and product formation of simple (e.g. H_2/CO_2 conversion to either methane or acetate, competition between sulfate reducing bacteria and methanogens) and more complex substrates, and the consequent effect on for instance the population dynamics in the (granular) sludge consortium is still an almost untouched but very important field of research. It might open perspectives to steer the process in bioreactors with mixed cultures in the desired direction.

Even when any metal limitations can not be deduced from the metal composition of the inoculum sludge and from the response of the SMA of the sludge to metal addition, the results of this study clearly show the relevance of sludge characterization on the basis of the assessment of its metal content (combined with the influent metal concentration) and the response of its SMA to metal supply to the feed/system. It is a valuable tool to monitor the metal stock of the (granular) sludge and the trace metal requirements.

CONCLUSIONS

- Significant positive responses of the SMA to the supply of metals were observed, although merely when the sludges were fed with methanol, no response manifested on acetate or H_2/CO_2.
- The SMA of the Nedalco and Hoogeveen sludge with methanol increased significantly when cobalt was supplied, while another trace element increased the SMA of the Heineken sludge.
- The response of the SMA to cobalt addition cannot be related to the initial cobalt content of the sludges.
- Sludge characterization on the based on the assessment of the metal content and the response of its SMA to metal supply to feed/system is a valuable tool to monitor the metal stock and the trace metal requirements of (granular) sludge.

REFERENCES

Deppenmeier U, Lienard T, Gottschalk G (1999). Novel interactions in energy conservation by methanogenic archaea. *FEBS letters*, **457**, 291-297

Eekert MHA, Schröder TJ, Stams AJM, Schraa G, Lens PNL (1998). Degradation and fate of carbon tetrachloride in unadapted methanogenic granular sludge. *Appl Environ Microbiol*, **64**, 2350-2356

Ferry JG (1999). Enzymology of one-carbon metabolism in methanogenic pathways. *FEMS Microbial Rev*, **23**, 13-38

Florencio L, Jeniček J, Field JA, Lettinga G (1993). Effect of cobalt on anaerobic degradation of methanol. *J Ferment Bioeng*, **75**, 368-374

Florencio L, Field JA, Lettinga G (1994). The importance of cobalt for individual trophic groups in an anaerobic methanol degrading consortium. *Appl Environ Microbiol*, **60**, 227-234

Gonzalez-Gil G, Kleerebezem R, Lettinga G (1999). Effects of nickel and cobalt on kinetics of methanol conversion by methanogenic sludge as assessed by on-line CH_4 monitoring. *Appl Environ Microbiol*, **65**, 1789-1793

Hullebusch van ED, Peerbolte A, Zandvoort MH, Lens PNL (2005). Sorption of cobalt and nickel on anaerobic granular sludges: isotherms and sequential extraction. *Chemosphere*, **58**, 493-505

Kida K, Shigematsu T, Kijima J. Namaguchi M, Yoshitaka M, Abe N, Morimura S (2001). Influence of Ni^{2+} and Co^{2+} on methanogenic activity and the amounts of coenzymes involved in methanogenesis. *J Biosci Bioeng*, **91**, 590-595

Kryzycki J, Zeikus JG (1980). Quantification of corronoids in methanogenic bacteria. *Curr Microbiol*, **3**, 243-245

Inoue K, Kageyama S, Miki K, Moringana T, Kamagat Y, Nakamura K, Mikami E (1992). Vitamin B_{12} production by *Acetobacterium sp.* and its tetrachloromethane-resistent mutants. *J Ferment Bioeng*, **73**,76-78

Meijden Van de P, van der Drift C,Vogels GD (1984). Methanol conversion by *Eubacterium limosum. Arch Microb*, **138**, 360-364

Osuna MB, van Hullebusch E, Zandvoort MH, Iza J, Lens PNL (2004). Effect of cobalt sorption on metal speciation in Anaerobic Granular Sludge, *J Environ Qual*, **33**, 1256-1270

Osuna MB, Zandvoort MH, Iza M, Lettinga G, Lens PNL (2003). Effects of trace element addtion on fatty acid conversions in anaerobic granular sludge reactors. *Environ Technol*, **24**, 573-587

Sauer K, Thauer RK (2002). Methyl-conzyme M formation in methanogenic archea. *Eur J Biochem*, **267**, 2498-2504

Smith MR, Mah RA (1978). Growth and methanogenesis by *Methanosarcina Strain* 227 on acetate and methanol. *Appl Environ Microbiol*, **36**, 870-879

Zandvoort MH, Geerts R, Lettinga G, Lens PNL (2002a). Effect of long-term cobalt deprivation on methanol degradation in a methanogenic granular sludge reactor. *Biotechnol Progr*, **18**, 1233-1239

Zandvoort MH, Osuna MB, Geerts R, Lettinga G, Lens PNL (2002b). Effect of nickel deprivation on methanol degradation in a methanogenic granular sludge reactor. *J Ind Microbiol Biotechnol*, **29**, 268-274

Zandvoort MH, Geerts R, Lettinga G, Lens PNL (2003) Methanogenic versus acetogenic degradation of methanol in granular sludge reactors at sub-optimal metal concentrations; role of iron, nickel and cobalt. *Enz Microbial Technol*, **33**, 190-198

Zandvoort MH, Van Hullebusch ED, Peerbolte A, Golubnic S, Lettinga G (in prep.) Dual metal limitation (nickel and cobalt) limitation in a methanol fed methanogenic granular sludge reactor

Zhang Y, Zhang Z, Suzuki K, Maekawa K (2003). Uptake and mass balance of trace elements for methane producing bacteria. *Biomass Bioenergy*, **25**, 4227-4233

Chapter 3

Effect of long-term cobalt deprivation on methanol degradation in a methanogenic granular sludge bioreactor

The effect of the trace metal cobalt on the conversion of methanol in a Upflow Anaerobic Sludge Blanket (UASB) reactor was investigated by assessing the effect of cobalt deprivation from the influent on the reactor efficiency and the sludge characteristics. A UASB reactor (30°C; pH 7) was operated for 261 days at a 12 h hydraulic retention time (HRT). The loading rate was increased stepwise from 2.6 g COD.l reactor^{-1}.d^{-1} to 7.8 g COD.l reactor^{-1}.d^{-1}. Cobalt deprivation had a strong impact on the methanogenic activity of the sludge. In batch tests, the methanogenic activity of the sludge with methanol as the substrate increased 5.3 (day 28) and 2.1 (day 257) times by addition of 840 nM of cobalt. The sludge had an apparent K_m for cobalt of 948 nM after 28 days of operation and 442 nM at the end of the run. Cobalt deprivation during 54 days of operation led to a methanol conversion efficiency of only 55%. Continuous addition of cobalt (330 nM) for 33 days improved the methanol removal efficiency to 100%. In this period of cobalt dosing, the cobalt concentration in the sludge increased with a factor 2.7 to 32 μg. g TSS^{-1}. Upon omission of the cobalt addition, the cobalt content of the sludge decreased at a stable rate of 0.1 μg.g VSS^{-1}.d^{-1}. At the end of the run, the cobalt content of the sludge was similar to that of the seed sludge.

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INTRODUCTION

Although methanol is a direct substrate for methanogens [Nishio et al., 1992; Smith and Mah, 1978], it is far less studied than the other direct methanogenic substrates such as hydrogen/carbondioxide (H_2/CO_2) and acetate. Latter compounds received much more attention, as they are common intermediates during anaerobic digestion. Methanol can be converted to methane via a number of pathways (Fig. 1), i.e. direct conversion to methane by methylothrophic methanogens [Nishio et al., 1992; Smith and Mah, 1978], indirect conversion to acetate by acetogens [van der Meijden et al., 1984; Zeikus et al., 1980] coupled to acetoclastic methanogenesis [Huser et al., 1982] or indirect conversion to H_2 and CO_2 [Cord-Ruwisch et al., 1986; Cord-Ruwisch et al., 1988] coupled to hydrogenotrophic methanogenensis [Braum et al., 1981; Whitman et al., 1982].

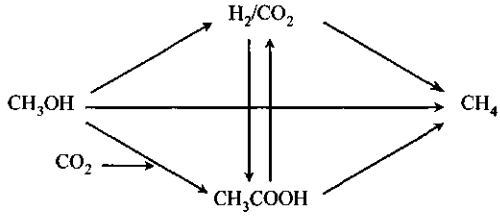

Figure 1 Possible pathways of methanol conversion to methane.

The main concern for stable operation of methanol fed reactor systems under mesophillic conditions is to prevent volatile fatty acids (VFA) accumulation. In earlier research dealing with the factors affecting methanol conversion, it was found that bicarbonate (HCO_3^-), pH and trace elements were key factors in the methanol degradation pathway and the stable operation of methanol fed bioreactors [Lettinga et al., 1979]. Among the trace elements, cobalt plays a key role, it regulates the methanol degradation pathway and competition between the different trophic groups (acetogens or methanogens) involved in methanogenic methanol conversion. If a sludge is capable to convert methanol to CH_4, cobalt addition may stimulate methane production because it is an essential building unit of enzymes involved in methane formation [Florencio et al., 1993]. Moreover, it has been observed that low cobalt levels during start-up can prevent reactor instability due to acetate build-up by acetogenic activity [Florencio et al., 1993]. With respect to the dosing strategy, continuous dosing of cobalt was found to improve the bioavailability better than compared to single pulse additions [Gonzalez-Gil et al., 1999]. Pulse dosing leads to an initial exponential increase of the methane formation rate but with time, when cobalt availability becomes limited in the medium due to e.g. sulfide formation, the increase in methane production rate becomes linear.

These problems could be overcome by dosing cobalt continuously at rates of 0.05 and 0.2 μmol/h [Gonzalez-Gil et al., 1999]. Despite the fact that the importance of cobalt for the methanol conversion pathway is well known, little is known about the fate, storage and dynamics of this trace metal in anaerobic granular sludge. Also the long-term effects of cobalt deprivation on the total cobalt pool in anaerobic granules are not known.

In this study, the effects of cobalt depletion and uptake by omitting and/or continuously dosing cobalt in the influent on the performance of a mesophillic UASB reactor was investigated. The methanol conversion into VFA and methane was monitored as a function of time. Using batch tests, the metabolic properties of the sludge that developed in the reactor were also characterized.

MATERIAL AND METHODS

Source of biomass

The methanogenic granular sludge was obtained from a full scale UASB reactor treating alcohol distillery wastewater of Nedalco (Bergen op Zoom, the Netherlands). The sludge was elutriated to remove the fines. The total suspended solids (TSS) and volatile suspended solids (VSS) concentration of the sludge were 10.03 (±0.2)% and 9.54 (0.2)%, respectively. The initial cobalt conctent of the sludge was relatively low, 17 μg.g TSS^{-1}.

Basal medium

The reactor was fed with a basal medium consisting of methanol, macro nutrients and a trace element solution dissolved in tap water. The inorganic macro nutrients content (in milligrams per litre of basal medium) was: NH_4Cl (280), K_2HPO_4 (250), $MgSO_4.7H_2O$ (100) and $CaCl_2.2H_2O$ (10). In addition 0.1 millilitre of both acid and base trace nutrient solution, from which cobalt was omitted (Table 1), was added per litre of basal medium. 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, the influent was composed of 4 streams: basal medium without K_2HPO_4, methanol with bicarbonate, K_2HPO_4 and dilution water.

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 in a temperature-controlled room of 30 (±2) °C. The UASB reactor was inoculated with 8.7 g $VSS.l^{-1}$ anaerobic granular sludge and operated at a hydraulic retention time (HRT) of 12h. The conical bottom of the reactor was filled with glass marbles (1cm in diameter) to distribute

the influent evenly over the sludge bed. For the influent flow, peristaltic pumps (type 505S, Watson and Marlow, Falmouth UK) were used. No effluent recycle was applied and the superficial upflow velocity was 0.08 $m.h^{-1}$.

During start-up (period I), methanol was fed to the reactor at a concentration of 1.4 g $COD.l^{-1}$, corresponding to an organic loading rate (OLR) of 2.6 g $COD.l^{-1}$ $reactor.d^{-1}$. The methanol loading rate was increased on day 154 to 5.2 g $COD.l^{-1}$ $reactor.d^{-1}$ (period II) and at day 199 to 7.8 g $COD.l^{-1}$ $reactor.d^{-1}$ (period III) until the end of the experiment (Fig. 2). Based on the initial sludge content of the reactor, this correspond to sludge loading rates (SLR) of 0.3, 0.6 and 0.9 g COD. g $VSS^{-1}.d^{-1}$ during period I, II and III, respectively.

The produced biogas was led through a waterlock filled with concentrated NaOH (15%) solution and then through a column with soda lime pellets to remove CO_2 and H_2S. The produced methane volume was measured with a wet gas meter (Schlumberger Industries Dordrecht, The Netherlands).

Table 1. Composition of the trace element solution.

Compound added	**Element**	**Concentration**	
		Compound ($mg.l^{-1}$)	Metal ($mg.l^{-1}$)
$FeCl_2.4H_2O$	Fe	2000	562
$ZnCl_2$	Zn	50	24
$MnCl_2.4H_2O$	Mn	500	139
$CuCl_2.2H_2O$	Cu	38	14
$NiCl_2.6H_2O$	Ni	92	23
$(NH_4)_6Mo7O24.4H_2O$[a]	Mo	50	27
$Na_2SeO_2.5H_2O$[a]	Se	164	49

1 ml of 36% HCl was added to 1 litre of trace acid element solution.; 1 ml of 33% NaOH was added to 1 litre of base trace element solution.; [a] Nutrient present in the base trace element solution

Specific maximum methanogenic activity test

Approximately 1.2 g (wet weight) of granular sludge was transferred to 120 ml serum bottles containing 60 ml of basal medium with the same composition as the reactor basal medium, supplemented with either methanol (4 g $COD.l^{-1}$) or acetate (2 g $COD.l^{-1}$) as the substrate. In the first activity experiment methanol was added in two feeds of 2 g $COD.l^{-1}$. The second feed was added 4 days after the first feed. The bottles were closed with butyl

rubber stoppers (Rubber b.v., Hilversum, the Netherlands) and flushed with a N_2/CO_2-gas mixture (70/30 v/v). The experiments were done at 30 (±2) °C in duplicate. The activity was determined by on-line measurement of the increase of headspace pressure as a result of methane production in the serum bottles using pressure transducers (Honeywell 26PCDFA1G). In order to measure the pressure in the serum bottle, a butyl rubber tube was connected to the outlet of the pressure transducer. At the other end of the tube, a needle was connected using a luer lock adapter (outer diameter 3.1mm, Unimed; Geneva, Switzerland). The needle was put through the butyl rubber stopper of the serum bottle. The signal of the transducer was send to a computer with a data acquisition card (PCL-818-HD) via a multiplexer card (PCLD-789D) on which data were stored by the data acquisition program Visidaq 3.11 (Advantech Europe, Advantech Europe, Eindhoven, the Netherlands). Data were acquired every 30 minutes. The data were plotted in a rate *vs.* time curve, using moving average trend lines with an interval of 15 data points.The methanogenic activity was measured at the beginning of the experiment, at day 28 (before cobalt addition to the influent), at day 147 (after cobalt addition and before the first loading rate increase) and at day 257 (end of the experiment).

Metal analyses

Total dissolved metal concentrations in the influent and effluent were determined by inductively coupled plasma-mass spectrometry i.e. ICP-MS (Perkin-Elmer, Elan 6000) in samples acidified with 0.1 M HNO_3. The samples were centrifuged at 9500 g to remove particles from the liquid. The total metal content of the sludge was determined after microwave destruction (CEM 2100, Matthews, USA) of pre-dried sludge (105°C) in a mixture of 2,5 ml of HNO_3 (65%) and 7,5 ml HCl (37%). After destruction, the samples were paper-filtered (Schleicher & Schuell 589^I, Germany) and diluted to 100 ml, 1ml of this solution was transferred to 9 ml of 0,1M of HNO_3 and subsequently analysed for their metal content by ICP-MS.

Other analytical methods

The concentration of methanol, VFA and the composition (CO_2, CH_4 and N_2) of the biogas were determined by gas chromatography according to the procedure described by Weijma et al. [2000]. The total sulfide concentration was determined colorimetrically using the methylene blue method [Trüper and Schlegel, 1964]. The total suspended solids (TSS) and volatile suspended solids (VSS) concentration were determined according to Standard Methods [APHA, 1985]. All chemicals were of analytical or biological grade and purchased from E. Merck AG (Darmstadt, Germany).

RESULTS

Methanol conversion

During the first 15 days of reactor operation the methanol conversion steadily increased until day 15 when it stabilized at 55±8%, remaining at this value until day 54. The removed methanol was fully converted to methane. In order to see whether or not the methanol conversion rate could be stimulated, a low concentration of cobalt (0.33 μM) was dosed to the influent from day 54 to 85. Indeed, from day 60 day onwards (6 days after cobalt dosing started), the methanol concentration in the effluent decreased sharply and any methanol was found in the effluent from day 69 until day 106 (Fig. 2). Following day 85, the reactor was operated again with a feed without cobalt till the end of the experiment. From day 106 to 152 some methanol (97 (±81) mg COD.l^{-1}) and VFA (75 (±35) mg COD.l^{-1}) could be detected in the effluent (Table 2).

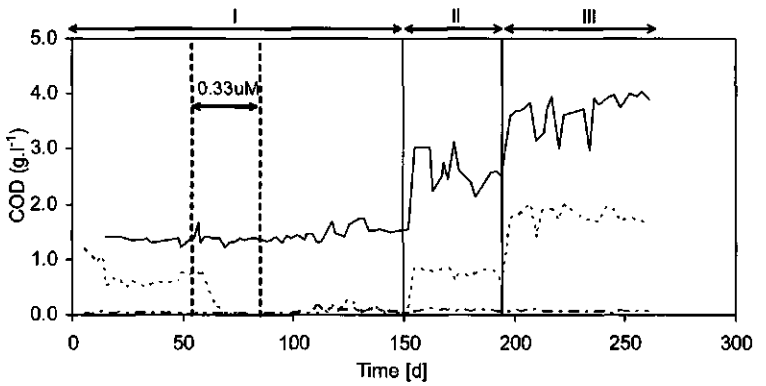

Figure 2 Evolution of reactor performance with time. Influent methanol (—), effluent methanol (····), and the effluent VFA (---) concentration.

In order to asses if the sludge had accumulated enough cobalt to cope with an increase in the loading rate, the organic loading rate was doubled to 5.2 g COD.l reactor^{-1}.d^{-1} on day 154. Following this OLR increase, the methanol concentration in the effluent immediately increased and stabilized at a concentration of 0.78 g COD.l^{-1} (Fig. 2), corresponding to a removal of only 37% of extra supplied methanol. The extra methanol introduced in the UASB reactor with the second loading rate increase (day 199) remained completely unconverted, resulting in a COD removal efficiency of only 50% (Fig. 2, Table 2).

The percentage methane in the biogas was around 85% throughout period I and II, but during period III it was not determined, because of technical problems with the gas collection system. A slight increase in the CO_2 concentration from 5% to 8% was observed when the loading rate was increased in period II (Table 2). The 0.41 mM sulfate in the influent was reduced to sulfide. The effluent sulfide concentration was measured from day 11-78 and amounted to 0.23 (±0.03) mM.

Table 2. Mean performance characteristics of the UASB reactor.

Parameter	**Period I**	**Period II**	**Period III**
Day	0-154	155-198	199-259
Effluent composition			
pH	7.4±0.2	7.1±0.1	7.1±0.1
COD Methanol influent ($mg.l^{-1}$)	1420±117	2642±316	3669±326
COD Methanol effluent ($mg.l^{-1}$)	243±273	774±68	1762±135
COD VFA ($mg.l^{-1}$)[a]	40±33	75±19	60±18
COD acetate ($mg.l^{-1}$)	29±29	57±21	42±11
COD propionate ($mg.l^{-1}$)	6±9	13±8	14±11
Methanol conversion route			
MeOHacc (%)[b]	15.7±19.9	29.7±4.3	46.4±8.3
VFAacc (%)[c]	2.9±2.6	3.0±0.9	1.8±0.5
Biogas composition			
CH_4	84.24±1.86	85.25±0.66	n.d.
CO_2	5.12±1.90	8.27±0.23	n.d.

[a] Total amount of VFA in the effluent including butyrate and valerate;[b] Percentage of unmetabolised methanol present in the effluent;[c] Percentage of methanol converted to VFA; n.d. not determined

Metals dynamics in the bioreactor

Before cobalt was added to the influent 5±1 nM cobalt washed out from the reactor. A slight increase in the effluent cobalt concentration (18±10 nM) was observed after cobalt addition to the influent. During the period of cobalt addition, the average influent concentration was 307±13 nM (days 54 to 85), this corresponds to a total amount 7.6 mg of cobalt added to the system. From day 56 until day 217 approximately 2.5 mg cobalt washed

out from the reactor based on the effluent concentrations. The cobalt content of the inoculum sludge (17 μg.g TSS^{-1}) corresponded to an initial total amount of 1.14 mg cobalt present in the bioreactor. During the first 54 days of operation, the cobalt content of the sludge decreased by 5 μg.g TSS^{-1} (Fig. 3). Cobalt addition to the influent (days 54 to 85), increased the cobalt content of the sludge 2.7 times (from 12 to 32 μg.g TSS^{-1}), which corresponds to an increase of only 1.34 mg based on the initial sludge content of the reactor. When cobalt was omitted from the influent again, the content in the sludge steadily decreased at a rate of 0.1 μg.g $TSS^{-1}.d^{-1}$.

Apparently iron and nickel were not well retained in the reactor, although it should be noted that the influent and effluent iron concentration and the nickel effluent concentration (Fig. 3) varied considerably. A similar linear decrease in the nickel and iron sludge content was observed, i.e. of 0.3 μg Ni. g $TSS^{-1}.d^{-1}$ and 7.1 μg Fe. g $TSS^{-1}.d^{-1}$. The content of nickel and iron in the sludge upon termination of the experiments were respectively, 38 and 28 % of that of the inoculum sludge (Fig. 3). The decrease in the content of these metals in the sludge seems primarily caused by leaching of the metals from the sludge, but also partially can be attributed to some "dilution" effect due to the formation of new sludge granules, because the sludge bed height increased by 30% (Fig. 4). The VSS/TSS ratio of the sludge decreased from 0.95 at the start of the experiment to 0.85 at termination of the experiment.

Metabolic characteristics of the sludge

During the reactor run, the methanogenic activity of the sludge on methanol doubled. The maximum methanogenic activity (in the absence of cobalt) of the seed sludge was similar to the activity after 28 days of operation. The methanogenic activity had doubled at day 147 and then remained almost unchanged until day 257 (Table 3).

The effect of cobalt addition on the methanogenic activity on methanol was determined with the sludge sampled on day 28 and day 257 (Fig. 5, Table 3). Cobalt addition clearly stimulated the methanogenic activity in both cases.

Addition of 8.4 μM of cobalt to the sludge sampled at day 28 increased the activity from 110 mg CH_4-COD.g $VSS^{-1}.d^{-1}$ in the absence of cobalt to 1094 mg CH_4-COD.g $VSS^{-1}.d^{-1}$ (calculated from the second feed). The same dose of cobalt (8.4 μM) increased the activity of the sludge to 507 mg CH_4-COD.g $VSS^{-1}.d^{-1}$ at the end of the experiment (day 257), methanol was added in one feed of 4 g $COD.l^{-1}$. Cobalt concentrations as low as 100 nM increased the methanogenic activity on methanol of the sludge sampled on day 257, but with only 10 nM of cobalt supplied no increase in the activity could be observed.

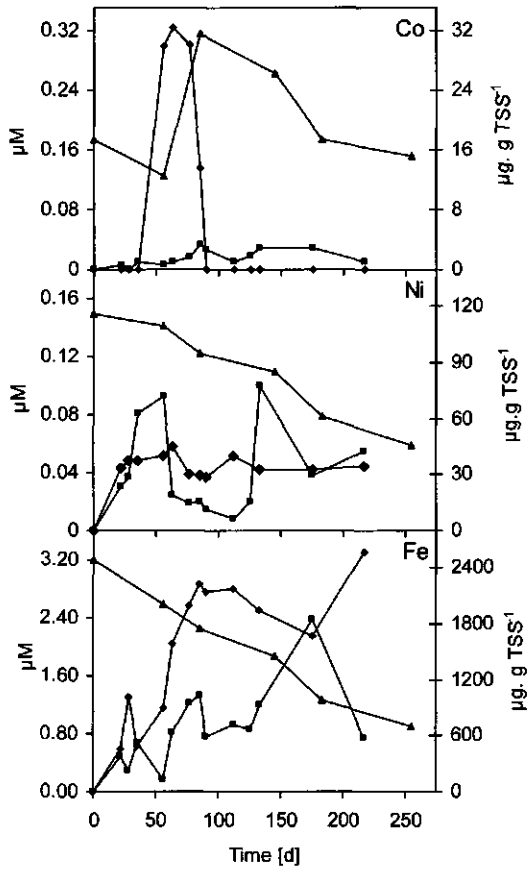

Figure 3 Evolution of the cobalt, nickel and iron concentration in the influent (♦), effluent (■) and granular sludge (▲) as a function of time.

From the response of the methanogenic activity on methanol to the cobalt addition, apparent K_m values of the sludge for cobalt were calculated using Michaelis-Menten kinetics (Table 3, Fig. 5d). The sludge sample of day 28 had an apparent K_m of 364 nM for the first feed and 938 nM for the second feed, respectively. The sludge sample of day 257 had an apparent K_m of 442 nM (first feed).

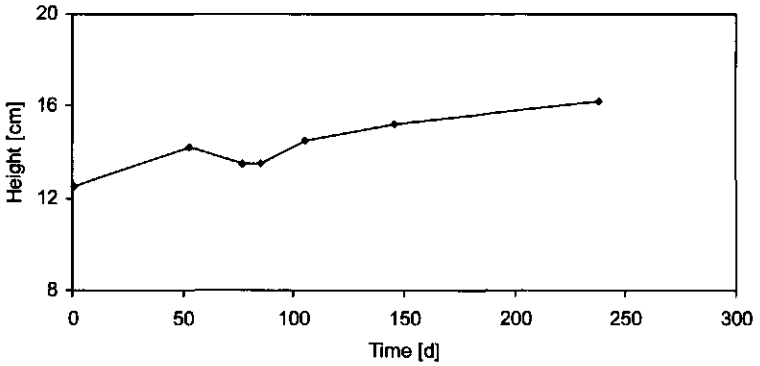

Figure 4 Evolution of the sludge bed size as a function of time

Table 3. Evolution of the specific methanogenic activity of the sludge (mg CH_4-COD.g $VSS^{-1}.d^{-1}$) on methanol during the reactor operation and as a function of the cobalt concentration.

	Day			
cobalt μM	0[a]	28[b]	147	257[b]
0.00	108	115/110	210	215 (28)
0.01	-	-	-	217
0.10	-	-	-	271
0.33	-	361/432	-	348
0.84	- (271)	491/582	-	453 (48)
2.10	-	-	-	490
4.20	-	-	-	512
8.40	-	620/1094	-	507
16.80	-	-	-	489

[a] Methanogenic activity on acetate is presented between brackets.; [b] Methanogenic activity after first and second feed, respectively

The reactor sludge was also tested for its response to iron (no cobalt added) with sample take at day 147. Addition of iron (10μM) increased the activity on methanol from 164 mg CH_4-COD.g $VSS^{-1}.d^{-1}$ to 210 mg CH_4-COD.g $VSS^{-1}.d^{-1}$ (data not shown).

The methanogenic activity of the seed sludge on acetate in a medium containing cobalt (8.4 μM) amounted to 271 mg CH_4-COD.g $VSS^{-1}.d^{-1}$ (Table 3). The methanogenic activity on acetate of the sludge sampled on day 257 was considerably lower, viz. 28 mg CH_4-COD.g $VSS^{-1}.d^{-1}$, addition of 0.84 μM cobalt increased the maximum specific methanogenic activity on acetate slightly to 49 mg CH_4-COD.g $VSS^{-1}.d^{-1}$.

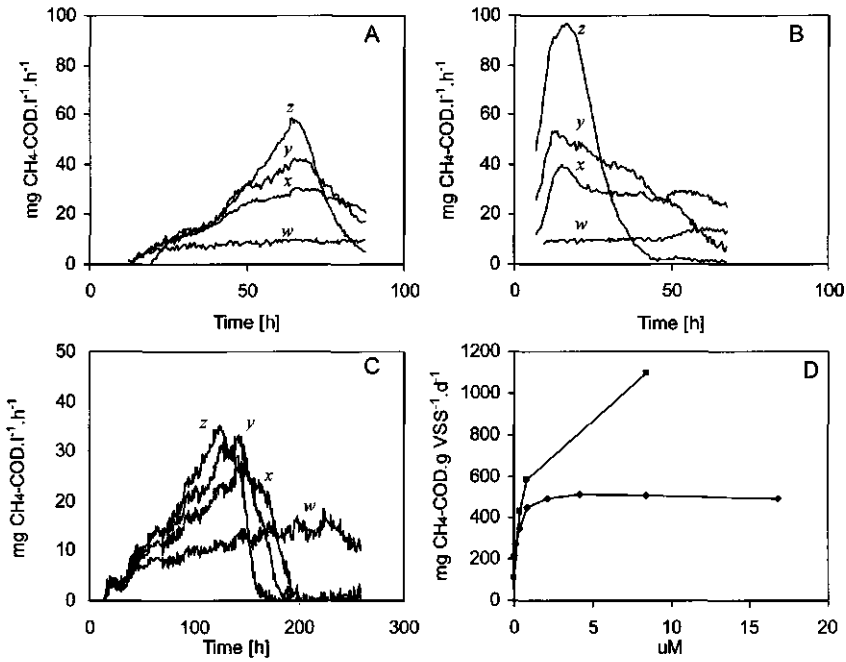

Figure 5 Methane production rate and methanogenic activity on methanol (pH 7; 30°C). (**A**) Methane production rate assessed for the sludge sampled at day 28, first feed. (**B**) Methane production rate assessed for the sludge sampled at 28 days, second feed. (**C**) Methane production rate assessed for the sludge sampled 257 days. (**D**) Methanogenic activity after second feed (2 g $COD.l^{-1}$) of the sludge sampled on day 28 (■) and 257 (♦) days (one feed of 4 g $COD.l^{-1}$) of operation. w, x, y and z represent concentrations of 0, 0.33, 0.84 and 8.4 μM cobalt in the test medium, respectively.

DISCUSSION

This study showed that the continuous supply of a very low concentration of cobalt (0.33 μM) considerably improves the reactor performance of a methanol fed, cobalt deprived UASB reactor (Fig. 2). This improvement can be related to the positive effect of the increased

cobalt stock present in the granules (Table 3). During the 33 days of cobalt addition to the sludge, at least 1.34 mg cobalt accumulated in the sludge bed. This amount of cobalt is, in theory, suffices for the formation of 19 g of dry weight of *Methanosarcina barkeri* 'fusaro', assuming direct conversion of methanol to methane and that methanol grown *Methanosarcina barkeri* 'fusaro' cells contain 0.070 mg $Co.g^{-1}$ of dry cells [Sherer et al., 1983]. The 30% increase of the sludge bed volume indicates that (likely) some new biomass may have been formed (Fig. 4), although this amount is relatively low compared to the amount produced in a nickel deprived reactor, operated in parallel with the same inoculum was used. The sludge bed volume increase amounted to 130%, which largely caused by growth of new biomass/granules [Chapter 4].

The methane formation rate curves in Fig. 5 show that at higher cobalt concentrations, the methane formation rate from methanol gradually increased over time and a plateau was not reached before the substrate became limiting. In contrast, methane production rates found in nickel deprived sludge cultivated in the parallel experiment did show a plateau [Chapter 4]. This indicates that at higher cobalt concentrations, either the uptake rate of cobalt, enzyme induction or the growth of new cells was the rate limiting factor, and not the cobalt concentration present in the medium. The estimated values of the apparent K_m of the sludge for cobalt (with methanol as the substrate), i.e. 364 nM and 442 nM after 28 and 257 days of operation, respectively are comparable to the K_m for nickel of nickel deprived sludge (678 nM) in a parallel experiment with the same inoculum [Chapter 4].

The very low and even decreasing methanogenic activity of the sludge on acetate suggests that methanol was directly converted to methane by methylothrophic methanogens and not via the intermediate formation of acetate. Overloading of the reactor with methanol did not result in the accumulation of VFA in the effluent (Period II and III, Fig. 2), which is in agreement with the results of Florencio et al. [1993], with the same inoculum sludge. According to Florencio et al. [1993] under cobalt limiting conditions, acetogens are not able to outcompete methylothrophic methanogens.

The supply of cobalt to the system induced the build up of a 2.7 times larger cobalt stock in the sludge. In cobalt binding experiments conducted by Schneider et al. [1995] with *Propionibacterium arabinosum*, cobalt from the medium was concentrated by several hundred times at the bacterial surface at medium concentrations of 9 to 100 nM. This binding is reversible and fast, i.e. it occurs in the first 5 to 8 minutes after cobalt addition. When exposed for longer period of time, cobalt internalisation starts and the binding is no longer reversible. The cobalt inside the cells is present in the form corrinoids [Schneider et al., 1995]. Adsorptive stripping voltammetry measurements on the effluent of a nickel deprived reactor operated in parallel with the reactor in the present study [Steffen et al., 2001], showed that cobalt was washed out in the form of corrinoids or molecules with comparable binding strengths. After terminating the supply of cobalt to the reactor, the washout of cobalt was

considerable and the cobalt content of the sludge decreased at a rate of 0.10 μg.g $TSS^{-1}.d^{-1}$. In order to be able to further optimise the dosing of cobalt, more research is needed with respect to the chemical form in which cobalt leaves the reactor. For instance, whether it was first internalised by microorganisms and subsequently lost in the form of corrinoids or if the cobalt washes out as another chemical species.

CONCLUSIONS

- Cobalt deprivation has a strong negative impact on the performance of mesophilic (30 °C) methanol fed granular sludge bed reactors.
- The methanol removal efficiency of an UASB reactor can be improved considerably by continuously dosing a relatively low concentration (0.33 μM) of cobalt for a period of 31 days to the influent of the bioreactor.
- The cobalt supplied to the system was retained in the sludge bed, i.e. the cobalt content of the sludge increase by a factor 2.7. However, due to small losses of cobalt from the sludge ultimately the cobalt content of the sludge reaches values similar to that of the inoculum.
- A low supply of cobalt during the reactor run results in a relatively low sludge production, indicating that restricting the supply of cobalt even may prevent excess sludge production.
- The methanogenic activity of the sludge can be controlled by the supply of cobalt, demonstrating that it can be used as a tool to control the reactor performance with respect to both sludge production and methanol conversion capacity.

REFERENCES

APHA (1985). Standard methods for the examination of water and wastewater. American Public Health Administration, Washington, D.C.,USA.

Braum MF, Mayer F, Gotschalk G (1981). *Clostridium aceticum* (Wieringa) a microorganism producing acetic acid from molecular hydrogen and carbon dioxide. *Arch Microbiol,* **128**, 288-293

Cord-Ruwisch R, Ollivier B (1986). Interspecific hydrogen transfer during methanol degradation by *Sporomusa acidovorans* and hydrogenothrophic anaerobes. *Arch Microbiol*, **144**, 163-165

Cord-Ruwisch R, Seitz H, Conrad R (1988). The capacity of hydrogenothrophic anaerobic bacteria to compete for trace of hydrogen depends on the redox potential of the terminal electron acceptor. *Arch Microbiol*, **14**, 350-357

Florencio L, Field JA, Lettinga G (1994). The importance of cobalt for the individual trophic groups in an anaerobic methanol degrading consortium. *Appl Environ Microbiol*, **60**, 227-234

Florencio L, Jenicek P, Field JA, Lettinga G (1993). Effect of cobalt on the anaerobic degradation of methanol. *J Ferment Bioeng*, **75**, 368-374

Gonzalez-Gil G, Kleerebezem R, Lettinga G (1999). Effects of nickel and cobalt on kinetics of methanol conversion by methanogenic sludge as assessed by on-line CH_4 monitoring. *Appl Environ Microbiol*, **65**, 1789-1793

Huser BAK, Wuhrman K, Zehnder AJB (1982) *Methanotrix soehngenii* gen. nov. sp., a new acetototrophic non-hydrogen oxidising methane bacterium. *Arch Microbiol*, **132**, 1-9

Lettinga G, van der Geest AT, Hobma S, van der Laan J (1979). Anaerobic treatment of methanolic wastes. *Wat Res*, **13**, 725-737

Meijden van der P, Drift van der C, Vogels, GD (1984). Methanol conversion in *Eubacterium limosum*. *Arch Microbiol*, **138**, 369-364

Nishio N, Kakizono T, Silveira RG, Takemoto S, Nagai S (1992). Nutrient control by the gas evolution in methanogenesis of methanol by *Methanosarcina barkeri*. *J Ferment Technol*, **73**, 481-485

Schneider Z, Trojanowska K, Jaszewski B, Nowak T (1995). cobalt binding by *Propionibacterium arabinosum*. *Lait*, **75**, 379-389

Scherer P, Lippert H, Wolff G (1983). Composition of the major elements ands trace elements of 10 methanogenic bacteria determined by inductive coupled plasma emission spectrometry. *Biol Trace Element Res*, **5**, 149-163

Smith MR, Mah RA (19780 Growth and methanogenesis by *Methanosarcina* strain 227 on acetate and methanol. *Appl Environ Microbiol*, **36**, 870-879

Steffen F (2001). Speciatie van nikkel en cobalt in sulfiderijk afvalwater. Msc. Thesis. Department of Physical and Colloidal Chemistry. Wageningen University, Wageningen, the Netherlands, In Dutch

Trüper HG, Schlegel HG (1964). Sulphur metabolism in *Thiordaceae* I. Qauntative measurment on growing cells of *Chromatium okenii*. *J Microbiol Ser*, **30**, 225-238

Weijma J, Stams AJM, Hulshoff Pol LW, Lettinga G (2000). Thermophillic sulfate reduction and methanogenesis with methanol in a high-rate anaerobic reactor. *Biotechnol Bioeng*, **67**, 354-363

Whitman W, Ankwanda E, Wolfe RS (1982). Nutrition and carbon metabolism of *Methanococcus voltae*. *J Bacteriol*, **149**, 852-863

Zandvoort MH, Osuna MB, Geerts R, Lettinga G, and Lens P (2002). Effect of nickel deprivation on methanol degradation in a methanogenic granular sludge reactor. *J Ind Microbiol Biotechnol*, **26**, 268-274

Zeikus JG, Lynd LH, Thompson TE, Krzycki JA, Weimer PJ, Hegge PW (1980). Isolation and characterization of a new methylothrophic, acidogenic anaerobe, the Marburg strain. *Curr Microbiol*, **3**, 381-386

Chapter 4

Induction of nickel and iron limitations in methanol fed granular sludge bioreactors

The effect of long term trace metal deprivation on the performance of methanol fed Upflow Anaerobic Sludge Bed (UASB) reactors was investigated. One UASB reactor received all trace metals except nickel (R1), whereas the second UASB reactor (R2) received all trace metals. The UASB reactors (30°C; pH 7) were operated for 261 days at a 12 h hydraulic retention time (HRT) and at organic loading rates (OLR) from 2.6 g to 7.8 g COD.l reactor^{-1}.d^{-1}. Nickel deprivation initially had a strong impact on the specific methanogenic (SMA) activity of the sludge on methanol, e.g. the activity of a sample taken after 89 days of operation doubled by adding 2 μM nickel. R1 could not cope with the second increase in OLR on day 57 (period III), the effluent methanol and VFA concentrations increased. Upon prolonged operation during period III, effluent methanol and VFA concentrations in R1 slowly decreased again, whereas the sludge lost its response to nickel addition in activity tests. Apparently a less nickel dependent methanol converting sludge had developed in the UASB reactor. Although, the lack of a nickel response might have also resulted from an increasing iron deficit that gradually manifested in the R1 sludge. In contrast to R1 the second OLR increase imposed to R2 fully converted to methane. After 92 days of operation the reactor efficiency of R2 suddenly deteriorated and both the concentration of methanol and volatile fatty acids (VFA) in the effluent increased. The SMA of the sludge on methanol had dropped from 1517 mg CH_4-COD. g VSS^{-1}.d^{-1} (on day 28) to 152 mg CH_4-COD. g VSS^{-1}.d^{-1} (on day 111). Testing the response of the SMA of the sludge to individual metals (iron, nickel and cobalt) revealed that only iron significantly increased the SMA on methanol.

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Zandvoort MH, Geerts R, Lettinga G, Lens PNL (2003) Methanogenic versus acetogenic degradation of methanol in granular sludge reactors at sub-optimal metal concentrations; role of iron, nickel and cobalt. *Enzyme and Microbial Technology*, **33**, 190-198

INTRODUCTION

The trace element requirement of anaerobic microorganisms is specific because many cobalt, nickel and iron containing enzymes are involved in the biochemistry of fermentation and methane production. Therefore, a lack of a single metal can severely impair the overall anaerobic conversion process [Shen et al., 1993a; Shen et al., 1993b, Kelly and Schwitzenbaum, 1984; Speece et al., 1983; Callandar and Barford, 1983; Florencio et al., 1993; Oleszkiewicz and Romanek, 1989]. Methanol represents a good model substrate to study the influence of trace elements on anaerobic conversions. The big importance of trace metals on the conversion of methanol to methane (CH_4) in an upflow anaerobic sludge bed (UASB) was first observed by Lettinga et al. [1979]. Following these investigations it was found that cobalt plays a key role in the methanogenesis of methanol [Florencio et al., 1993, Chapter 3].

Besides cobalt, nickel and iron are important elements in anaerobic microorganisms as well as they are structural elements in several enzymes involved in methane formation. Methanogenic *Archaea* (MA) use several pathways to reduce the various carbon substrates (e.g. methanol, acetate and H_2/CO_2) but all pathways converge to the common intermediate methyl-S-CoM [Thauer, 1988]. Methyl-S-CoM contains a nickel harbouring tetrapyrolic structure, coenzyme F_{430}, present in all methanogens and exclusively found in methanogens [Fukuzaki and Nishio, 1997]. Scherer *et al.* [1983] found that *Methanosarcina barkeri* Fusaro cells grown on methanol contain 2 times more nickel than cobalt when grown in a medium containing respectively 5 and 1 μM of nickel and cobalt. Nickel is present in enzymes involved in the metabolic pathways of anaerobic micro-organisms, both acetogens and methanogens. In addition, many hydrogenase enzymes used to form or consume hydrogen posses nickel [Deppenmeier et al., 1992; Kemnaer and Zeikus, 1994]. Carbon monoxide dehydrogenase (CODH), which possesses two nickel containing metallocenters is present in both acetoclastic methanogens and acetogenic microorganisms [Hausinger, 1987]. Nickel may also play a role in the stability of some methanogens, for instance in maintaining the wall stability [Jarrel and Sprott, 1982].

The role of iron in anaerobic methanol conversion has not received a lot of attention with respect to methanol conversion, despite its requirement as a macronutrient. However the nutritional requirement of iron for anaerobic microorganisms is clear: iron is present in iron-sulfur clusters, responsible for electron transport, and in various enzymes and cytochromes. For instance the iron content of *Methanosarcina barkeri* 'fusaro' is 2150 μg.g dry cells^{-1}, while nickel and cobalt concentrations are only 135 and 60 μg.g dry cells^{-1}, respectively [Scherer et al., 1983].

In this study, the effect of long term trace metal deprivation on the performance of methanol fed UASB reactors was investigated. One UASB reactor received all trace metals except nickel (R1), whereas the second UASB reactor (R2) received all trace metals. The two reactors were operated in parallel with a third reactor, from which cobalt was omitted from the feed [Chapter 3]. The fate and removal efficiency of methanol and sludge metal content were monitored as a function of time. Using batch tests, the metabolic properties and possible metal deficiencies of the sludge that developed in the UASB reactors were characterized as well.

MATERIAL AND METHODS

Source of biomass

The methanogenic granular sludge was obtained from a full-scale UASB reactor treating alcohol distillery wastewater of Nedalco (Bergen op Zoom, the Netherlands). The sludge was elutriated to remove the fines. The total suspended solids (TSS) and volatile suspended solids (VSS) concentration of the sludge were 10.0 (±0.2)% and 9.5 (±0.2)%, respectively.

Basal medium

The reactors were fed using a basal medium consisting of methanol, macro nutrients and a trace element solution dissolved in tap water. The inorganic macro nutrient solution contained (in milligrams per litre of basal medium): NH_4Cl (280), K_2HPO_4 (250), $MgSO_4.7H_2O$ (100) and $CaCl_2.2H_2O$ (10). In addition 0.1 millilitre of both acid and base trace element solution were added (Table 1). It should be noted that, in order to trigger metal limitations, the trace elements were supplied at concentrations 10 times lower than normally applied. 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, the influent was composed of 4 streams: basal medium without K_2HPO_4, methanol with bicarbonate, K_2HPO_4 and dilution water. Tap water was used to prepare the influent and was used as dilution water. When the medium was made without the addition of nickel (R1), it contained only traces of nickel (2 ± 4 nM), comparable to the nickel contamination present in demineralised water (3 nM). Also the chemicals to make up the macro nutrient solution contain some nickel contamination, resulting in a final nickel influent concentration of 9 (±5) nM.

UASB reactor operation

The experiment was performed using two Plexiglas cylindrical UASB reactors with a working volume of 7.25 l and an inner diameter of 0.1 m. One of the reactors (R1) was supplied with the full metal cocktail except nickel, while a second reactor (R2) received the full metal cocktail (Table 1). The reactors were operated in a temperature-controlled room at a temperature of 30 (±2) °C. The UASB reactors were inoculated with 8.7 g VSS.l^{-1} anaerobic granular sludge and operated at a hydraulic retention time (HRT) of 12h. The conical bottom of the reactors was filled with glass marbles (1cm in diameter) to evenly distribute the influent over the sludge bed. For the influent flow, peristaltic pumps (type 505S, Watson and Marlow, Falmouth UK) were used. No effluent recycle was applied, the superficial upflow velocity was 0.08 $m.h^{-1}$.

During start-up (period I), methanol was fed to the reactors at a concentration of 1.4 g COD.l^{-1}, corresponding to an organic loading rate (OLR) of 2.6 g COD.l^{-1} reactor.d^{-1}. The methanol loading rate was increased on day 33 to 5.2 g. COD.l^{-1} reactor.d^{-1} (period II) and at day 57 to 7.8 g. COD.l^{-1} reactor.d^{-1} (period III) until the end of the experiment (Fig. 1). Based on the initial sludge content of the reactors these OLR's correspond to sludge loading rates (SLR's) of 0.3, 0.6 and 0.9 g COD.g $VSS^{-1}.d^{-1}$, during period I, II and III, respectively.

Table 1. Composition of the trace element solution.

Compound added	Element	Concentration	
		Compound ($mg.l^{-1}$)	Metal ($mg.l^{-1}$)
$FeCl_2.4H_2O$	Fe	2000	562
$CoCl_2.6H_2O$	Co	2000	495
$ZnCl_2$	Zn	50	24
$MnCl_2.4H_2O$	Mn	500	139
$CuCl_2.2H_2O$	Cu	38	14
$NiCl_2.6H_2O$	Ni	92	23
$(NH_4)_6Mo7O_{24}.4H_2O$[a]	Mo	50	27
$Na_2SeO_2.5H_2O$[a]	Se	164	49

1 ml of 36% HCl was added to 1 litre of trace acid element solution.; 1 ml of 33% NaOH was added to 1 litre of base trace element solution.; [a] Nutrient present in the base trace element solution

Specific maximum methanogenic activity test

Approximately 1.2 g (wet weight) of granular sludge was transferred to 120 ml serum bottles containing 60 ml of basal medium with the same composition as the reactor basal medium, supplemented with either methanol (4 g $COD.l^{-1}$) or acetate (2 g $COD.l^{-1}$) as the substrate. In the first activity experiment (day 28) methanol was added in two feeds of 2 g $COD.l^{-1}$. The second feed was added 4 days after the first feed. The SMA was determined on-line as described in Chapter 3.

Analyses

Total dissolved metal concentrations in the influent and effluent were determined by inductively coupled plasma-mass spectrometry i.e. ICP-MS (Perkin-Elmer, Elan 6000) in samples acidified with 0.1 M HNO_3. The samples were centrifuged at 10,000 rpm to remove particles from the liquid. The total metal concentration in the sludge was determined after microwave destruction (CEM 2100, Matthews, USA) of pre-dried sludge (105°C) in a mixture of 2.5 ml of HNO_3 (65%) and 7.5 ml HCl (37%). After digestion, the samples were paper-filtered (Schleicher & Schuell 589[1], Germany) and diluted to 100 ml, 1ml of this solution was transferred to 9 ml of 0.1M of HNO_3 and subsequently analysed for their metal content by ICP-MS.

The concentration of methanol, VFA and the composition (CO_2, CH_4 and N_2) of the biogas were determined by gas chromatography using the method as described by Weijma *et al.* [2000]. The total sulfide concentration was determined colorimetrically using the methylene blue method [Trüper and Schlegel, 1964]. The total suspended solids (TSS) and volatile suspended solids (VSS) concentration were determined according to Standard Methods [APHA, 1985]. All chemicals were of analytical or biological grade and purchased from E. Merck AG (Darmstadt, Germany).

RESULTS

Methanol conversion (period I and II)

After a start-up period of 14 days at an OLR of 2.6 g $COD.l^{-1}$ $reactor.d^{-1}$, all methanol from the influent was converted to methane and biomass in both reactors (Fig. 1A, B), and after doubling of the loading rate on day 33, all methanol was immediately removed as well. The pH of the effluent was 7.5 (±0.3) during period I and 7.1 (±0.1) during period II in both reactors. The biogas composition was similar as well in both reactors during the first two operational periods, i.e. it consisted of 87 % and 84 % of CH_4 and 7 and 12 % of CO_2, during period I and II respectively.

Methanol and VFA accumulation in R1 (period III)

The nickel deprived reactor could not cope with the second increase of the organic loading rate imposed on day 57 (period III), as the methanol started to accumulate in the effluent (Fig. 1A). During period III the SLR based on the initial sludge content in the reactor was 0.9 g COD. g $VSS^{-1}.d^{-1}$. This methanol accumulation was accompanied by a gradual increase in the VFA concentration of the effluent to an average value of 316 (±162) mg $COD.l^{-1}$ during period III (Fig. 1C). The VFA consisted mainly of acetate and propionate viz. an average value of 185 (±80) and 101 (±65) mg $COD.l^{-1}$, respectively. Period III can be divided in three sub-periods. The VFA concentration in the effluent gradually increased from day 57 until day 102 after which it stabilised at 450 (±65) mg $COD.l^{-1}$ (256 (±42) mg $COD.l^{-1}$ and 153 (±34) mg $COD.l^{-1}$ for acetate and propionate, respectively). After this stable period the VFA concentration gradually decreased again from day 196 onwards, to reach a total concentration of ±200 mg $COD.l^{-1}$ at the end of the experiment. The methanol concentration in the effluent already started to decrease at an earlier stage (from day 134 onwards). The CO_2 content of the biogas remained similar to period II at approximately 12 %. At the start of period III, the pH (day 58-139) averaged 7.1 (±0.1) but it slowly decreased from day 100 onwards averaging 6.9 (±0.1) from day 139 until termination of the experiment.

The methanogenic activity on methanol was assessed on day 89 (Table 2), when the performance of the reactor had deteriorated upon the second increase in OLR, in order to asses whether the sludge was nickel limited, nickel was added this clearly increased the methanogenic activity on methanol of the sludge sample of day 89, viz. from 292 mg CH_4-COD.g $VSS^{-1}.d^{-1}$ in the absence to 546 mg CH_4-COD.g $VSS^{-1}.d^{-1}$ in the presence of 2 μM nickel (Table 2, Fig. 2). The results in Figure 2C show that the supply of only 20 nM nickel already improved methanogenic activity: the apparent K_m found for the supply of nickel was 678 nM. The maximum gas production rate for this sludge was reached after 50h and then remained unchanged until the substrate was depleted (Fig 4A).

Methanol and VFA accumulation in R2 (period III)

In contrast to R1 the imposed methanol load was immediately and completely removed by R2 from the start of period III. However, the methanol removal efficiency started to declined at day 92 (Fig. 1B), accompanied with an increase in the VFA concentration, i.e. to an average value of 1077 (±686) mg $COD.l^{-1}$ during period III (Fig. 1C). The VFA consisted mainly of acetate and some propionate, viz. with an average value of 981 (±626) and 67 (±58) mg $COD.l^{-1}$, respectively (Table 2). The pH decreased in period III due to the accumulation of VFA and averaged 6.6 (±0.3). Especially, after the onset of the VFA and methanol accumulation (from day 110 until day 145) the pH was occasionally as low as 6.0 (Fig. 1C). The CO_2 concentration of the biogas increased from 12% period II to and average

16% in period III. Before the start of the acidification (day 57 until day 92) the biogas contained 14% CO_2, but it further increased to 17% at day 92 until the termination of the experiment.

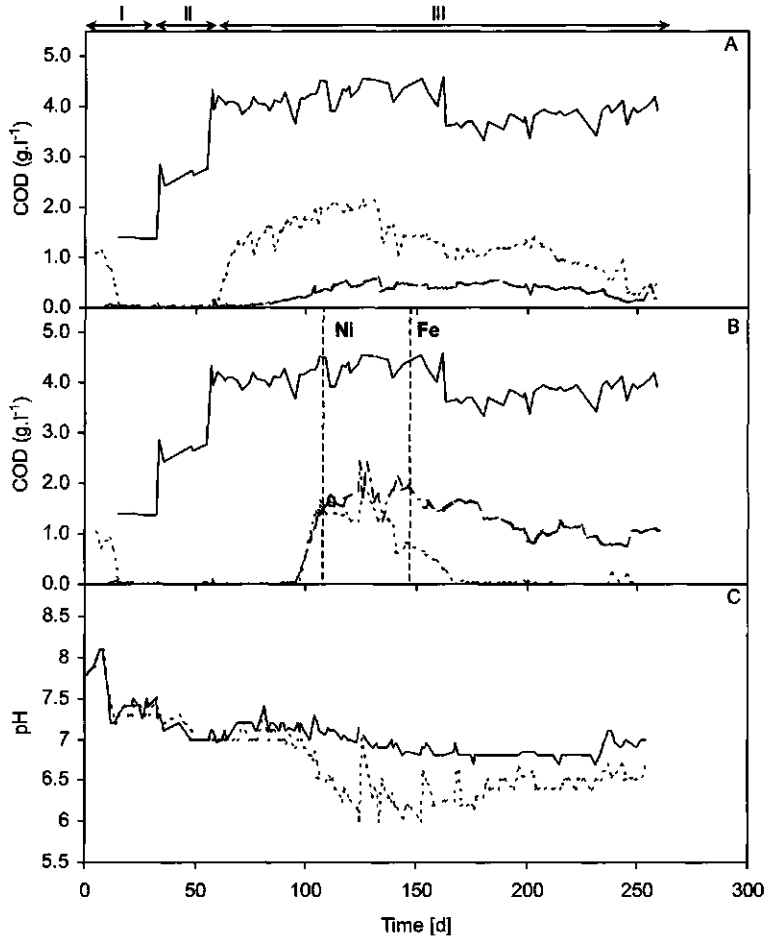

Figure 1 Reactor performance R1 (A) and R2 (B), methanol concentration in the influent (—), methanol concentration in the effluent (····), VFA concentration in the effluent (---). (C) Evolution of pH as a function of time of R2 (—) and R3 (····).Vertical lines indicate periods with increased nickel concentration (0.4 μM) in the influent (first dashed line) and increased iron concentration (10 μM) in the influent (second dashed line).

The methanogenic activity of the R2 sludge on methanol was assessed several times throughout the experiment, viz. at day 0, 28, 111, 147 and 259. The methanogenic activity considerably increased during the start up period (day 0 to 28), i.e. from 108 to 1517 mg CH_4-COD.g $VSS^{-1}.d^{-1}$ (Table 3). However, coinciding with the deterioration of the reactor performance on day 92, also the methanogenic activity of the sludge dramatically declined, viz. to a value of only 291 mg CH_4-COD.g $VSS^{-1}.d^{-1}$ (with the supply of the full metal cocktail) on day 111.

The reason for this deterioration might be a nickel limitation of the sludge, because a serious nickel limitation manifested in the parallel operated R1 reactor where the addition of nickel to the influent was omitted. In R2 the amount of dosed nickel was relatively low, viz. at a concentrations of 47 nM, at least compared to the amount of other essential elements added such as iron (2197 nM) and cobalt (779 nM). In an effort to recover the reactor performance, the nickel concentration in the influent was increased with a factor 10 to 409 nM at day 113. The results in Fig. 1B reveal that this did not immediately improve the methanol removal efficiency. Also the results of the methanogenic activity assay on methanol (conducted at day 111) did not show a clear positive response of the addition of 2μM nickel on the methanogenic activity. The assessed value of 107 mg CH_4-COD.g $VSS^{-1}.d^{-1}$ was even slightly lower than the value found in the absence of trace metals, viz.152 mg CH_4-COD.g $VSS^{-1}.d^{-1}$.

Another reason for the relatively poor performance of R2 might be iron limitation. Apparently this indeed is the case, because increasing the iron concentration in the medium of the assay conducted with the sludge sample of day 111 with a factor 10 to 10 μM, resulted in a significant higher methanogenic activity, i.e. from 152 to 291 mg CH_4-COD.g $VSS^{-1}.d^{-1}$. The effect of the iron supply manifested after 50 h and as shown in Fig. 3A a lag phase was absent. In contrast, the supply of nickel and cobalt at 10 times higher concentration than the reactor influent concentration did not improve the methanogenic activity of the sludge (data not shown). Dosing of the full metal cocktail at a 10 times higher concentration resulted in a methanogenic activity of 326 mg CH_4-COD.g $VSS^{-1}.d^{-1}$, a value very similar to that in the presence of solely 10 μM iron. This indicates that iron clearly improved the methanogenesis and therefore, the iron concentration in the reactor feed was increased to 10 μM at day 153. Although this resulted in an improved methanol removal efficiency, it is rather doubtful whether this really can be attributed to the iron dosing, since the increased methanol removal already started from day 139 onwards. Moreover, the acidified fraction in the effluent remained the same. A complete methanol removal was achieved again after day 167, coinciding with a slow decrease of the acidified fraction (Fig. 1A).

Table 2. Evolution of the specific methanogenic activity on methanol (mg CH_4-COD.g $VSS^{-1}.d^{-1}$) of the R1 sludge as a function of time and Nickel concentration.

	Day			
Nickel μM	0	89	147	261
0.00	n.d.	292 (111)[a]	315	245 (21)[a]
0.02	n.d.	313	n.d.	265
0.04	n.d.	327	n.d.	196
0.20	n.d.	380	n.d.	310
0.40	108 (271)[a]	401	n.d.	321
2.00	n.d.	546	n.d.	312 (21)[a]
4.00	n.d.	n.d.	n.d.	304

[a] Methanogenic activity on acetate is presented between brackets.; n.d. not determined

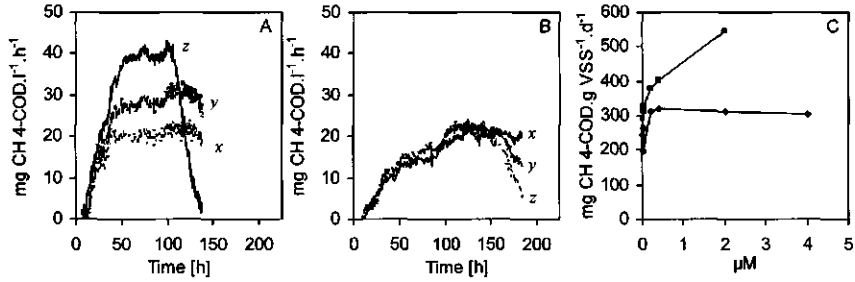

Figure 2 The response of the methane production rate and methanogenic activity to the addition of nickel (pH 7; 30°C) of the R1 sludge fed with methanol (**A**) Methane production rate of the sludge sampled on day 89. (**B**) Methane production rate of the sludge sampled on day 261. (**C**) Methanogenic activity of the sludge sampled on day 89 (■) and 261 (♦). (*x*) no nickel addition, (y) 0.4 μM nickel, (*z*) 2 μM Nickel.

The methanogenic activity of the sludge sampled on day 147 in the presence of 10 μM iron was similar (288 mg CH_4-COD.g $VSS^{-1}.d^{-1}$) to that assessed for the sludge sampled on day 111. An arithmetic increase in the gas production rate manifested and a plateau was not reached (Fig 3B(*z*)). The SMA in the absence of iron amounted to 27 mg CH_4-COD.g $VSS^{-1}.d^{-1}$ over a 250 h period (Fig 3B(*y*), Table 3). Thereafter, the rate of gas production increased

exponentially and the calculated specific methanogenic activity from the maximum rate observed amounted to 361 mg CH_4-COD.g $VSS^{-1}.d^{-1}$. At the end of the activity assay, ±50% of the methanol was converted to VFA and ±50% was converted to methane independent of the iron concentration.

Metabolic characteristics of the final sludges

In contrast to the sludge sampled from R1 on day 89, the supply of 2 μM nickel to the sludge sample of day 261 only slightly improved the activity on methanol, viz. from 245 CH_4-COD.g $VSS^{-1}.d^{-1}$ to 312 CH_4-COD.g $VSS^{-1}.d^{-1}$. Moreover, similar methane formation rates were found for all nickel concentrations tested (Fig. 2B). The maximum methanogenic activities (in the absence of nickel) on methanol at days 89 and 261 were similar and amounted to 292 and 245 mg CH_4-COD.g $VSS^{-1}.d^{-1}$, respectively, viz. only 126 to 170 % higher than of the seed sludge (108 mg CH_4-COD.g $VSS^{-1}.d^{-1}$).

Table 3. Evolution of the specific methanogenic activity of the R2 sludge (mg CH_4-COD.g $VSS^{-1}.d^{-1}$) on methanol as a function of time and iron concentration.

	Day				
Iron μM	0	28	111	147	259
0.00	n.d.	n.d.	152	27 (361)[b]	193
1.00	n.d.	n.d.	n.d.	n.d.	236
10.00	108 (271)[a]	1517	291	288	321
20.00	n.d.	n.d.	n.d.	n.d	412

[a] Methanogenic activity on acetate (between brackets); [b] after 250 h lag phase (between brackets); n.d. not determined

The R1 sludge sampled on day 147 and day 261 were also tested for their response on iron (no nickel added). Addition of 10 μM iron to the sludge sample of day 147 increased the activity from 244 mg CH_4-COD.g $VSS^{-1}.d^{-1}$ to 315 mg CH_4-COD.g $VSS^{-1}.d^{-1}$ (data not shown) and the response of the R1 sludge sample of day 261 to the supply of 10 μM iron, was even larger, the methanogenic activity increased from 211 to 346 mg CH_4-COD.g $VSS^{-1}.d^{-1}$, which indicates that an increasing iron deficit manifested in the sludge.

The methanogenic activity on methanol of the R2 sludge in the absence of iron increased from 27 mg CH_4-COD.g $VSS^{-1}.d^{-1}$ at day 147 to 193 mg CH_4-COD.g $VSS^{-1}.d^{-1}$ at day 259. The response of the methanogenic activity to a range of iron concentrations was

determined on day 259, the methanogenic activity increased almost linearly as a function of the added iron concentration (Fig. 3C) by adding 20 μM of iron a twice as high value was reached compared to the activity of the sludge in the absence of iron (Table 3; Fig. 3B). At the end of the activity test the acetate concentration in the batches was 983, 794 and 718 mg $COD.l^{-1}$ and the rates of acetate formation in the batches were 5.8, 5.1 and 5.1 mg $COD.l^{-1}.h^{-1}$ in the presence of 0, 1 and 10 μM of iron, respectively. The slightly lower conversion rates to VFA in the presence of elevated iron concentrations can be attributed to the prevailing higher methane formation rates at higher iron concentrations, because then less methanol is available to be converted to VFA.

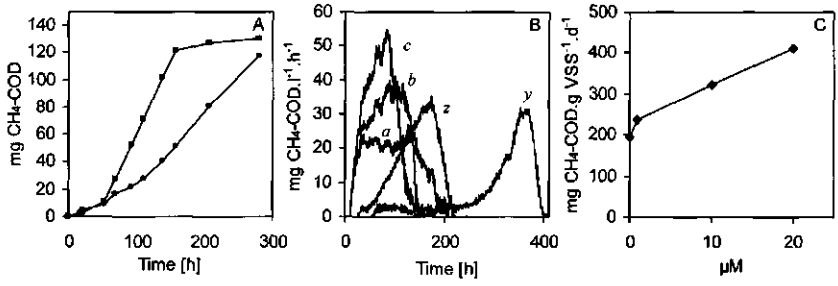

Figure 3 Metabolic characteristics of the R2 sludge samples when fed with methanol (pH 7; 30°C). (A) Methane production of the R2 sludge sampled at day 111, no iron (•), 10μm (■) of iron.(B) Methane production rate and response to iron addition of the R2 sludge sampled at day 147 ((*y*) 0 μM, (*z*) 10 μM) and day 259 ((*a*) 0 μM, (*b*) 10 μM, (*c*) 20μM). (C) Maximum methanogenic activity of the sludge sample taken at 259 assessed at different iron concentrations.

Specific activity on acetate

The methanogenic activity of the seed sludge on acetate in a medium containing both nickel and iron amounted to 271 mg CH_4-COD.g $VSS^{-1}.d^{-1}$ (Table 2). In earlier experiments conducted with the seed sludge, the supply of the full trace element cocktail to the assay medium did not affect the methanogenic activity on acetate. The methanogenic activity on acetate of the R1 sludge in the absence of nickel was 111 mg CH_4-COD.g $VSS^{-1}.d^{-1}$ at day 89, while at the end of the run hardly any activity on acetate was left (21 mg CH_4-COD.g $VSS^{-1}.d^{-1}$) regardless whether nickel was absent (not supplied) or present (2μM) in the medium. No SMA on acetate could be determined for the R2 sludge at the end of the run.

Nickel and iron dynamics in the sludge

The nickel and iron contents of the sludges clearly declined over time (Fig. 4). In addition to a loss of these elements with the effluent, also a certain dilution effect should be taken into account due to the formation of new granules, because at termination of the experiment at least 80 and 90% more sludge was present in R1 and R2, respectively. The VSS/TSS ratio of the R1 and R2 sludge remained around 94% throughout the experiment.

Increasing the nickel concentration in the R2 influent 10 times (to 390 nM) at day 113 resulted in a clear increase of the nickel content of the sludge from 60 to 120 μg.g TSS^{-1}, i.e. similar to that of the seed sludge (Fig. 4). In contrast, a 10 times increase of the influent iron concentration (to 16,8 μM) did not lead to an elevated iron content of the sludge; (Fig. 4) even the contrary was found, despite the fact that iron was dosed at significantly higher concentrations than nickel (Fig. 4).

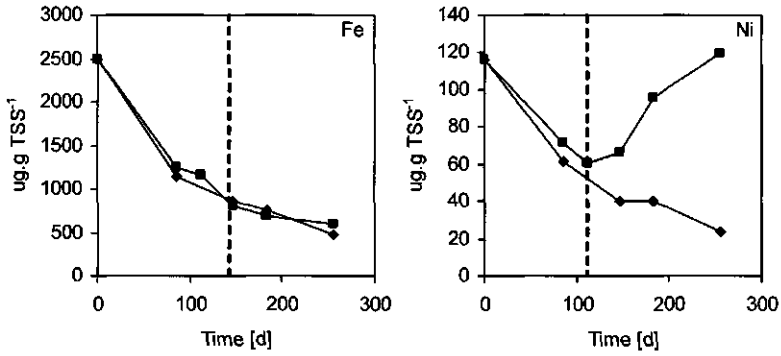

Figure 4 Iron and nickel content of the R1 (♦) and R2 (■) sludge. Vertical dashed lines indicate the start of the period with increased iron (10 μM) and nickel (0.40 μM) concentrations in the R2 influent.

DISCUSSION

Effect of nickel limitation on bioreactor performance

The operation of a UASB reactor under nickel deprivation (R1) resulted in a clearly nickel limited sludge, as evidenced by the almost two times increase of the SMA when 2μM nickel was supplied to the sludge sample of day 89 (Fig, 2A, Table 2). The reactor could not handle the second OLR increase, which corresponded to a SLR of 0.9 g COD. g $VSS^{-1}.d^{-1}$ based on the initial sludge content. The system was clearly overloaded with methanol, as indeed appeared from the SMA assessed on day 89, being only 292 mg CH_4-COD. g $VSS^{-1}.d^{-1}$

(Fig. 2A, Table 2), i.e. significantly lower than the estimated SLR. On the other hand, it should be noted that growth of new biomass had occurred during reactor operation, as the amount sludge in R1 had at least increased by 80% at termination of the experiment (data not shown). Therefore the actual SLR was very likely lower than the estimated value.

In a reactor with the same inoculum sludge operated under similar conditions but without the supply of cobalt to the influent [Chapter 3], a cobalt limitation was observed within in a period of only 28 days operation and the reactor then only removed 55 % of the supplied methanol (SLR=0.3 g COD. g $VSS^{-1}.d^{-1}$). Such a cobalt limitation has been prevented in this research by the supply of 0.84 μM cobalt. Both reactors removed all supplied methanol and could even deal with significantly higher loads in period I.

The absence of nickel in the R1 feed clearly altered the metabolic properties of the sludge, as evidenced by the slow increase of the VFA accumulation along with the methanol build up (R1, Period III, Fig. 1A), the loss of response of the sludge to nickel in activity tests (Fig. 2B) and the slow decrease in the R1 effluent methanol concentration at the end of the reactor run (from day 134 onwards, Fig. 1A). Feeding the reactor with a nickel free influent to may have induced adaptation of the sludge, i.e. making it less nickel dependent (Fig. 1A, Fig. 2B). This might be attributed to a change in the (enzymatic) methanol conversion pathway or growth of new less nickel dependent biomass (Fig. 4). It is known that for instance the hydrogenotrophic archeon *Methanobacterium marburgensis* possesses two hydrogenase systems that catalyze the reduction coenzyme F_{420} [Afting et al., 1998]. One system consists of a nickel-containing enzyme, while the other system is nickel free, which develops under nickel limited conditions. On the other hand it should be noted, however, that also some other trace element may have become limiting, e.g. iron, because the content of this element in the sludge also declined (Fig. 4). Indeed, iron addition (10μM) clearly improved the methanogenic activity of the sludge sampled at termination of the experiment.

During the SMA assay on methanol conducted with the R1 sludge sampled at day 89 after 50h a remarkable plateau was reached in the rate of gas production (Fig. 2A). As the nickel concentration was the only variable, it seems that uptake of the available nickel by the biomass took place within the first 50 h. Pusheva *et al.* [1989] showed that incorporation of ^{63}Ni by *C. thermoautotrophicum*, grown in the presence 3μM Ni, occurred during the first 20h of growth.

Effect of iron limitation on bioreactor performance

In contrast to R1, R2 with 47 nM nickel supplied to the feed, could well accommodate the second OLR increase (period III), at least initially, because all methanol was converted to methane (SLR of 0.9 g COD. g $VSS^{-1}.d^{-1}$ based on the initial sludge content). However at day 92 (period III) an abrupt transition of the performance occurred, i.e. from full methanogenesis

to a partial acidogenesis. Apparently, the capacity of the sludge to directly convert methanol into methane had slowly decreased during this period. The sludge present in R2 after 28 days of operation (Period I) would have been capable to convert the in period III supplied methanol to methane, at least based on the assessed SMA of 1517 mg CH_4-COD. g $VSS^{-1}.d^{-1}$.

A similar abrupt deterioration of the reactor performance (after 75 days of operation), as found in R2, was previously observed by Florencio et al. [1995] in experiments with a UASB reactor operating at similar SLR with synthetic methanol wastewater containing moderate $NaHCO_3$ concentration (15 mM). The reactors were supplied with the same trace metal solution as applied in the present research, but at a ten times higher concentration. The pH dropped to nearly 5 and remained below 6 for 60 days. In contrast to R2, where the methanogesis from methanol recovered spontaneously (Period III, Fig. 1), no recovery of the methanogenesis was observed by Florencio et al. [1995] in their experiments within 40 days, even not when the pH was restored to above 6 by the supply of 50 mM $NaHCO_3$.

The response of the reactor to the increased iron supply to R2 was less pronounced than observed in the batch tests. Moreover, in contrast to the nickel concentration, the iron concentration of the sludge did not increase upon dosing. Most probably this is due to partial wash-out of the extra dosed iron with the effluent for instance due to the formation of iron sulfide colloids making the iron unavailable for the granular sludge biomass. The reactor pH is also an important factor in the retention of iron, e.g. considerable losses/release of iron from the sludge bed was observed when the pH was temporarily lowered (30 h) to 5 [Chapter 7]. This indicates that, especially for iron, the chemical form in which it is supplied to the reactor is important for its retention and bioavailability within the sludge bed. Further research is required to study how the bioavailability and retention of iron in bioreactors can be increased e.g. by supplementation of iron bound to ligands.

Acetogenesis versus methanogenesis in methanol fed UASB reactors

This study showed that a serious deficit of one or more essential trace elements (nickel and/or iron) was gradually developed by operating methanol fed UASB reactors with sub-optimal metal concentrations. This deficit resulted in a decrease in the capacity of the sludge to convert methanol into methane, ultimately leading to a deterioration of the performance of the UASB reactors and the creation of favourable conditions for acetogenesis.

The increase in VFA concentration in the R1 effluent occurred rather slowly after onset of methanol accumulation. The accumulation of VFA increased from day 57 and reached a maximum on day 102 in R1 (Fig 1A). Nickel plays an essential role in the pathway of acetogenenis, because it is present in carbon monoxide dehydrogenase [Wood et al., 1986]. Therefore, the nickel deprivation of the sludge may have limited the development of the acetogens in R1. This phenomenon of VFA-formation has been well documented for UASB

reactors operating under cobalt deprived conditions [Florencio et al., 1993; Chapter 3]. Low cobalt concentrations (< 0.0001 mg $Co.l^{-1}$), enhance the competitive edge of methanogens over acetogens, because the growth rate of methanogens exceeds that of the acetogens over a broad methanol concentration range, and thus the reactor remains methanogenic [Florencio et al., 1994]. In contrast, at higher cobalt and methanol (80 mM) concentrations, the growth rate of acetogens exceeds that of methanogens, and thus these conditions lead to a predominantly acidifying reactor. Reactor performance data of Florencio et al., [1995] show that acidification occurs at methanol concentrations exceeding 1000 mg $COD.l^{-1}$, and this was confirmed by the results obtained in the present reactor run (Fig. 1).

The SMA on methanol of R2 sludge decreased after deterioration of the reactor performance (Table 2 and 3) viz. from 152 mg CH_4-COD. g $VSS^{-1}.d^{-1}$ on day 111 to only 27 mg CH_4-COD. g $VSS^{-1}.d^{-1}$ on day 147 (Table 3, Fig. 3A, B). While in R1, the SMA and pH remained relatively constant after overloading of the system (Table 2), the significantly higher formation of VFA in R2 was accompanied by a drop in the pH to values as low as 6, which as such is not toxic for methylotrophic methanogens [Florencio et al., 1993]. However, lower pH-values increase the fraction of undissociated VFA, and this can lead to inhibitory concentrations for methanogens. In acetate and glucose fed reactors containing flocculent sludge, 50% inhibition of methanogens was found at undissociated acetate concentrations as low as 10 $mg.l^{-1}$ and almost complete inhibition occurred from 40 $mg.l^{-1}$ onwards [Duarte et al., 1982]. At the start of the acidification in R2, when the highest effluent acetate concentrations and lowest pH values occurred (day 110 to 145), the undissociated acetate concentrations were around 40 $mg.l^{-1}$, and consequently an inhibition may have contributed to the further decrease of the SMA.

The abrupt methanol accumulation in the R2 effluent was accompanied by a significant increase in the VFA concentration and decrease of the effluent pH to 6. As a result more CO_2 was made available for acetogenesis compared to R1, and CO_2 is essential for acetate formation from methanol, according to:

$$4CH_3OH + 2HCO_3^- \rightarrow 3CH_3COO^- + H^+ + 4H_2O \qquad (1)$$

$$4CH_3OH \rightarrow 3CH_4 + HCO_3^- + H^+ + H_2O \qquad (2)$$

Based on the stoichiometry of methanol conversion to acetate (Reaction 1; Heijthuijsen and Hansen, 1986), ± 43 mM CO_2 would be required to fully convert the methanol supplied with the influent to acetate. Thus, based on the available CO_2/bicarbonate (30 mM) supplied to the reactors, only part of the methanol can be converted in VFA-COD under the conditions prevailing in both R1 and R2. Indeed, only 17 (±3) mM of CO_2 was

needed for the formation of the amount of acetate present in the R2 effluent from day 110-145. It should be noted that the actual CO_2/bicarbonate budget becomes higher as a result of the CO_2 formed during methanogenesis (Reaction 2). On the other hand part of the formed CO_2 will not become available for acetogenesis, because it is lost with the biogas.

The very low and decreasing methanogenic activity on acetate (Table 2) indicates that methanol has been directly converted to methane [Fig. 1 in chapter 3], which is conform the findings of Florencio et al., [1994], who found that under mesophilic conditions direct methanogenesis is the main pathway. Apparently, the methanogenic population looses its ability to convert acetate to methane. In view of the fragile equilibrium between methanogenesis and acetogenesis, further research should focus on methods that retain acetotrophic populations in methanol fed UASB reactors.

CONCLUSIONS

- Nickel deprivation (R1) leads to an earlier deterioration/overloading and less pronounced acidification compared to a parallel operated reactor (R2) supplied with 47 nM nickel.
- Nickel deprivation of R1 initially had a strong impact on the SMA of the sludge with methanol, e.g. after 89 days of operation the SMA almost doubled by adding 2 μM nickel. The response of the SMA on nickel was no longer observed at termination of the experiment.
- The R2 sludge was clearly iron limited after 111 days of operation, addition of iron to the batch medium significantly increased the methane formation rate of the R2 sludge.
- Nickel dosing at 390 nM (day 113) increased the nickel content of the sludge from 60 to 120 μg. g TSS^{-1} at termination of the experiment.

REFERENCES

Afting C, Hochheimer A, Thauer RK (1998). Function of the H_2-forming methylenetetrahydromethanopterin dehydrogenase from *Methanobacterium thermoautotrophicum* in coenzyme F_{420} reduction with H_2. *Arch Microbiol,* **169**, 206-210

APHA (1985). Standard methods for the examination of water and wastewater. American Public Health Administration, Washington, D.C., USA.

Callendar IJ, Barford JP (1983). Precipitation, chelation and availability of metals as nutrients in anaerobic digestion. II Application. *Biotechnol Bioeng*, **25**, 1959-1972

Deppenmeier JM, Blaut M, Schimdt B, Gotschalk G (1992). Purification and properties of F_{420}-nonreactive membrane bound hydrogenase from *Methanosarcina mazei* strain Göl. *Arch Microbiol*, **157**, 505-511

Duarte AC, Anderson GK (1982). Inhibition modelling in anaerobic digestion. *Wat Sci Tech*, **14**, 749-763

Florencio L, Jenicek P, Field JA, Lettinga G (1993). Effect of Cobalt on the anaerobic degradation of methanol. *J Ferment Bioeng*, **75**, 368-374

Florencio L, Field JA, Lettinga G (1994). The importance of Cobalt for the individual trophic groups in an anaerobic methanol degrading consortium. *Appl Environ Microbiol*, **60**, 227-234

Florencio L, Field JA, Lettinga G (1995) Substrate competition between methanogens and acetogens during the degradation of methanol in UASB reactors. *Wat Res*, **29**, 915-922

Fukuzaki S, Nishio N (1997). Methanogenic fermentation and growth of granular methanogenic sludge on a methanol-propionate mixture. *J Ferment Bioeng*, **84**, 382-385

Hausinger P (1987). Nickel utilization by microorganisms. *Microbiol Rev*, **51**, 22-42

Heijthuijsen JHFG, Hansen TA (1986). Inter-species hydrogen transfer in co-cultures of methanol utilizing acidogens and sulfate reducing or methanogenic bacteria. *FEMS microbial Lett*, **38**, 57-64

Jarrel KF, Sprott D (1982). Nickel transport in *Methanobacterium bryantti*. *J Bacteriol*, **151**, 1195-1203

Kelly CR, Schwitzenbaum M (1984). Anaerobic treatment and nutrient effects. *Agric Wastes*, **10**, 135-154

Kemnaer JM and Zeikus JG (1994). Purification and characterization of membrane-bound hydrogenase from *Methanosarcina barkeri* MS. *Arch Microbiol*, **161**, 47-54

Lettinga, G.; van der Geest, A. T.; Hobma, S. van der Laan, J (1979). Anaerobic treatment of methanolic wastes. *Wat Res*, **13**, 725-737

Oleszkiewicz JA, Romanek A (1989). Granulation in anaerobic sludge bed reactors treating food industry waste. *Biol Wastes*, **27**, 217-235

Pusheva MA, Berestovskaya YY, Borudulina NP (1989). Effect of Nickel on homoacetate bacteria. *Micriobioligiya*, **2**, 206-211

Scherer P, Lippert H, Wolff G (1983). Composition of the major elements and trace elements of 10 methanogenic bacteria determined by inductive coupled plasma emission spectrometry. *Biol Trace Element Res*, **5**, 149-163

Shen CF, Kosarik N, Blasczyk R. (1993a). The effect of selected heavy metals (Ni, Co and Fe) on anaerobic granules and their extracellular polymeric substances (EPS). *Wat Res*, **27**, 25-33

Shen CF, Kosarik N, Blasczyk R. (1993b). Properties of anaerobic granular sludge as affected by yeast extract cobalt and iron supplements. *Appl Microbial Biotech*, **39**, 132-137

Speece RE, Parkin GF, Gallagher D (1983). Nickel stimulation of anaerobic digestion. *Wat Res*, **17**, 677-683

Thauer RK (1998). Biochemistry of methanogenesis: a tribute Majory Stephenson. *Microbiology*, **144**, 2377-2406

Trüper HG, Schlegel HG (1964). Sulphur metabolism in *Thiorrodaceae* I. Qauntative measurment on growing cells of *Chromatium okenii*. *J Microbiol Ser*, **30**, 225-238

Wood HG, Ragsdale W, Pezacka E (1986). The acetyl-CoA pathway of autotrophic growth. *FEMS Microbiol Rev*, **39**, 345-362

Weijma J, Stams AJM, Hulshoff Pol LW, Lettinga G (2000). Thermophilic sulfate reduction and methanogenesis with methanol in a high-rate anaerobic reactor. *Biotechnol Bioeng*, **67**, 354-363

Chapter 5

Stimulation of methanol degradation in UASB reactors: in situ versus pre-loading cobalt on anaerobic granular sludge

The effect of pre-loading and in situ loading of cobalt onto a cobalt limited granular sludge on the performance of methanol fed bioreactors was investigated. One Upflow Anaerobic Sludge Bed (UASB) reactor was inoculated with cobalt pre-loaded sludge (24h; 30°C; 1 mM $CoCl_2$) and a second UASB with unloaded sludge. The UASB reactors (30°C; pH 7) were operated for 77 days at 8h hydraulic retention time and organic loading rates ranging from 5 to 20 g COD.l reactor^{-1}.d^{-1}. Cobalt pre-loading clearly stimulated the methanogenic activity of the sludge with methanol as the substrate, e.g. after 30 days of reactor operation this activity was 5.8 times higher than that of the cobalt unloaded sludge. During the experiment, part of the cobalt leached from the pre-loaded sludge, i.e. 54% of the cobalt content was lost during the 77 days of reactor operation. Sequential metal extraction showed that losses mainly occurred from the exchangeable and carbonate fraction and that the in the sludge remaining cobalt was mainly present in the organic/sulfide fraction of the sludge. In situ loading of cobalt in the unloaded UASB reactor on day 57 by adding 31 μM cobalt to the influent for a 24h period (16% of the cobalt present in the loaded sludge at day 11) resulted in a 4 times increase of the methanogenic activity on methanol. This study showed that both pre-loading sludge and in situ loading are adequate for achieving an increased reactor performance of methanol fed UASB reactors operating under cobalt limitation. However, the in situ dosing procedure needs substantially lower amounts of cobalt, while it also gives significantly smaller losses of cobalt with the effluent.

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INTRODUCTION

Trace elements such as cobalt are essential for good performance of anaerobic bioreactors [Shen et al.1993; Kelly and Schwitzenbaum 1984; Florencio et al. 1993; Speece et al. 1983]. In full scale practice, trace elements are generally supplemented with the influent solution of the anaerobic bioreactors to ensure good reactor performance. The dosed trace metals accumulate in the anaerobic sludge granules through physical, chemical or biological processes [Callender and Barford, 1983; Chen et al. 2000; Shen et al. 1993]. Their bioavailability for the microorganisms present in the granules is determined by the distribution of the metals over different fractions viz. organic, free ion or chelated [Alibhai et al. 1985]. However, so far very little is known about the storage dynamics of the various trace metals in anaerobic granular sludge and the effect of the metal dosing procedure on the metabolic activity.

Cobalt plays a key role in methanol degradation, as it regulates the methanol degradation pathway by affecting the different trophic groups (acetogens or methanogens) involved in methanogenic methanol conversion [Florencio et al. 1993]. In the presence of high bicarbonate and methanol concentrations, high influent cobalt concentrations can result in acetate build up due to a relatively high acetogenic activity [Florencio et al. 1993]. As this can lead to reactor acidification, cobalt concentrations should be kept lower, and thus appropriate cobalt dosing strategies need to be developed. Gonzalez et al. [1999] showed that supplying cobalt continuously in a batch methanol degradation test was more effective in overcoming and cobalt limitations than dosing the total amount of cobalt at once to the batch medium at the beginning of the test. The increase in methane formation remained exponential during the continuous cobalt dosing experiment while in the batches supplemented with cobalt at the beginning of the experiment it became arithmetic with time.

An alternative for the continuous supply of cobalt to reactors operated in continuous mode might be to load cobalt onto the granules prior to their inoculation in a UASB reactor. This can be achieved by contacting anaerobic sludge granules with a cobalt rich solution [Osuna et al. 2004]. It was found that by contacting UASB granules to a 1 mM cobalt solution for a 4 day period the cobalt content was increased from 16 to 1558 $\mu g.g\ TSS^{-1}$. Sequential extraction of the cobalt from the sludge revealed that 40% of the cobalt was present in the exchangeable fraction of the sludge. However still little is known about the dynamics of the cobalt stock during reactor operation and how it affects the reactor performance with time.

This study investigated the effect of a cobalt pre-loading strategy of granular sludge and compares it to the effect of in situ cobalt loading in a UASB reactor operated in parallel with unloaded inoculum. The objective was not merely to assess the effect on the reactor performance, but also to assess the effect on important sludge characteristics. Based on these

parameters it is possible to find the most economical and sustainable strategy of metal dosing in anaerobic bioreactors.

MATERIAL AND METHODS

Source of biomass

The UASB reactors were inoculated with 20 g VSS.l^{-1} anaerobic granular sludge originated from a full scale UASB reactor treating alcohol distillery wastewater of Nedalco (Bergen op Zoom, the Netherlands). Before use the sludge was elutriated to remove the fines. The total suspended solids (TSS) and volatile suspended solids (VSS) content of the sludge amounted to 10.0 (±0.2)% and 9.5 (±0.2)%, respectively.

Experimental design

The Nedalco sludge was selected as inoculum because of its relatively low cobalt content (17 μg. g TSS^{-1}, Chapter 3). One of the two reactors (R1) was inoculated with untreated sludge, while the inoculum of the other reactor (R2) had been pre-loaded with cobalt by contacting it with a 1 mM cobalt solution ($CoCl_2.6H_2O$) for 24h at 30°C in a 1litre serum bottle in the absence of substrate. The sludge of R1 was loaded in situ after 57 days of operation by supplying cobalt to the influent of the reactor for 24 h at a concentration of 31 μM (16% of the mass of cobalt present in the loaded sludge on day 11).

The methanol-organic loading rate (OLR) of both UASB reactors was increased stepwise. The reactor was started up at an OLR of 5 g MeOH COD.l $reactor^{-1}.d^{-1}$ (period I), and the OLR was increased for the first time at day 17 to 10 g MeOH COD.l $reactor^{-1}.d^{-1}$ (period II, day 17-50) and increased a second time to 20 g MeOH COD.l $reactor^{-1}.d^{-1}$ on day 50 (period III, day 51-57). The OLR was reduced back to 10 g MeOH COD.l $reactor^{-1}.d^{-1}$ on day 57 and remained unchanged until the termination of the experiment (period IV, day 58-77).

Influent composition

The influent of the reactors contained methanol, macro nutrients as described in Chapter 3 and a trace element solution dissolved in demineralised water. The trace elements (nickel, zinc, manganese, copper, tungsten, molybdenum and selenium) except cobalt were added to the influent at a concentration of 5μM. Iron was added at a concentration of 50 μM. To ensure pH stability, 2.52 g (30 mM) of $NaHCO_3$ was added per litre of influent. To avoid precipitation in the storage vessels, the influent was composed of three different streams: macro nutrients without K_2HPO_4; methanol with bicarbonate and K_2HPO_4; and the trace

element solution. The trace element solution was kept anaerobic under a nitrogen atmosphere in order to prevent oxidation. Note that cobalt was omitted from the trace element solution.

Specific maximum methanogenic activity tests

Specific methanogenic activity (SMA) of the sludge developing in the reactor was determined in duplicate at 30 (±2)°C using on-line gas production measurements as described in Chapter 3. Approximately 1g (wet weight) of granular sludge was transferred to 120 ml serum bottles containing 50 ml of medium with the same composition as the reactor influent with respect to macro nutrients, $NaHCO_3$ and trace element concentration. The medium was supplemented with either methanol (4 g $COD.l^{-1}$) or acetate (2 g $COD.l^{-1}$) as the substrate and different amounts of cobalt.

The SMA with methanol as the substrate of the sludge present in both reactors was determined after 30 days of operation and at the end of the reactor run (77 days of operation). The SMA with acetate as the substrate was determined only in the sludge sampled at the termination of the experiment.

Metal analyses

Total and dissolved cobalt concentrations in the influent and effluent were determined by inductively coupled plasma mass spectrometry (ICP-MS) as described in Chapter 3. The total cobalt concentration in the sludge was determined by ICP-MS, after microwave destruction of the sample, as described by in Chapter 3.

The sequential extraction procedure of the cobalt from the sludge was performed at the beginning and the termination of the reactor run using an extraction scheme as described by Osuna et al. [2004]. This scheme consists of four extraction steps, which become more stringent with each subsequent step. The following nomenclature is applied for the subsequent extraction steps, viz. the exchangeable fraction (1M NH_4CH_3COO), the carbonate fraction (1M CH_3COOH), organic/sulfide fraction (30% H_2O_2) and the residual fraction (3:1 HCl/HNO_3)

Chemical analyses

The concentration of methanol and volatile fatty acids (VFA) was determined by gas chromatography as described by Weijma *et al.* [2000]. The total sulfide concentration was determined colorimetrically using the methylene blue method [Trüper and Schlegel, 1964]. The total suspended solids (TSS) and volatile suspended solids (VSS) concentrations were determined according to Standard Methods [American Public Health Association, 1985]. All

chemicals were of analytical or biological grade and purchased from E. Merck AG (Darmstadt, Germany).

RESULTS

Effect of cobalt loading on methanol degradation

During the start-up (period I), ultimately all methanol was removed and converted to methane in both reactors (Figure 1A, B). In period II, a complete removal of methanol could not be achieved anymore in R1 (Figure 1A), and even a slight but persistent decrease in the gas production of R1 occurred during period II. This drop in gas production can likely be attributed to the removal of the sludge from the reactor due to the weekly sampling for metal analyses and activity tests. After doubling the OLR again (period III), also the cobalt-loaded sludge was unable to convert all the methanol. At the same time, VFA appeared in the effluent of R2 up to a concentration of 675 $mg.l^{-1}$ on day 57 (Fig. 1B). None of the 'extra' methanol, 3.5 $g.COD.l^{-1}$, introduced in both R1 and R2 after the second OLR increase was converted as the effluent methanol concentration on average was 3.6 and 3.4 g $COD.l^{-1}$ higher compared to the previous period in R1 and R2, respectively (Table 1), indicating that both reactors were overloaded. Based on the sludge volume still present in the reactor at the time of the overloading, the sludge loading rates were 1.95 and 1.69 g COD. g $VSS^{-1}.d^{-1}$ for R1 and R2, respectively. The gas production in both reactors decreased during period III (Figure 1C), suggesting that methanol may actually have been toxic for the methanogenic biomass at the applied concentrations (about 7.3 g $COD.l^{-1}$ influent and 5.4 and 3.4 g $COD.l^{-1}$ in the mixed liquor of R1 and R2, respectively).

After returning the OLR back to 10 g MeOH $COD.l^{-1}.d^{-1}$ in period IV, the performance of R2 restored and all methanol was removed but still some VFA remained present in the effluent, which explains the slightly lower gas production compared to period II. In order to elucidate the difference between sludge pre-loading and in situ sludge loading procedure on the reactor performance, cobalt was added to R1 during a 24 hour period at a concentration of 31 μM at day 57, i.e. at the beginning of period IV. This 31 μM corresponds to roughly 16% of the amount of cobalt present in the R2 sludge on day 11 of the experiment. The effect is immediate and remarkable, viz. the methanol removal efficiency improved significantly, i.e. and within 4 days no methanol could be detected in the effluent of this reactor anymore (Fig. 1A). Also remarkable is that the formation of some VFA coincided with the cobalt addition. The on-line measurement of the CH_4 production rate showed first a rapid increase in the methane production (Fig. 1C). Once the VFA concentration started to decrease at day 63, a further increase in the methane production was observed corresponding

with this VFA decrease. Interestingly, both reactors produced the same amount of methane at the end of the experiment.

A slight decrease of the pH occurred along with the occurrence of the significant VFA formation from day 59 and 52 onwards for R1 and R2, respectively (Fig. 1A, B and 2A). In that period the pH dropped from 7.2 to 6.7 and 7.0 to 6.7 for R1 and R2, respectively. This indicates that the $NaHCO_3$ buffer capacity supplied with the influent was still sufficient to maintain the pH in the proper range.

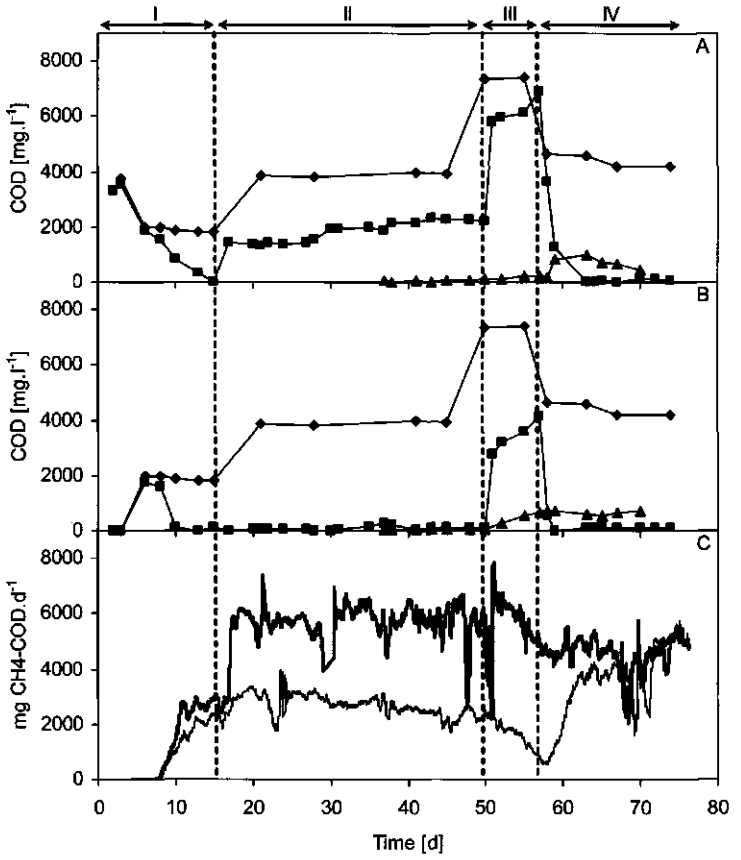

Figure 1 Evolution of the reactor performance of R1 and R2 with time. (A) R1 Influent methanol (♦), effluent methanol (■), and effluent VFA (▲) concentration. (B) R2 Influent methanol (♦), effluent methanol (■), and effluent VFA (▲) concentration. (C) Methane production of R1 (—) and R2 (▬).

Table 1. Mean operational and performance characteristics of the cobalt deprived (R1) and cobalt loaded (R2) UASB reactor.

Cobalt deprived reactor (R1)				
Parameter	Period I	Period II	Period III	Period IV
Day	0-16	17-50	51-57	58-77
PH	7.5±0.3	7.1±0.1	7.1±0.1	6.7±0.2
Methanol influent (mg COD.l^{-1})	1903±75[a]	3904±54	7373[b]	4404±253
Methanol effluent (mg COD.l^{-1})	919±789[a]	1791±113	5411±489	583±1218
VFA effluent (mg COD.l^{-1})	0	15±23	167±53	636±285
Cobalt loaded reactor (R2)				
Parameter	Period I	Period II	Period III	Period IV
Day	0-16	17-50	51-57	58-77
PH	7.2±0.5	7.0±0.1	6.8±0.1	6.8±0.2
Methanol influent (mg COD.l^{-1})	1903±75[a]	3904±54	7373[b]	4404±253
Methanol effluent (mg COD.l^{-1})	710±887[a]	87±72	3424±601	140±150
VFA effluent (mg COD.l^{-1})	0	23±8	381±290	657±82

[a] first 2 data points not included; [b] average of 2 data points.

Sulfate reduction in the reactor

The amount of sulfide in the effluent of R1 exceeded that of R2 i.e. amounting to values of 23±0.02 mM and 0.10±0.02 mM during period II, respectively. An increase in the effluent sulfide concentration can be observed from day 24 until day 52 (Fig. 2B). The difference in effluent sulfide concentrations remained during period III, with values of 0.30 mM and 0.13 mM for R1 and R2, respectively. In period IV, after cobalt had been supplied and the performance of R1 had been recovered, the effluent sulfide concentrations became approximately 0.17 mM in both reactors.

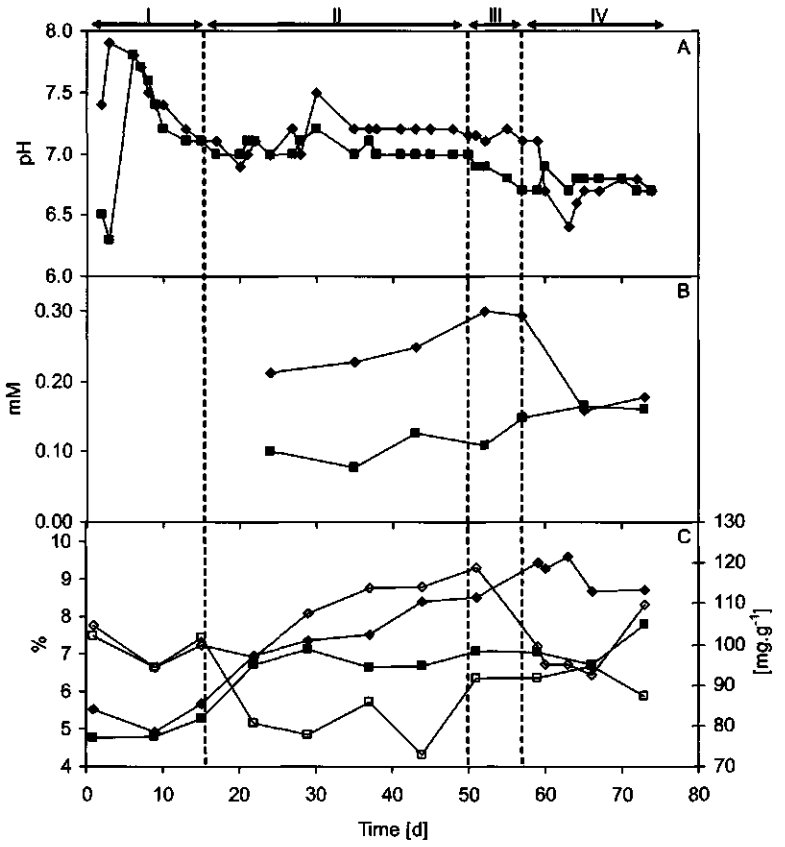

Figure 2 Evolution of the effluent characteristics of R1 and R2 with time. (**A**) pH of R1 (♦) and R2 (■). (**B**) Sulfide concentration of R1 (♦) and R2 (■). (**C**) The TSS (open symbols) concentration and ash content (closed symbols) of the sludge granules growing in R1 (♦) and R2 (■).

Evolution of the sludge characteristics as a function of time

A large difference was found in the TSS content of the sludge in the two reactors. After start up, the TSS content of the sludge in R1 increased from 105 $mg.g^{-1}$ to 119 $mg.g^{-1}$ on day 51 while it decreased in R2 to 73 $mg.g^{-1}$ on day 44. After cobalt was supplied to R1 (day 57), the TSS content of the sludge dropped from 119 $mg.g^{-1}$ on day 51 to 95 $mg.g^{-1}$ on day 60 (Fig. 2C). During the first 20 days of reactor operation, the ash content of the sludge increased in both reactors and subsequently it more or less increased linear in R1 (until day 66), while it remained constant in R2 (Fig. 2C).

The average TSS content of the effluent of R1 (66 $mg.l^{-1}$) was considerably higher than that of R2 (10 $mg.l^{-1}$). Although it distinctly increased when R2 became overloaded with methanol, i.e. then reaching 38 $mg.l^{-1}$ during period III.

Cobalt retention by the granular sludge during the reactor operation

The total cobalt content in the loaded sludge (R2) decreased significantly during the reactor operation, from 1667 $\mu g.g\ TSS^{-1}$ on day 11 to 763 $\mu g.g\ TSS^{-1}$ on day 73 (Fig. 3B). The total cobalt content of the R2 sludge initially decreased rather rapidly with 22 $\mu g.g\ TSS^{-1}.d^{-1}$ (between days 11 and 44), but following day 44 the leaching rate dropped to 5 $\mu g.g\ TSS^{-1}.d^{-1}$ (Fig. 3B). Most cobalt present in the sludge sampled at day 11 could be extracted in the first three extraction steps. The cobalt extracted from the freshly loaded inoculum (at day 1) during the first two extraction steps (sludge sample was lost after second extraction step) was considerably higher compared to that from sludge sampled at day 11, i.e. amounting to 39 and 37% respectively (data not shown). Apparently a considerable decrease of the cobalt content in these fractions occurred during the first days of operation. This was reflected by the initial cobalt loss as dissolved cobalt in the effluent of R2, which decreased fast from 11 μM on day 1 and to 4.82 to 0.08 μM on day 2 and 5, respectively as well as by the fast decrease in total effluent cobalt concentration from day 17 onwards (Fig. 3D). Although, the cobalt content of the sludge further decreased till the termination of the experiment, a significant amount of cobalt is still retained (Fig. 4). The relative contribution to the loss of cobalt from the sludge is higher for the exchangeable and carbonate fraction, 78% and 67%, respectively, compared to 45 and 42% for the organic/sulfide and the residual fraction (Fig. 4). Most of the cobalt is retained in the organic/sulfide fraction. From the difference in the total and soluble cobalt concentrations in the effluent it can be concluded that cobalt is mostly (approximately 80%, from period II onwards) present in a particulate or colloidal form and maybe associated with cells. The soluble cobalt concentrations were relatively low averaging 34 (±27) nM from period II onwards.

Cobalt addition to the influent of R1 at a concentration of 31 μM for a 24 h period on day 59 resulted in cobalt accumulation in the sludge up to 107 $\mu g.g\ TSS^{-1}$ on day 63 (Fig. 3A). This concentration is still considerably lower than the concentration of the sludge from the cobalt pre-loaded reactor at termination of the experiment. After start-up some soluble cobalt was found in the effluent of R1, 68 nM on day 1 decreasing to 12 nM on day 5 (Fig. 3C). On average the effluent of R1 contained 10(±2) nM cobalt during the first 58 days of operation. After dosing cobalt, the cobalt concentration in the effluent of R1 increased up to a maximum cobalt concentration of 0.45 μM (Fig. 3C), showing that more than 95% of the dosed cobalt was retained within the reactor. The maximum soluble cobalt concentration

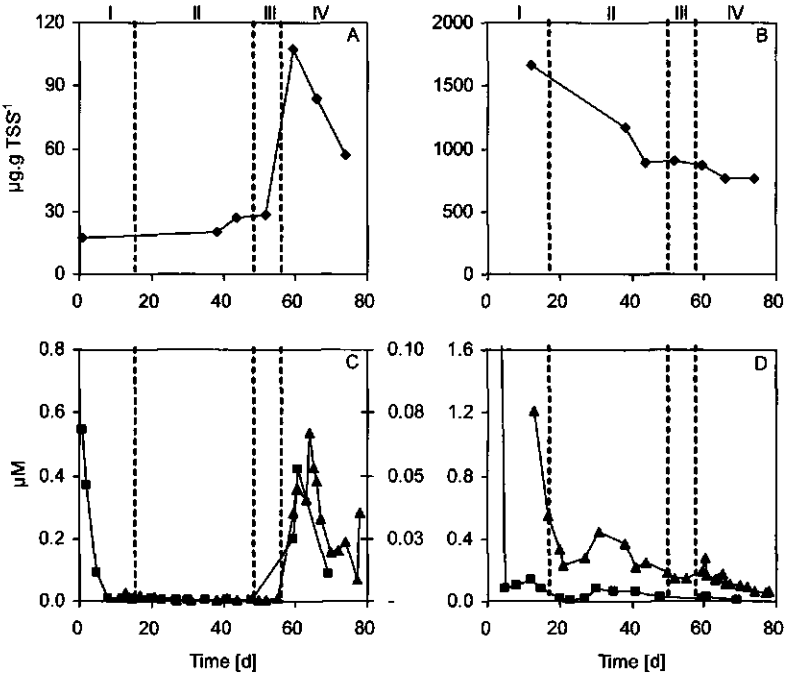

Figure 3 Evolution of the cobalt content in the sludge of R1 (A) and R2 (B) and effluent cobalt concentration of R1 (C) and R2 (D) with time. Cobalt concentration in the sludge (♦), total effluent cobalt concentration (▲) and dissolved effluent cobalt concentration (■).

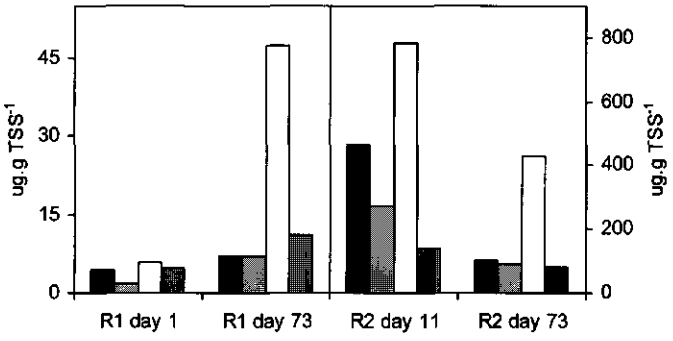

Figure 4 Sequential extraction of cobalt form the sludge in R1 (left axis) and R2 (right axis) at the beginning and at the end of the experiment. Bars represent, from left to right, the exchangeable, carbonate, organic/sulfide and residual fraction.

in the effluent after dosing amounted to 50 nM (Fig. 3C). The total cobalt concentration in the sludge of R1 decreased from day 63 onwards from 107 μg.g TSS^{-1} to 57 μg.g TSS^{-1} on day 73 at a rate of 3.5 μg.g $TSS^{-1}.d^{-1}$. This approximately corresponds with the losses found via the effluent of R1 during this period, i.e. amounting to 0.7 mg cobalt based on the total effluent concentrations of 0.30 ± 0.12 μM. Cobalt was mainly present in the organic/sulfides fraction of the sludge (65%). In contrast to the pre-loaded reactor, a relatively low accumulation of cobalt in the exchangeable fraction of the R1 sludge was observed (Fig. 4).

Methanogenic activity of the granular sludge

The SMA of the sludge in R1 with methanol as the substrate, after 30 days of operation, was considerably lower than the SMA of the sludge present in R2. The SMA of the R1 sludge increased 3 times in the presence of 0.5 μM cobalt in the medium. This clearly demonstrates the positive effect of the presence of small amounts of cobalt. At higher cobalt concentrations, the SMA increased only slightly further (Table 2, Fig. 5). After the in situ cobalt dosing procedure at day 57, the SMA of the sludge from R1 could still be slightly increased by supplying cobalt to the assay medium, i.e. the activity increased from 631 to 828 mg CH_4-COD.g^{-1}VSS.d^{-1} by in the addition of 5μM cobalt. From the response curve of the SMA with methanol as the substrate on cobalt addition (Table 2, Fig. 5), apparent K_m values of the sludge for cobalt were estimated using Michaelis-Menten kinetics. After 30 days of operation, the apparent K_m of the sludge of R1 was 304 nM. At the end of the experiment (day 77), after the in situ dosing of cobalt, the apparent K_m amounted to only 81 nM.

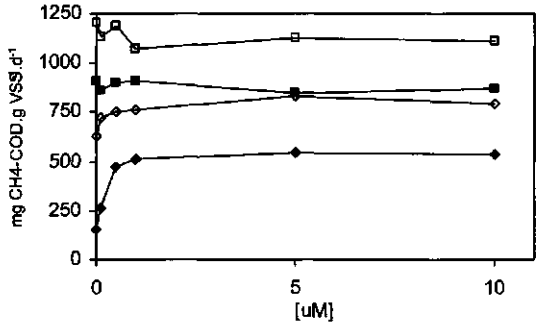

Figure 5 Maximum specific methanogenic activity (SMA) with methanol as the substrate (pH 7; 30°C) of UASB sludge as a function of the cobalt concentration in the test medium. Activity of R1 (♦) and R2 (■) after 30 days (closed symbols) and after 77 days (open symbols) of operation.

The SMA of the R2 sludge was not affected by the addition of cobalt to the batch medium (Fig. 5). The activities measured after 77 days of operation were on average 39% higher than the activities found at day 30 (Table 2). The SMA at the termination of the experiment was still considerably higher in R2, i.e. amounting to 1200 mg CH_4-COD.g $VSS^{-1}.d^{-1}$ compared to 631 mg CH_4-COD.g $VSS^{-1}.d^{-1}$ for the sludge from R1.

On the other hand, the SMA with acetate as the substrate at the termination of the reactor run was approximately twice as high for the sludge of R1 compared to the sludge from R2. Cobalt addition to the medium (5μM) did not affect the SMA with acetate as the substrate (Table 2).

Table 2. Maximum specific methanogenic activities (mg CH_4-COD.g^{-1}VSS.d^{-1}) with methanol and acetate as the substrate at day 30 and 73 and with and without the supply of different cobalt concentrations.

		Reactor 1		Reactor 2	
Cobalt		Day 30	Day 77*	Day 30	Day 77*
μM	μg.batch^{-1}				
0	0	155	631 (224)	906	1200 (156)
0.1	0.3	265	721	861	1130
0.5	1.5	475	756	899	1185
1	3.0	509	763	909	1074
5	14.7	540	828 (229)	843	1126 (145)
10	29.5	536	792	876	1108

*Values between brackets refer to the methanogenic activity on acetate.

DISCUSSION

Effect of cobalt loading on reactor performance

This study shows that providing Nedalco granular sludge with a cobalt stock by contacting it to a cobalt solution prior to inoculation represents an effective measure to overcome cobalt limitations when treating wastewater consisting of methanol as the main pollutant (Fig. 1 A, B). The SMA of the sludge immediately increased and subsequently much higher organic loading rates could be applied to the cobalt pre-loaded sludge without acidification of the reactor system (Fig. 6, Fig. 1A, B). The results also show that this SMA with methanol as the substrate could be maintained and even improved during the reactor

operation, as the methanogenic activity of the sludge in R2 increased with time from 906 on day 30 to 1200 mg CH_4 COD. g $VSS^{-1}.d^{-1}$ on day 73 (Table 2, Fig. 5).

Gonzalez-Gil et al. [1999] recently proposed a dosing strategy where cobalt is supplemented continuously at low concentrations, thus increasing the direct bioavailability of the cobalt and overcoming limitations due to precipitation-dissolution kinetics of metal sulfides. The in situ cobalt dosing strategy applied in this work is also effective, i.e. the methanogenic activity of R1 increased by a factor of 4 when cobalt was dosed for one day (16% of the cobalt amount present in the sludge of R2 at day 11), still this activity was 50% lower than that of sludge growing in R2. This four time increase is still quite significant and the methanogenic activity may be increased further by repeating the same in situ loading procedure. Figure 5, however, suggests that this increase is expected to be less efficient than after the first addition and activities similar to R2 at day 30 may be achieved only after prolonged periods of time. This warrants further research to determine the optimal dosing frequency of low concentrations of cobalt to increase the reactor performance and minimize cobalt losses from the sludge.

Effect of cobalt loading on the cobalt retention

The sequential extraction of cobalt from the pre-loaded sludge (day 11) showed that a large fraction of the cobalt was present in the exchangeable and carbonate fraction (Fig. 4), while in the in situ cobalt loaded sludge cobalt is mainly present in the organic/sulfide fraction (Fig. 4). The latter may be due to either the direct uptake of cobalt by the biomass or to preferential sorption of cobalt by the organic/sulfide fraction of the sludge. According to Mazumder et al. [1987] the maximum cobalt content in *Methanosarcina barkeri* cells, based on their corrinoid content, is approximately 330 μg.g dry $cell^{-1}$. Assuming that the VSS present in R2 consists of viable *M. barkeri* biomass, which is certainly a large overestimation, the maximum concentration internalized in the reactor biomass can only be 5 mg. This is much less than the actual amount of cobalt in the reactor, i.e. 25 mg on day 11, based on the amount of inoculum added to the reactor and its cobalt content at that day. Therefore, it can be concluded that a large fraction of this 25 mg cobalt is present outside the cells within the granular matrix, e.g. as cobalt sulfide.

The cobalt supplemented to the sludge leached from the granules during reactor operation (Fig. 4). The cobalt depletion from the sludge occurred in two phases, viz. at a relatively high rate of 22 μg.g $TSS^{-1}.d^{-1}$ during the first 40 days of operation and at a much slower rate of 5 μg.g $TSS^{-1}.d^{-1}$ from day 40 onwards. Even after dosing cobalt in situ, the cobalt content of the sludge decreased at a rate of 3.5 μg.g $TSS^{-1}.d^{-1}$, i.e. very similar to the rate observed with the pre-loaded sludge used in R2 from day 40 onwards. In practice any loss of cobalt from the sludge is obviously undesirable both from an economical and

environmental point of view. By dosing cobalt at 0.33 μM for a period of 30 days to a reactor containing cobalt deprived sludge (upflow velocity of 0.01 $m.h^{-1}$) using the same Nedalco inoculum as was used in the present experiments [Chapter 3], the cobalt concentration increased from 12 to 32 $\mu g.g\ TSS^{-1}$. During the subsequent 150 days when no cobalt was supplied, the cobalt was lost from the sludge at quite a low and more or less constant rate of 0.1 $\mu g.g\ TSS^{-1}.d^{-1}$. The effluent cobalt concentration of this reactor amounted to only 18±10 nM. According to Mazumder et al, [1987], *M. barkeri* excretes more than 40% of its synthesised corrinoid structures extracellularly during growth condition, irrespective of the cobalt concentration of the medium. This indicates that during reactor operation continuous losses of cobalt in the form of corrinoids might be expected. In the present research, the cobalt losses with the effluent were mainly in a non-soluble form. Nevertheless, the soluble cobalt fraction, although present at very low concentrations (Fig. 3), might have contained corrinoid structures. Indeed, a labile cobalt fraction could not be detected in the filtered effluent using adsorptive stripping voltametry (AdSV) (data not shown), as also observed in previous work [Zandvoort et al. 2002]. AdSV measures the free metal with the ligand dimethylglyoxime (DMG) which forms complexes with the free metal. The lack of AdSV binding indicates that this cobalt is present in a molecule with a higher binding strength than sulfides and the ligand (DMG), e.g. in corrinoid structures.

Methanol conversion

Based on the methanogenic activity with methanol as the substrate (900-1200 mg CH_4-COD. g $VSS^{-1}.d^{-1}$) and the amount of sludge (<15 g VSS) present in R2, it is clear that the imposed OLR of 20 g COD.l $reactor^{-1}.d^{-1}$ during period III comprised a distinct overloading of the system. However, this overloading resulted in a similar decrease in the methane production and methanol removal efficiency (Fig. 1C) in both reactors, suggesting inhibition due to methanol. The drop cannot be attributed to the VFA accumulation, because no significant VFA accumulation occurred in R1 during period III. Nevertheless an inhibition by methanol at concentrations of 7 g $COD.l^{-1}$ would be surprising, as pure cultures of *M. Barkeri* have been grown at concentrations as high as 12 g $COD.l^{-1}$ without any clear detrimental effect [Silveira *et al.* 1991]. In a UASB operated under similar conditions as the present research, the methanol loading was increased in two steps to influent methanol concentrations of 11.6 g $COD.l^{-1}$ and an OLR of 21.6 g COD.l $reactor^{-1}.d^{-1}$ [Florencio et al., 1995], without any apparent inhibition of the methanogens upon the increase in the methanol load. However, this reactor was operated at significantly lower sludge loading rates (i.e. 1.08 g COD. g $VSS^{-1}.d^{-1}$) compared to that R1 at the time of overloading (estimated 1.95 g COD. g $VSS^{-1}.d^{-1}$). Methanol inhibition might thus represent, together with the factors like a high cobalt concentration, high methanol in the effluent and high bicarbonate as found by Florencio [1993], an important trigger for the prevalence of acetogenesis.

The formation of significant amounts of VFA confirms the findings of Florencio [1993], as all the conditions stimulating the formation of VFA were met in period III in R2 and after cobalt is dosed for one day (31 μM) at the beginning of period IV in R1. Apparently the VFA formation in R1 is suppressed once no methanol is present in the effluent (Fig. 1A), which is also in agreement with the results of Florencio [1993]

CONCLUSIONS

- Both pre-loading and in situ dosing are adequate for achieving an increased reactor performance of methanol fed UASB reactors operating under cobalt limitation.
- Pre-loading increased the SMA on methanol of the R2 sludge considerably, the relatively high SMA on methanol could be maintained during reactor operation. Cobalt addition to the assay medium did not increase the SMA.
- Pulse dosing of cobalt to R1 (16% of the cobalt present in the R2 on day 11) increased the SMA on methanol 4 times, but was 50% lower than the SMA of the R2 sludge. Cobalt addition to the assay medium could still increase the SMA slightly.
- In situ dosing requires much lower amounts of cobalt, while it also gives substantial smaller losses of cobalt with the effluent.
- The cobalt content of the pre-loaded sludge decreased at two rates, during the first 40 days of operation at a fast rate (22 μg. g $TSS^{-1}.d^{-1}$), followed by a slower rate (5 μg. g $TSS^{-1}.d^{-1}$). After pulse dosing to R1, the cobalt content of the sludge decreased at a rate similar to the slower rate observed for the R2 sludge (3.5 μg. g $TSS^{-1}.d^{-1}$).

REFERENCES

Alibhai KRK, Mehrotra I, Forster CF (1985). Heavy metal binding to digested sludge, *Wat Res*, **19**, 1483-1488

APHA (1985). Standard methods for the examination of water and wastewater. American Public Health Administration, Washington, D.C., USA

Callendar IJ, Barford JP (1983). Precipitation, chelation and availability of metals as nutrients in anaerobic digestion. II Application. *Biotechnol Bioeng*, **25**, 1959-1972

Chen B, Utgikar VP, Harmon SM, Tabak HH, Bishop DF (2000). Govind R. Studies on biosorption of zinc (II) and copper (II) on *Desulfovibrio desulfuricans. Int Biodeter Biodegr*, **46**, 11-18

Florencio L, Jenicek P, Field JA, Lettinga G (1993). Effect of cobalt on the anaerobic degradation of methanol. *J Ferment Bioeng*, **75**, 368-374.

Florencio L, Field JA, Lettinga G (1995). Substrate competition between methanogens and acetogens during the degradation of methanol in UASB reactors. *Wat Res*, **29**, 915-922

Gonzalez-Gil G, Kleerebezem R, Lettinga G (1999). Effects of nickel and cobalt on kinetics of methanol conversion by methanogenic sludge as assessed by on-line CH_4 monitoring. *Appl Environ Microbiol*, **65**, 1789-1793

Kelly CR, Schwitzenbaum M (1984). Anaerobic treatment and nutrient effects. *Agri Wastes*, **10**, 135-154

Mazumder TK, Nishio N, Fukazaki S, Nagai S (1987). Production of extracellular vitamin B_{12} compounds from methanol by *methanosarcina barkeri*. *Appl Microbiol Biotech*, **26**, 511-516

Osuna, MB, Hullebusch van ED, Zandvoort MH, Iza J, Lens PNL (2004). Effect of cobalt sorption on metal fractionation in anaerobic granular sludge. *J Env Qual*, **33**: 1256-1270

Shen CF, Kosarik N, Blasczyk R (1993). Properties of anaerobic granular sludge as affected by yeast extract cobalt and iron supplements. *Appl Microb Biotech*, **39**, 132-137

Silveira RG, Nishio N, Nagai S (1991). Growth characteristics and corronoid production of *Methanosarcina barkeri* on methanol-acetate medium. *J Ferment Bioeng*, **71**, 28-34

Speece RE, Parkin GF, Gallagher D (1983). Nickel stimulation of anaerobic digestion. *Wat Res*, **17**,677-683

Trüper HG, Schlegel HG (1964). Sulphur metabolism in *Thiordaceae* I. Qauntative measurment on growing cells of *Chromatium okenii*. *J Microbiol Ser*, **30**, 225-238

Weijma J, Stams AJM, Hulshoff Pol LW, Lettinga G (2000). Thermophillic sulfate reduction and methanogenesis with methanol in a high-rate anaerobic reactor. *Biotechnol Bioeng*, **67**, 354-363.

Zandvoort MH, Osuna MB, Geerts R, Lettinga G, Lens P (2002). Effect of nickel deprivation on methanol degradation in a methanogenic granular sludge reactor. *J Ind Microbiol Biotechnol*, **29**, 268-274

Chapter 6

Effect of sulfur source on the performance and metal retention of methanol fed UASB reactors

The effect of a sulfur source on the performance and metal retention of methanol fed upflow anaerobic sludge bed (UASB) reactors was investigated. For this purpose, two UASB reactors were operated with cobalt pre-loaded granular sludge (1 mM $CoCl_2$; 30°C; 24 h) at an organic loading rate (OLR) of 5 g COD.l reactor^{-1}.d^{-1}. One UASB reactor (R1) was operated without a sulfur source in the influent during the first 37 days. In this period the methanol conversion to methane remained very poor, apparently due to the absence of a sulfur source, once cysteine, a sulfur containing amino acid, was added to the influent of R1 (day 37) a full conversion of methanol to methane occurred within 6 days. The second reactor (R2) was operated with sulfate (0.41 mM) in the influent during the first 86 days of operation, during which no limitation in the methanol conversion to methane manifested. Cobalt was released from the sludge at similar rates in both reactors, a process that appeared to be independent of the presence and/or type of sulfur source. The leaching of cobalt occurred at two distinct rates, first at a high rate of 22 μg.g TSS^{-1}.d^{-1}, which proceeded mainly from the exchangeable and carbonate fraction and later at a relatively slow rate of 9 μg.g TSS^{-1}.d^{-1} from the organic/sulfide fraction. The other trace metals (Zn, Cu, Mn, Mo, Se, Fe) were dosed continuously from day 0 until day 86 at a concentration of 5 μM, except iron (50 μM). They accumulated in the sludge of the reactors, although in different chemical fractions and to a different extent. When after 86 days of operation the supply of a sulfur source and of metals to the influent to both reactors were terminated, all these metals leached from the sludge. This study showed that the supply of L-cysteine has a pronounced positive effect on the methanogenic activity and the retention of metals like iron, zinc and molybdenum.

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INTRODUCTION

Trace metals such as cobalt, nickel and iron are essential for anaerobic micro-organisms, because they are present in many enzymes involved in the biochemistry of fermentation and methane (CH_4) production. Therefore, lack of even a single trace metal may severely limit anaerobic conversion processes [Kelly and Schwitzenbaum, 1984; Speece et al., 1983; Florencio, 1993; Chapter 3 and 4]. Consequently, trace elements are generally supplied to the influent of full-scale anaerobic bioreactors to maintain a good reactor performance. In order to minimize the cost of this metal dosing and to reduce the release of trace metals into the environment, these metals should be dosed to the bioreactor in such a way that they are retained within the sludge bed. On the other hand, the retained metals should be present or become present in a bioavailable form in the sludge in order to provide their stimulating effect on the biological activity of the sludge.

Little is known about the influence of the prevailing reactor conditions on the dynamics of the trace metal stock present within the granular sludge. For instance sulfide, which is ubiquitously present in anaerobic bioreactors because of the occurrence of sulfate reduction or organic matter mineralization, is an important factor for the retention of the supplied trace metals because of the production of insoluble sulfides. This property of most metal sulfides is used for their removal from wastewater [Kaksonen et al., 2003, Jong and Perry, 2003]. Due to metal sulfide precipitation, it is likely that trace metals are retained better within the sludge bed, but consequently these metals may be no longer 'directly' available for the uptake by microorganisms [Gonzalez-Gil et al., 2003]. On the other hand, reduced sulfur is an essential macronutrient for the growth of methanogenic microorganisms [Mountfort and Asher, 1979]. For instance, *Methanosarcina barkeri* requires 36.8 mg S. mol^{-1} methanol, supplied as cysteine for optimal growth [Nishio et al., 1992]. This equals, based on their experimentally determined yield of 4.23 g cell.mol $methanol^{-1}$ to 8.8 mg S. g $cell^{-1}$, which corresponds quite well with the cell content of 11 mg S. g $cell^{-1}$ reported by Scherer *et al.* [1983] for this species.

Different sources of sulfur can be supplied to the microorganisms, e.g. sulfate or L-cysteine. Sulfate can be reduced by dissimilatory, but also by assimilatory sulfate reduction processes. The ability to (assimilatively) reduce sulfate seems to be specific for different methanogenic bacteria. For instance, Daniels *et al.* [1979] showed, using *Methanobacterium thermoautothrophicum*, *Methanococcus thermolithothrophicus* and *Methanospirillum hungatei* grown on H_2/CO_2, that only the first of these micro-organisms was capable to grow on sulfate. The amino acid L-cysteine can be used by *Methanosarcina barkeri* for assimilatory processes [Mazumder et al., 1986]. L-cysteine is not only a sulfur source, but it can possibly influence the metal precipitation as well, as L-cysteine is a ligand that will complex the metals present in the wastewater. Gonzalez-Gil et al. [2003] showed in batch

experiments using *Methanosarcina sp.* that the nickel and cobalt bioavailability could be improved by the addition of 1 mM L-cysteine compared to batches with 1 mM of sulfide.

In this paper, methanol fed upflow anaerobic sludge bed (UASB) reactors inoculated with cobalt pre-loaded granular sludge were used as a model system to investigate the effect of the presence of a sulfur source as well as the type of sulfur source (L-cysteine and sulfate) on the performance of and the metal retention dynamics in UASB reactors. The metal retention was studied by monitoring the distribution of the metals over different chemical fractions as a function of time using a sequential extraction scheme. Further, morphology and metabolic properties of the sludge that developed in the reactor were monitored using, respectively, microscopy and batch activity tests.

MATERIAL AND METHODS

UASB reactor operation

Two UASB reactors (working volume 0.75 l; inner diameter 5 cm) were operated in a temperature-controlled (30 ± 2°C) room. The conical bottom of the reactors was filled with glass marbles in order to achieve a more even distribution of the influent over the sludge bed. For introducing the influent in the reactors, peristaltic pumps (type 202, Watson and Marlow, Falmouth UK) were used. Effluent recycle was applied to obtain a superficial upflow velocity of 1 $m.h^{-1}$. The reactors were operated at a hydraulic retention time (HRT) of 8 h.

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 produced methane volume was measured by water displacement from mariot-flasks using an on-line balance system as described by Gonzalez et al. [1999].

Source of biomass

Each of the UASB reactors was inoculated with 20 g $VSS.l^{-1}$ anaerobic granular sludge originated from a full scale UASB reactor treating alcohol distillery wastewater of Nedalco (Bergen op Zoom, the Netherlands). The sludge was elutriated to remove the fines. The total suspended solids (TSS) and volatile suspended solids (VSS) content of this sludge were 10.0 (±0.2)% and 9.5 (±0.2)%, respectively. The initial cobalt content of the sludge was 18.7 $\mu g.g\ TSS^{-1}$. The inoculum of both reactors was pre-loaded with cobalt by contacting 400 g of sludge (wet weight) with 1600 ml of a 1 mM cobalt solution ($CoCl_2.6H_2O$) for 24h at 30°C in a 1 litre serum bottle in the absence of substrate. During the run, the sludge was examined for its morphology and microbial composition by stereo (Olympus SZ-PT, Tokyo, Japan) and light microscopy (Olympus BH2, Tokyo, Japan).

Experimental design

The methanol-organic loading rate (OLR) was maintained at 5 g methanol (MeOH) COD.l reactor^{-1}.d^{-1} during the entire experiment, corresponding to a sludge loading rate (SLR) of 0.25 g MeOH COD.g VSS^{-1}.d^{-1} when calculated based on the amount of VSS added to the reactor at the start of the experiment. During the first period of operation (Period I, day 1-37), no sulfur source was added to the influent of one of the UASB reactors (R1), while 0.41mM of sulfate was added to the influent of the other UASB reactor (R2). During period II (day 38-87), L-cysteine (0.41 mM) was added as a sulfur source to the influent of R1, while the operational condition of R2 remained unchanged. In period III (day 88-118), no sulfur source and no metals were added with the influent of both reactors.

Basal medium

The reactors were fed a medium containing methanol, macro nutrients ($MgCl_2$ instead of $MgSO_4$ for R1) and a trace element solution without cobalt, containing Fe (50μM), Ni, Zn, Mn, Cu, W, Mo, Se at a concentration of 5 μM. To ensure pH stability, 30 mM (2.52 g.l^{-1}) of $NaHCO_3$ was added to the basal medium. As mentioned above, from day 38 onwards, L-cysteine (0.41 mM; 13.1 mg.l^{-1}) was added as a sulfur source to the influent of R1, which is equal to the amount of sulfur dosed in the form of $MgSO_4$ to R2. To avoid precipitation in the storage vessels, the influent consisted of three different streams; viz. basal medium without K_2HPO_4; methanol with bicarbonate and K_2HPO_4; and the trace element solution. To prevent oxidation, the trace element solution was kept anaerobic under a nitrogen atmosphere.

Maximum specific methanogenic activity tests

Maximum specific methanogenic activity (SMA) of the sludge developing in the reactors was determined in duplicate at 30 (±2) °C using on-line gas production measurements as described in Chapter 3. Approximately 1g (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 either methanol (4 g COD.l^{-1}) or acetate (2 g COD.l^{-1}) as the substrate and different amounts of cobalt. The SMA of the sludge of both reactors with methanol as the substrate was assessed after 29, 71, 91 (only R2) and 118 days of operation, while the SMA with acetate as the substrate was determined only at the termination of the experiment.

Metal analyses

The total metal content of the granular sludge was determined as described previously [Chapter 3]. In order to assess the metal speciation in the granular sludge matrix, the metals were sequentially extracted using a four-step extraction scheme (Table 1), in which each next step becomes more stringent. The following nomenclature is applied for the subsequent extraction steps, viz. the exchangeable fraction (1M NH_4CH_3COO), the carbonate fraction (1M CH_3COOH), organic/sulfide fraction (30% H_2O_2) and the residual fraction (3:1 HCl/HNO_3).

Table 1. Sequential extraction procedure.

Fraction	Extracting agent[c]	Extraction conditions	
		Shaking Time[a]	Temp
1. Exchangeable	10 ml NH_4CH_3COO, (1 M, pH = 7)	1 hour	20°C
2. Carbonates	10 ml CH_3COOH, (1 M, pH = 5.5)	1 hour	20°C
3. Organic matter/Sulfide	5 ml H_2O_2, (30% pH = 2)	3 hours	35°C
4. Residual	10 ml demineralised water and 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 with the total extraction method; [c] References to the applied methods: Veeken, 1998; Tessier et al., 1979; Modak *et al.*, 1992.

Chemical analyses

The concentration of methanol and volatile fatty acids (VFA) were determined using gas liquid chromatography as described by Weijma *et al.* [2000]. The total sulfide concentration was determined colorimetrically using the methylene blue method (Trüper and Schlegel, 1964), and the total suspended solids (TSS) and volatile suspended solids (VSS) contnents were determined according to Standard Methods [American Public Health Association, 1985]. All chemicals were of analytical or biological grade and purchased from E. Merck AG (Darmstadt, Germany).

RESULTS

Methanol degradation

During period I most methanol was removed and converted to methane, within 13 days in the sulfate fed R2-reactor (Fig. 1B), while in R1 (no sulfur source) only a part of the methanol was converted both to methane and VFA. After 8 days of operation VFA started to accumulate in the R1 effluent, reaching a value of 412 mg $COD.l^{-1}$ on day 35 (Fig. 1A). After 12 days of operation some methane formation was observed in R1 (Fig 1C). The methane

formation did not further improve and it even tended to decline. The incomplete methanol conversion and the poor methanogenesis in R1 can likely be attributed to the absence of a sulfur source in the influent. This was tested by assessing the effect of the sulfur source L-cysteine on the SMA with methanol of the sludge (see paragraph: maximum specific methanogenic activity of the granular sludge). Because of the observed positive effect of L-cysteine on the SMA, this S-compound was supplied with the influent of R1 from day 38 onwards (period II) at a concentration (0.41 mM) similar to the sulfur content of the R2 influent. Initially, the response was an increase of the VFA concentration up to 945 mg $COD.l^{-1}$ on day 41, but then the VFA concentration started to decrease from that day onwards. At the same time, methane formation significantly increased reaching similar values as found in R2 on day 55 with only traces of VFA present in the effluent (Fig. 1B vs. 1A).

The performance of R2 remained unchanged until day 72. From that day onwards, a sudden drop in the methanol removal efficiency was observed during a 4 day period, without a simultaneous increase in VFA concentration (Fig. 1C). Surprisingly, the methanol removal had completely recovered on day 78 (Fig. 1B). The observed drop in COD removal coincided with a change in the granular sludge bed. After approximately 70 days of operation, a flocculent sludge bed had developed above the granular sludge bed, which filled up the reactor completely. This flocculent sludge likely resulted from either the disintegration of the granules in the sludge bed or new growth in flocs instead of granules. At the time of flocculent biomass development, the SMA of the sludge granules was determined and it was significantly lower than previously observed on day 29 (see paragraph; maximum specific methanogenic activity of the granular sludge). This corroborates with the temporary deterioration of the R2 performance (Fig. 1). On day 76, the amount of flocculent sludge in the reactor had decreased significantly due to its washout with the effluent. This washout continued until hardly any flocculent sludge could be observed in the reactor on day 83.

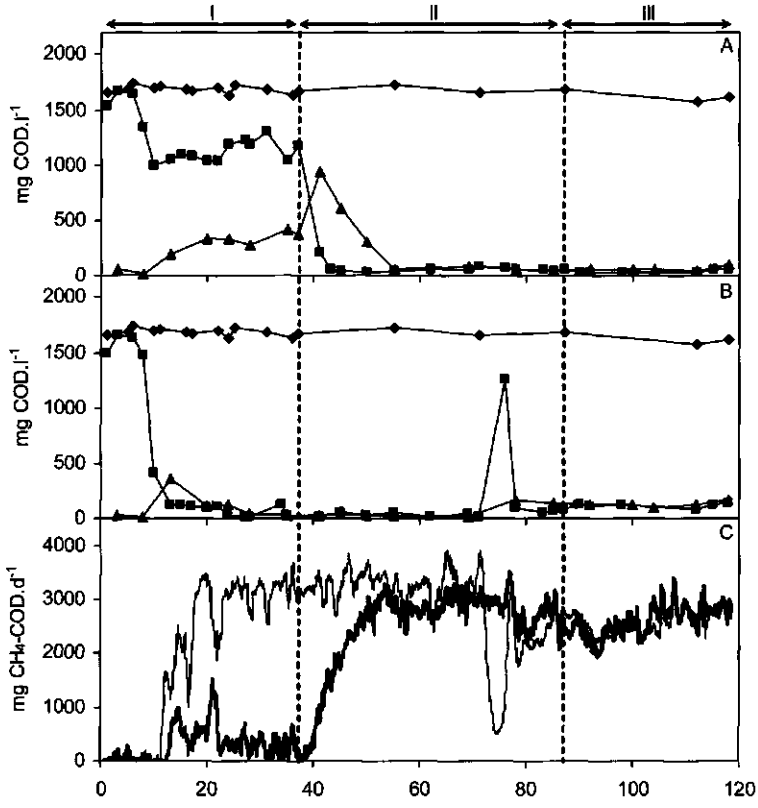

Figure 1 Evolution of the reactor performance of R1 and R2 with time. (**A**) R1 Influent methanol (♦), effluent methanol (■), and effluent VFA (▲) concentration. (**B**) R2 Influent methanol (♦), effluent methanol (■), and effluent VFA (▲) concentration. (**C**) Methane production of R1 (—) and R2 (—). Start of period II, L-cysteine addition to R1 (1st vertical broken line) and start of period III no sulfur source and trace element solution in both reactors (2nd vertical broken line)

Sulfide

The effluent sulfide concentration during the period a sulfur source was supplied to the influent amounted to 7.43 (±2.10) $mg.l^{-1}$ and 3.02 (±2.42) $mg.l^{-1}$ for R1 and R2, respectively (Fig. 2). Sulfide was first observed in the effluent of R2 after 10 days of reactor operation (Fig. 2) and its appearance was accompanied by a clear blackening of the reactor liquid, likely due to the formation of soluble and/or colloidal metal sulfides. Blackening of the sludge granules manifested already at day 7, which indicates that metal sulfide precipitation in the

sludge granules preceded its presence in the reactor liquid. Simultaneous with the onset of the sulfate reduction, methane formation occurred (Fig. 1C and 2A).

The sulfide concentration in the R2 effluent varied remarkably during the first 30 days of operation, viz. between 0.29 and 5.32 $mg.l^{-1}$ (Fig. 2). Following day 30, the effluent sulfide concentration of R2 stabilized at around 4.89 (±1.47) $mg.l^{-1}$ from day 31 to day 77. During the same period the sulfide concentrations in the effluent of R1 was slightly higher. From day 86 onwards (Period III), when no sulfur sources were supplied with the influent, the sulfide concentration in the effluent of both reactors decreased to very low values (Fig. 2).

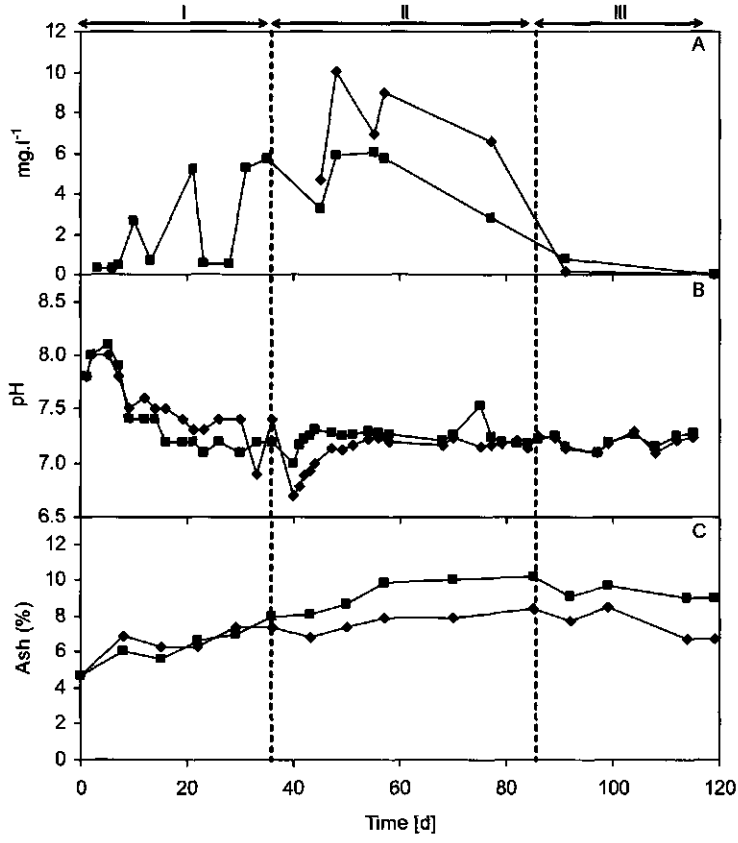

Figure 2 (A) Evolution of the sulfide concentration in the effluent of R1 (♦) and R2 (■) as a function of time. **(B)** pH of the effluent of R1 (♦) and R2 (■). **(C)** Ash content of the granular sludge of R1 (♦) and R2 (■) as a function of time. For explanation of the periods see Figure 1.

Sludge morphology

The periphery of the inoculum sludge was smooth and appeared to have clustered structure, viz. yellowish inclusions in a transparent black outside layer (Fig. 3A). Cross sections of the sludge granules revealed that the black outside layer was very thin, while the whole inside of the granules still was yellowish in color. The inclusions apparently were encapsulated in a light black marbled structure (Fig.3B). In the inoculum sludge, filamentous rods as well as blunt rods predominated. The flocculent sludge that accumulated in R2 during the first 70 days of operation consisted mainly of *Methanosarcina* type organisms. This was the dominant organism present in the sludge washing out with the effluent of R2. Some of the *Methanosarcina* clusters were black or dark in color, indicating that metal sulfides precipitated on the cell walls (Fig. 3E). On day 70, the granules present in both reactors had a similar appearance, i.e. a black and rough outside layer had developed on the periphery of the granules (Fig. 3C), without any clear visual changes on the inside of the granules.

At the end of the period without a sulfur source (period III), this rough layer was still present on most granules present in R2, but the appearance of some granules was similar to the seed sludge, indicating that the outside layer had been sloughed off (Fig. 3D). Filamentous rods were still dominant in the R2 sludge. However, the fraction of *Methanosarcina* like organisms clearly had increased relatively to the inoculum, suggesting their proliferation in the granular sludge (Fig. 3F). In the sludge of R1, rod and filamentous shaped organisms still dominated at the termination of the run, and it contained less *Methanosarcina* like organisms compared to the R2 sludge. In period III, the effluent of R1 contained a lot of blunt rods and hardly any *Methanosarcina*, the same was observed for the R2, although still some *Methanosarcina* were present in this effluent.

Maximum specific methanogenic activity of the granular sludge

After 29 days of operation, the SMA with methanol in R1 only amounted to 80 mg CH_4-COD.g $VSS^{-1}.d^{-1}$ (Fig. 4). Addition of L-cysteine (0.41 mM) resulted in only a slight increase of the SMA to 128 mg CH_4-COD.g $VSS^{-1}.d^{-1}$. Although, L-cysteine clearly positively affected the methane formation rate (data not shown), the occurrence of a rapid simultaneous acidification (mainly acetate, i.e. 1919 $mg.l^{-1}$) resulted in a pH drop of the medium to 5.3, which seriously hampered an equivocal evaluation of this positive effect on the methanogenesis. The continuous supply of L-cysteine to the influent of R1 resulted in an almost 8-fold increase of the SMA to 792 mg CH_4-COD.g $VSS^{-1}.d^{-1}$ on day 71, which even further increased to 1119 mg CH_4-COD.g $VSS^{-1}.d^{-1}$ at the termination of the experiment (Fig. 4).

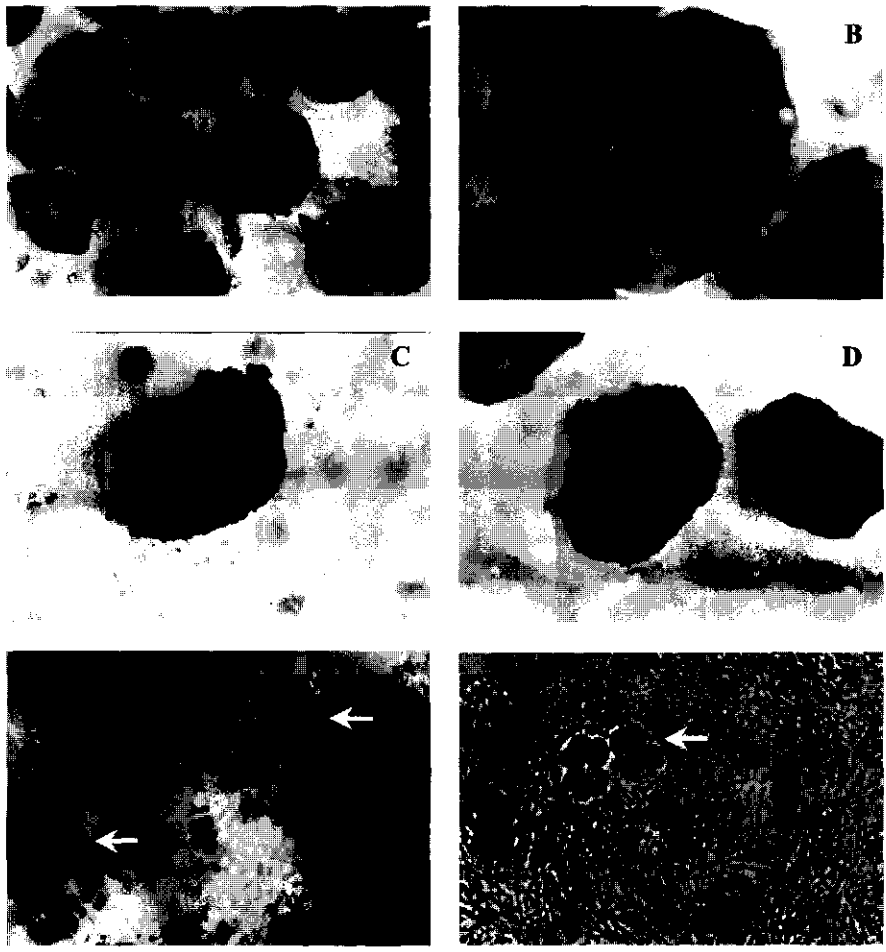

Figure 3. Sludge morphology. (**A**) Inoculum granules, (**B**) cross-section of the inoculum granules, (**C**) granule after 70 days of operation (**D**) rough granule (left) and smooth granule (right) at the end of the reactor run, (**E**) black *Methanosarcina* clusters (indicated by arrows) in the effluent R2, (**F**) sludge at the end of reactor operation (*Methanosarcina* cluster indicated by arrow).

The SMA of the sludge in R2 with methanol as the substrate amounted to 744 mg CH_4-COD.g $VSS^{-1}.d^{-1}$ on day 29. Regarding the accumulation of flocculent sludge in R2, the SMA of the granular and the accumulated flocculent sludge were determined separately (at day 71). The SMA of the flocculent sludge amounted to 706 mg CH_4-COD.g $VSS^{-1}.d^{-1}$, comparable to the SMA of the granular sludge on day 29, while the SMA of the granular

sludge had dropped to only 295 mg CH_4-COD.g $VSS^{-1}.d^{-1}$. One day following the sludge sampling a remarkable drop in methanol removal efficiency (Fig 1B) manifested in the reactor. Surprisingly, as the performance restored and the flocculent sludge washed out from the reactor, the SMA of the granular sludge restored and even became substantially higher than prior to the system disturbance viz. 1284 mg CH_4-COD.g $VSS^{-1}.d^{-1}$ and 1382 mg CH_4-COD.g $VSS^{-1}.d^{-1}$, at day 91 and 118, respectively (Fig. 4). Addition of cobalt (5μM) to the medium did not significantly improve the SMA with methanol of the R1 and R2 sludge at day 118 (data not shown).

At the termination of the reactor run (day 118), the SMA on acetate of the R1 (608 mg CH_4-COD.g VSS^{-1} $.d^{-1}$) and R2 (562 mg CH_4-COD.g $VSS^{-1}.d^{-1}$) sludge were very similar. In both cases methane production started after a short lag phase of 7-10 hours (data not shown).

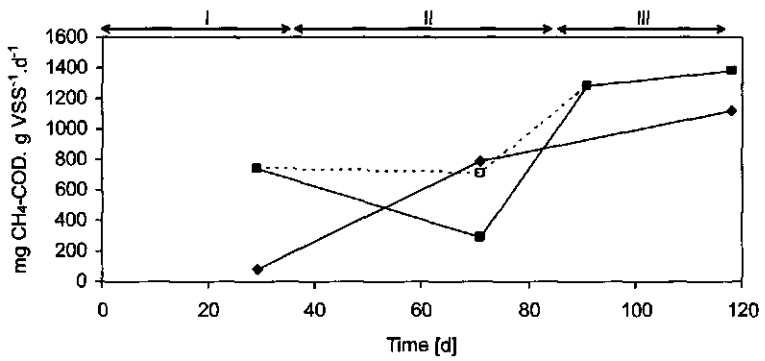

Figure 4 Maximum specific methanogenic activity (pH 7; 30°C) of UASB sludge with methanol as the substrate as a function of the cobalt concentration in the test medium. Activity of R1 (♦) and R2 (■) after 30 days (closed symbols) and after 77 days (open symbol) of operation. For explanation of the periods see Figure 1

Specific sludge loading rate

The ash content of the sludge in both reactors increased from 6.4 % at start-up to 7.9 and 9.8 % for the R1 and R2 sludge, respectively, after 57 days of operation. From then onwards, the ash content of the sludge stabilized in both reactors (Fig 2C). As sludge samples were taken regularly from the reactors, a gradual decrease of the sludge bed volume occurred and consequently an increase of the specific loading rate. Based on the amount of sludge removed as a result of sampling, only 25% of the original amount of inoculum sludge was still present in the reactors at the termination of the experiment. Consequently, the specific methanol sludge load increased from 0.25 g COD. g $VSS^{-1}.d^{-1}$ on day 1 to 0.98 and 1.19 g COD. g $VSS^{-1}.d^{-1}$ on day 114 for R1 and R2, respectively. Similarly, the total metal load of

the various supplied metals increased from 0.82 μmol. g $VSS^{-1}.d^{-1}$ on day 1 to 1.88 and 2.60 μmol. g $VSS^{-1}.d^{-1}$ on day 84 for R1 and R2, respectively.

Sulfur content of the sludge

The sulfur content of the sludge present in R1 and R2 was monitored during the first 43 days of operation. At day 7, the sulfide content was 7.8 and 8.9 mg.g TSS^{-1} for the sludge of R1 and R2, respectively. Once a sulfur source was present in the influent, the sulfur content of the sludge gradually increased to 13.9 and 15.4 mg.g TSS^{-1} for R1 and R2, respectively, at day 43. Sulfur did not accumulate in the exchangeable and carbonate fraction but clearly accumulated in the organic/sulfide fraction (Fig. 5). In R2, a linear increase in the sulfur content of the organic/sulfide fraction was observed from 4.84 mg.g TSS^{-1} on day 7 to 9.34 mg.g TSS^{-1} on day 49.

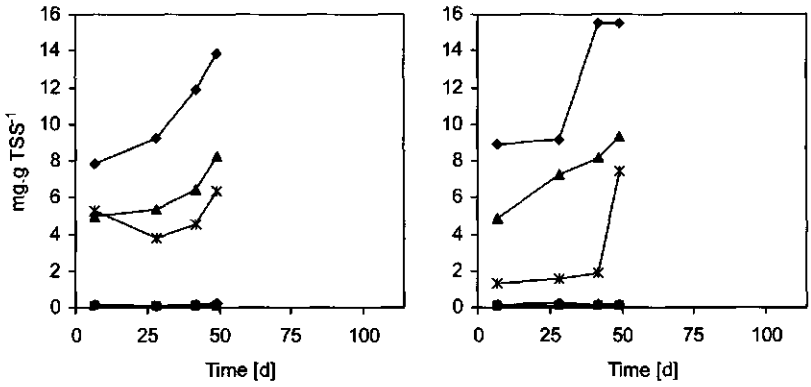

Figure 5. Evolution of the sulfur concentration in the sludge during the first 49 days of operation, Exchangeable fraction (◇), Carbonate fraction (■), Organic/sulfide fracion (▲), Residual fraction (×), Total content (♦).

Trace element retention by the granular sludge during reactor operation

The cobalt content of the inoculum sludge was elevated from 19 μg.g TSS^{-1} to 1.99 mg.g TSS^{-1} by the cobalt loading procedure. During the reactor operation, the cobalt content of the sludge decreased considerably at an overall rate of 14 and 12 μg.g $TSS^{-1}.d^{-1}$ for R1 and R2, respectively (Fig. 6, Table 3). At the termination of the experiment, the remaining cobalt content of the sludge amounted to 22 and 28% of the initial amount present in the R1 and R2 sludge, respectively. At the start of the experiment, 62% of the cobalt was present in the exchangeable and carbonate fractions of both sludges. During the reactor operation, cobalt

particularly decreased significantly in these fractions and it only contributed 15 and 7% of the total cobalt content, of respectively, the R1 and R2 sludge on day 50. During the first 36 days of operation, the average cobalt depletion rates were 22 and 21 μg.g $TSS^{-1}.d^{-1}$ for the R1 and R2 sludge, respectively.

Table 3a. Rate of cobalt depletion (μg.g $TSS^{-1}.d^{-1}$) from the different sludge fractions of the R1 sludge.

L-cysteine reactor (R1)	**Overall**	**Period I**	**Period II+III**
Fraction	μg.g $TSS^{-1}.d^{-1}$	μg.g $TSS^{-1}.d^{-1}$	μg.g $TSS^{-1}.d^{-1}$
Total	14	22	9
Exchangeable	7	16	1
Carbonate	4	8	1
Organic/sulfide	3	1	6 (8)[a]
Residual	1	1	1

[a] The depletion rate of cobalt from day 50 onwards after the initial increase of cobalt in this fraction during period II

Table 3b. Rate of cobalt depletion (μg.g $TSS^{-1}.d^{-1}$) from the different sludge fractions of the R2 sludge.

Sulfate reactor (R2)	**Overall**	**Period I**	**Period II+III**
Fraction	μg.g $TSS^{-1}.d^{-1}$	μg.g $TSS^{-1}.d^{-1}$	μg.g $TSS^{-1}.d^{-1}$
Total	12	21	8
Exchangeable	7	20	0
Carbonate	4	11	0
Organic/sulfide	2	-5 (-18)[a]	7
Residual	1	-1	-1

[a] The rate of increase in this fraction from day 1 to 29.

The main part of the cobalt ultimately remaining in the sludge was present in the organic/sulfide fraction and the rate of release from this fraction was significantly lower compared to that from the exchangeable and carbonate fraction. In fact, only 52 and 40% of the cobalt in this fraction was lost during operation of R1 and R2, respectively (Fig. 6).

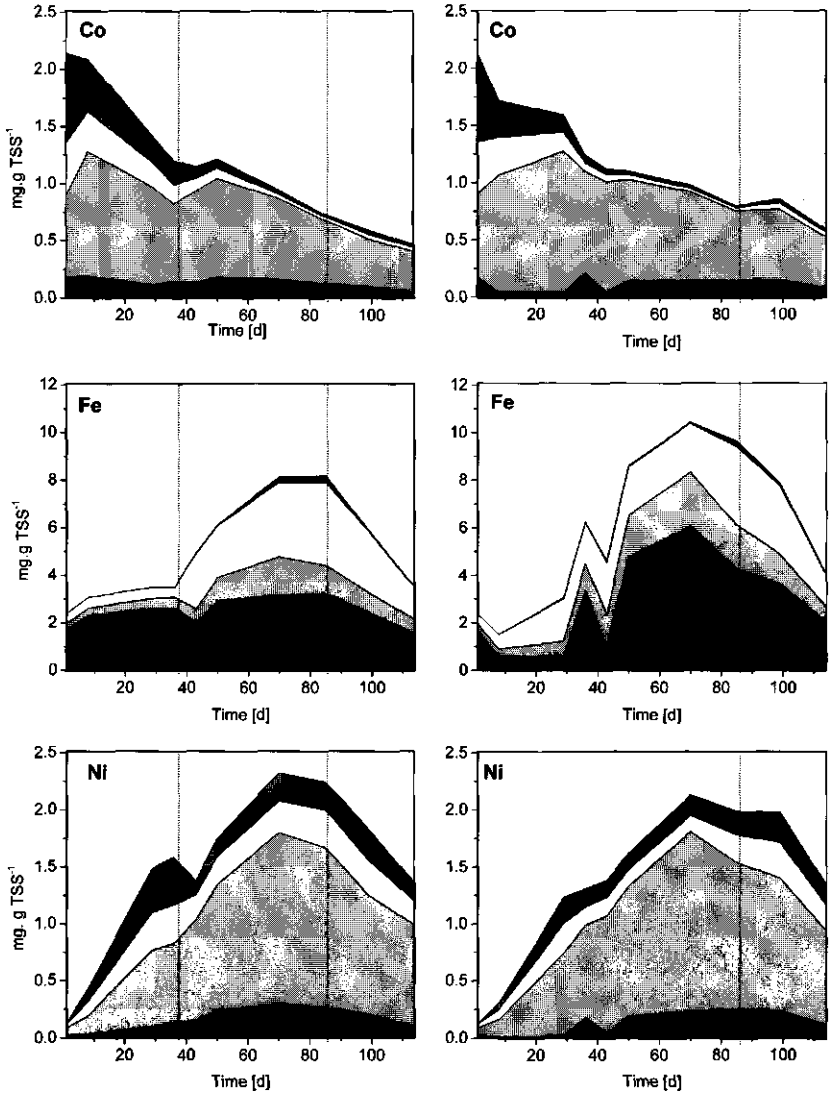

Figure 6 Evolution of the cobalt, iron and nickel concentration in the extracted metal fractions of R1 (left) and R2 (right). Residual fraction (Black), Organic/sulfide fraction (light grey), carbonate (white) and exchangeable (dark grey). For explanation of the periods see Figure 1

Initially, the cobalt content of the organic/sulfide fraction of the R2 sludge increased from 0.72 on day 1 to 1.23 μg.g TSS^{-1} on day 29 (Fig. 6, Table 3). A similar but lower (from 0.68

on day 36 to 0.86 μg.g TSS^{-1} on day 50) increase was observed in the R1 sludge once L-cysteine was added as the sulfur source (Fig. 6). Apparently, a transfer of cobalt from the exchangeable and carbonate fraction to the organic/sulfide fraction occurred in the presence of sulfide. From day 43 onwards, once the exchangeable and carbonate fraction were almost depleted, cobalt was released from the organic/sulfide fraction at a rate of 6 and 7 μg.g $TSS^{-1}.d^{-1}$ for R1 and R2, respectively.

Iron accumulation in absolute amounts exceeded that of any of the other metals, which can likely be attributed to a 10 times higher supply of iron (50 μM) to the influent. Accumulation of iron seems to occur only once significant methanol conversion and sulfide evolution occurred in the reactors (Fig. 1 and 2 vs. Fig. 5). Most of the iron was initially present in the residual fraction (71%) and showed a preference for accumulation in this fraction in R2. The total iron content of the sludge reached a maximum on day 70 in both reactors, i.e. 8.14 and 10.44 mg.g TSS^{-1} in R1 and R2, respectively. The higher iron content in the R2 sludge mainly results from the high accumulation in the residual fraction, which reached 6.07 mg.g TSS^{-1} on day 70 while it was only 3.17 mg.g TSS^{-1} in R1 on that day. In contrast, the carbonate fraction of the sludge of R1 showed a high iron accumulation, while hardly any iron accumulated in the residual fraction even after L-cysteine was supplied with the influent (Fig. 6). This indicates that the mechanism of iron accumulation in R1 is different compared to R2.

A constant increase in the nickel content, independent of the absence or presence of a sulfur source, occurred in the sludge of both reactors. Nickel mainly accumulated in the organic/sulfide fraction, reaching a maximum on day 70 of 1.50 mg.g TSS^{-1} and 1.57 mg.g TSS^{-1} in the sludge of R1 and R2, respectively. From that day onwards the nickel content of the sludge decreased at a rate of 22 and 18 μg.g $TSS^{-1}.d^{-1}$, for R1 and R2, respectively. Losses where mainly from the organic/sulfide fraction, the exchangeable and carbonate fraction remained almost unchanged.

The zinc fractionation was clearly influenced by the presence or absence of sulfide. In R1, in the absence of sulfide (period I), zinc accumulated in the carbonate (and exchangeable) fraction. This accumulation was absent in the sulfate fed R2. Clearly some copper was also present in the exchangeable and carbonate fractions of R1 (Fig. 7) Around day 50, zinc had accumulated to a significantly higher value in the sludge of R1 (2.46 mg.g TSS^{-1}) compared to the sludge in R2 (1.45 mg.g TSS^{-1}). Until day 36, before L-cysteine was supplied to the influent of R1, 43% of the zinc was present in the carbonate fraction, while it was mainly present in the organic/sulfide fraction of the R2 sludge (68%). After L-cysteine was supplied with the R1 influent, a shift in the zinc fractionation occurred towards the organic/sulfide fraction: 71% of the zinc had accumulated in this fraction at day 50, while it was only 37% at day 36.

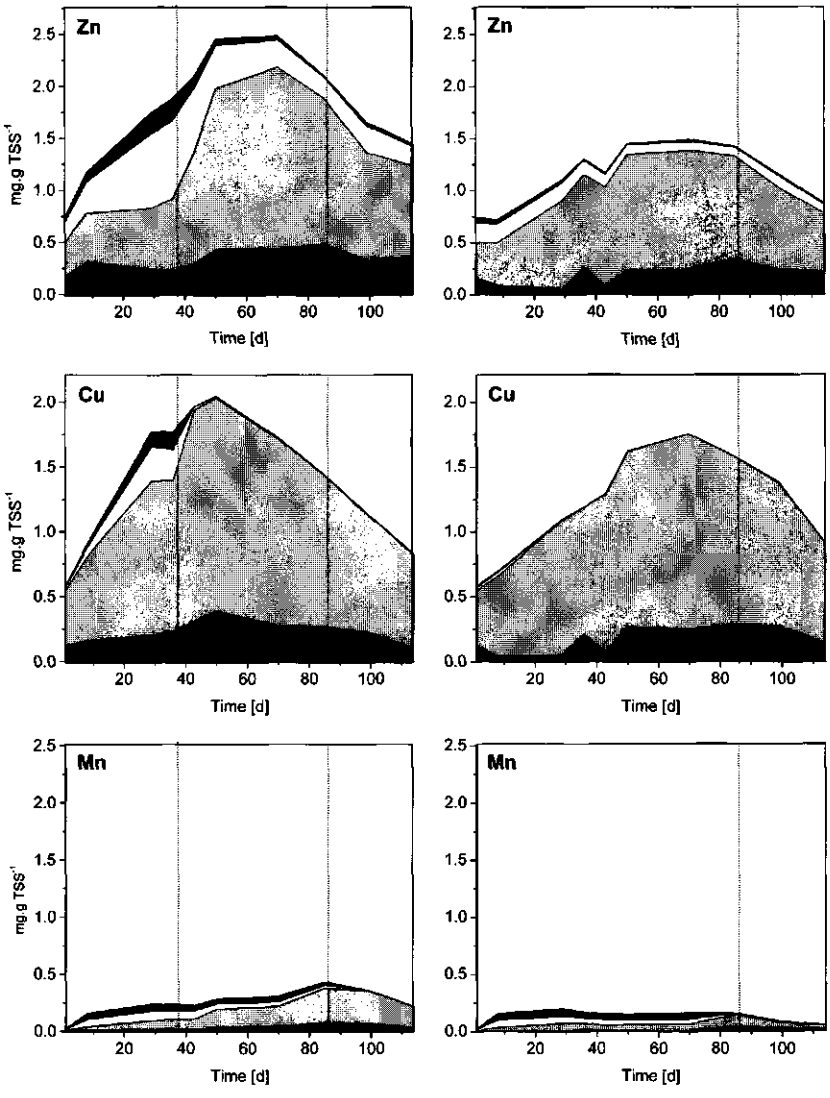

Figure 7 Evolution of the zinc, nickel and manganese concentration in the extracted metal fractions of R1 and R2. Residual fraction (Black), Organic/sulfide fraction (light grey), carbonate (white) and exchangeable (dark grey). For explanation of the periods see Figure 1.

A similar shift was observed for copper in R1, although the preceding accumulation in the carbonate fraction was less pronounced than found for zinc. Copper in general clearly preferred accumulation in the organic/sulfide fraction. Nickel had a similar preference for the organic/sulfide fraction, although it also slightly accumulated in the exchangeable and carbonate fraction.

Manganese did not accumulate significantly, although it accumulated better in the R1 sludge compared to the R2 sludge. Manganese is equally distributed over the exchangeable, carbonate and organic/sulfide fraction. After the supply of L-cysteine to the influent, the accumulation in the organic/sulfide fraction increased slightly, but remained low. At the termination of the experiment, the accumulation in the carbonate and exchangeable fraction of the R2 sludge were neglectable.

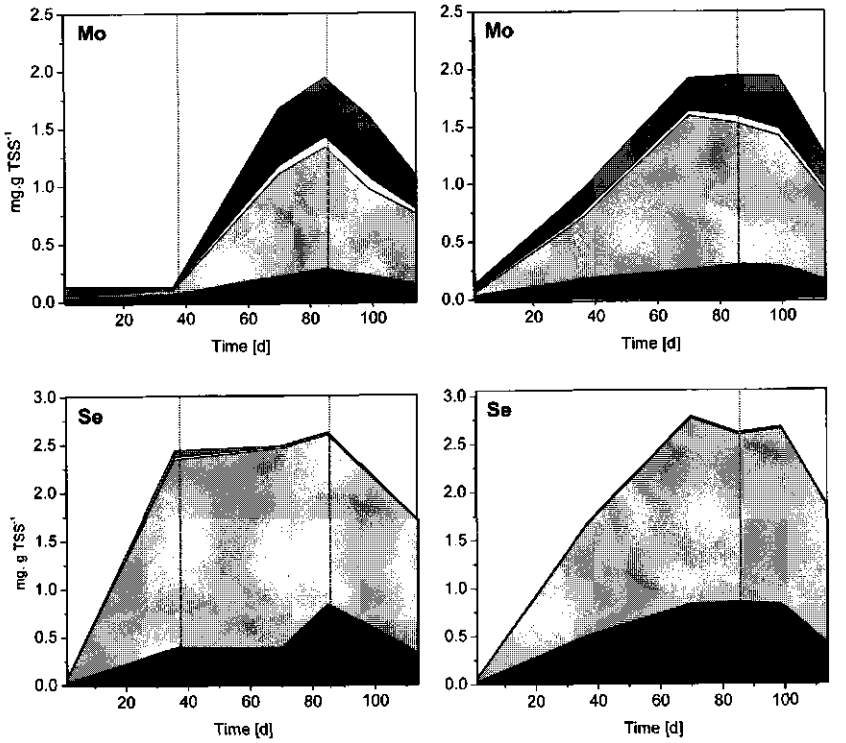

Figure 8 Evolution of the molybdenum and selenium concentration in the extracted metal fractions of R1 and R2. Residual fraction (Black), Organic/sulfide fraction (light grey), carbonate (white) and exchangeable (dark grey). For explanation of the periods see Figure 1

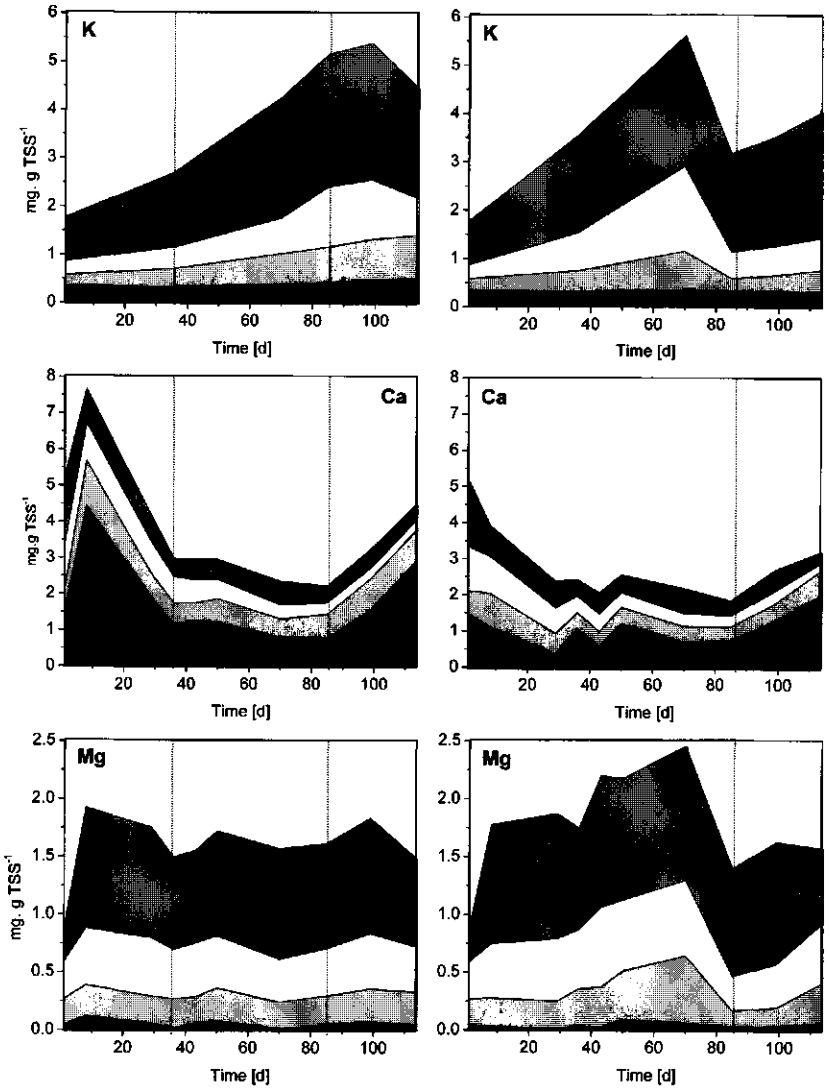

Figure 9 Evolution of the macro element concentration in the extracted metal fractions of R1 and R2. Residual fraction (Black), Organic/sulfide fraction (light grey), carbonate (white) and exchangeable (dark grey). For explanation of the periods see Figure 1.

Molybdenum and selenium, both dosed as oxyanions, were retained in the sludge in a different way (Fig. 8). Accumulation of molybdenum in the sludge of R1 started only following the supply of L-cysteine and the consequent start-up of the biological activity. In contrast, selenium accumulated from the start of operation in the sludge of both reactors and

was mainly present in the organic/sulfide fraction: 67 and 66% for R1 and R2, respectively. The remaining selenium was found in the residual fraction. Molybdenum, although being mainly present in the organic/sulfide fraction as well, was also found in the exchangeable fraction, 26 and 15% on day 85 for R1 and R2, respectively. Molybdenum is the only trace metal added with the influent with a relatively high accumulation in the exchangeable fraction. Once the supply of the S-source to the feed is terminated (day 83), both trace elements are released from the sludge, although with a delay of more than 14 days for the R2 sludge. The release also occurs from the residual fraction.

Macro nutrient retention by the granular sludge during reactor operation

The disturbed performance of R2 (from day 72-76) was accompanied by a drop in the potassium and magnesium content (Fig. 9). Remarkably, the trace metal content of the sludge of R2 remained unaffected. The total potassium content decreased from day 70 to day 85 from 5.6 to 3.2 mg.g TSS^{-1} and the magnesium content from 2.5 to 1.4 mg.g TSS^{-1}.

The calcium content in both the R1 and R2 sludge decreased during the first 36 days of operation. Thereafter, the calcium content more or less stabilized. Calcium was present in all extracted fractions.

DISCUSSION

Effect of sulfur source on UASB reactor operation

This work showed that the absence of a sulfur source severely limited methanol conversion to either methane or acetate in mesophilic methanol fed UASB reactors. Supply of L-cysteine to the influent of R1 immediately improved the methanol conversion to methane (Fig. 1a) and the acetate concentration in the effluent declined drastically. Apparently, an instant nutritional sulfur deficiency existed, despite the high sulfur content of the inoculum sludge (7.9 mg.g TSS^{-1}). This indicates that the endogenous sulfur was present in a non or poorly bioavailable form, e.g. as metal sulfide and/or organically bound sulfur present in the biomass. As the biomass was not adapted to convert methanol, new bacterial growth or enzymatic pathways, which both require sulfur, need to be induced in order to enable the system to convert the methanol [Zandvoort et al., 2002]. According to Scherer and Sahm [1981], *Methanosarcina barkeri* can use iron sulfide (FeS) and zinc sulfide (ZnS) as a sulfur source for growth on methanol at concentrations of 20 and 200 $mg.l^{-1}$, respectively. This suggests that the sulfur availability depends on the dissolution rate, as the solubility of ZnS ($K_{sp}=1.6*10^{-24}$, Sphalerite) [Quan et al., 2003] is lower than that of FeS ($K_{sp}=3.1*10^{-18}$, Mackinawite) [Davison, 1991]. The slow rate of dissolution of these metal sulfides and

relatively high sulfur requirements [Nishio et al., 1992; Sherer et al., 1983] can explain the observed sulfur limitation during start-up of R1.

The evolved sulfide in R2 likely originates from biological sulfate reduction and not from assimilatory reduction. Zhang and Maekawa [1996] showed that when sulfidogenic bacteria present in an acclimated mixed culture of H_2/CO_2 grown methanogens were inhibited by antibiotics, the assimilatory reduction by methanogens or syntrofic organisms produces only a small amount of hydrogen sulfide (70 µmol compared to 2485 µmol in the medium without inhibitor). The evolved sulfide in R1 after cysteine addition can originate from the direct uptake of cysteine by *M. Barkeri* Fusaro (DSM 804), which is capable to metabolize (with the enzyme cysteine desulfhydrase) and use L-cysteine as the sole sulfur source during growth on methanol. The uptake of L-cysteine proceeds fast and is accompanied with the simultaneous evolution of H_2S [Mazumder et al., 1986] (equation 1).

$$Cysteine + H_2O \rightarrow NH_3 + pyruvate + H_2S \qquad (1)$$

In contrast, according to Mountfort and Asher [1979], *Methanosarcina barkeri* strain DM fed with methanol does not grow on sodium sulfate or L-cysteine. Sulfide might also evolve from the use of L-cysteine as a carbon source by fermentative bacteria present in the sludge.

Metabolic route of methanol conversion

At the termination of the experiment the SMA of the sludge on acetate was relatively high (608 and 562 mg CH_4-COD.g $VSS^{-1}.d^{-1}$ for R1 and R2 respectively), contrary to the SMA found in previously conducted reactor experiments with the same inoculum and methanol feeding conditions. The highest value for the SMA on acetate in these experiments amounted to 224 mg CH_4-COD.g $VSS^{-1}.d^{-1}$ [Chapter 5] and in some cases the SMA on acetate was almost lost after prolonged (260 days) operation [Chapter 4]. This indicated that an acetotrophic methanogenic population was absent and consequently that methanol was converted directly to methane. However in the present study, the high SMA on acetate and the absence of a lag phase suggests that methanol may have been converted to methane via the intermediate formation of VFA. The fact that rod and cocci shaped organisms washed out with the effluent of R1, which might be microorganisms unable to directly convert methanol to methane point also in that direction. This warrants further research using specific inhibitors for methanogens (Bromosulfonic acid) and homoacetogens (vancomycin/high CO_2 partial pressure) to determine the methanol degradation pathway [Paulo et al., 2004].

Sludge morphology

The clustered structures observed in the inoculum sludge (Fig 3A) likely originate from the segregated growth of different metabolic types of microorganisms. Gonzalez-Gil et al. [2001] observed similar structures in granular sludge originating from an expanded granular sludge bed reactor (EGSB) treating brewery wastewater. Such clustered growth was postulated to be the optimal cell arrangement for synthrophic associations. Acetate utilizing *Methanosaeta sp.* present in white clusters were spatially separated from the synthrophic species and the hydrogenothrophic methanogens, mainly present outside these clusters. Rocheleau *et al.* [1999], observed that granules growing on methanol develop a layered structure; bacteria other than *Methanosarcina* and *Methanosaeta* occupied the outer layer of the granules. *Methanosarcina barkeri* was present in the underlying layer, followed by a layer of *Methanosaeta concilii* and an inner core composed of *M. concilii* and other bacteria. According to Rocheleau *et al.* [1999] the mechanism underlying this phenomenon is the out-competition of *Methanosarcina* by homoacetogens because conditions for this out-competition are favourable on the periphery of the granule, viz. high methanol and cobalt concentrations and ready available inorganic carbon [Florencio et al., 1994]. Similar conditions prevailed in the reactor in this research.

The distinct black layer, which had developed around the granule after 70 days of operation in reactor R2 (Fig. 3C) and upon the L-cysteine supply to R1 may suggest that sulfate reduction predominantly occurs on the periphery of the granule, a phenomenon also observed in thermophilic methanol fed reactors fed with sulfate (COD/SO_4^{2-} 10 and 5) [Vallero et al., 2003]. The dominant presence of sulfate reducers in the outer layers was also observed in mesophilic granules cultivated on sucrose [Sekiguchi et al.,1999] and in mesophilic granules cultivated on VFA [Santegoeds et al., 1999]. It is clear that the distribution of the microorganisms over the granule, especially of sulfate reducers, will strongly affect the location of metal precipitation

The occurrence of the sudden formation of a flocculent sludge blanket above the granular sludge bed, which is likely related to the partial disintegration of the granules, together with the accompanying deterioration of the reactor performance on day 70, obviously is a matter of crucial importance with respect to the stable operation of granular sludge bed reactor systems. Gonzalez [personal communications] previously also observed a sudden overnight disintegration of the sludge granules in a methanol fed expanded granular sludge bed (EGSB) reactor. Schmidt and Ahring [1993] observed a similar phenomenon for acetate-degrading *Methanosarcina* dominated thermophilic granular sludge, where the *Methanosarcina* clusters in the granules disaggregated as a result of a high Mg^{2+} (100 mM) concentration and 20% of the biomass washed out of the reactor in the form of single cells. The sudden release of flocculent biomass might be related to the complex 'life cycle' of certain *Methanosarcia* species. It is known that these organisms form cell aggregates, and that

the disintegration of these aggregates is governed by the enzyme disaggregatase, which is present in and active in certain *Methanosarcina* strains, while other strains do not disaggregate [Conway de Marco *et al.*, 1993]. Anyhow, when disaggregation occurs, it proceeds fast, i.e. is completed within 48h. According to Xun et al. [1988] and Boone et al. [1987] the morphology (single coccoid cells or aggregated growth) of *Methanosarcina mazei* is influenced by factors such as e.g. pH, divalent cation and substrate concentration. Therefore, a similar abrupt change in the structure of the sludge by such a mechanism might have occurred in this reactor as well. More research e.g. microscopic techniques in combination with fluorescent in situ hybridization (FISH) is required in order to improve the insight in the dynamics of the biomass development.

The R2 removal efficiency recovered within 2 days after the sudden drop in COD removal efficiency, while during start-up approximately 6 days were needed to achieve an almost complete methanol removal and methanogenesis occurred (Fig. 1B and 1C). The recovery of the methanol removal efficiency could originate from new growth during the 2 days of system recovery or from the increase or recovery of the activity of the remaining granular sludge. Assuming a yield of 4.23 g cell.mol methanol^{-1} [Nishio et al., 1992] an overestimated (as not all methanol is converted) 0.66 g cells could grow from the methanol provided to the system during these 2 days. In order to remove the methanol present in the effluent on day 76, this new growth should have an SMA as high as 5750 mg CH_4-COD. g cells^{-1}.d^{-1}. This seems rather high at first sight but flocculent sludge grown in a methanol fed cobalt deprived UASB was found to have a SMA of 4937 mg CH_4-COD. g VSS.d^{-1} (unpublished data). Applying similar reasoning to the start-up of the reactor, which occurs within 6 days, 1.98 g cells would grow when all methanol was converted during these 6 days, with an SMA of approximately 1900 mg CH_4-COD.g cells^{-1}.d^{-1}. This is an activity that is in the range of SMA's observed in granular sludge (Fig. 5). This merely indicates that the granular biomass serves as support material for new growth of *Methanosarcina* and that a relatively small amount of VSS in the granular biomass is active in the methanol conversion.

Metal retention

Despite the different operational conditions in R1 and R2 with respect to the presence and the type of sulfur source, no clear differences in the cobalt retention of the sludge were observed. The leaching of cobalt from the sludge occurred at more or less similar rates and from the same fractions for both reactors even when sulfur and metal were absent in the influent. This indicates that the leaching of cobalt is not related to the sulfur chemistry or a sulfur source. The cobalt leaching proceeds in two phases: a fast washout in which the exchangeable and carbonate fraction is depleted followed by a slower depletion from the

organic/sulfide fraction (Table 3), i.e. at similar rates as observed previously with cobalt loaded sludge [Chapter 5]

Van Hullebusch et al., [2004] investigated the sorption of cobalt and nickel on the different extractable fractions of the same sludge used in the present research. They found that both nickel and cobalt had the highest affinity for sorption in the organic/sulfide fraction similar to what was observed in the present study (Fig. 6), i.e. a maximum sorption (Q_{max}) in the organic/sulfide fraction of 3.08 and 1.50 mg.g TSS^{-1} for cobalt and nickel, respectively. In the presence of other metals both the sorption capacity and affinity decrease, e.g. for nickel in the presence of an equimolar cobalt concentration the Q_{max} in the organic sulfide fraction was reduced to only 0.90 mg.g TSS^{-1} [van Hullebusch et al., 2005]. On day 70 of the experiment, the nickel content in the organic sulfide/fraction approached the theoretical maximum sorption of the inoculum and it even exceeded this observed maximum in the presence of cobalt. This is probably the reason for the observed plateau in nickel sorption from this day onwards. The fact that the theoretical maximum in the absence of competing metals is reached indicates that the sorption capacity of the organic/sulfide fraction increased during reactor operation, either due to H_2S formation, but also as a result of growth of new biomass and associated extracellular polymers formation.

Quan *et al.* [2003] found that metal ions present in the influent of an anaerobic bioreactor inoculated with a mix of granular sludge and cow manure in the presence of sulfide mainly precipitated as metal sulfides. The sequence of precipitation could be related to the solubility products (K_{sp}) of the metals. The metal with the lowest K_{sp}, viz. Cu ($K_{sp}=6.3*10^{-36}$), precipitated first, followed by Zn, Fe and Mn with a Ksp of $1.6*10^{-24}$, $3.1*10^{-18}$ and $2.5*10^{-10}$, respectively. The K_{sp} of amorphous cobalt is $5.1*10^{-22}$ [Martell and Smith, 1981], indicating that cobalt would precipitate following the precipitation of zinc. However, during the first 38 days of operation of R1, all metals except molybdenum were retained in a similar way as in reactor R2 (Fig. 6,7 and 8), despite the fact that no sulfur source was supplied to the influent of R1. This indicates that these metals are either sorbed to existing (sulfide) precipitates [Morse and Luther, 1999] or onto the biomass and/or extracellular polymeric substances (EPS). Considering that metals hardly accumulated in the carbonate fraction (except zinc) and that the amount and/or availability of sulfur present in the granules of R1 is likely to be insufficient for precipitation with the metals in the influent, the metals that accumulated in the sludge of R1 during the first 38 days of operation likely are bound to the biomass and EPS present. Copper, for instance, is known to form very strong complexes with organic matter [Morse and Luther, 1999]. On the other hand the blackening of the sludge, which manifested already on day 7 of operation in R2, clearly points to a precipitation of – at least part – of the metals as sulfides. Application of a different sequential extraction scheme in which these fractions are separated, such as e.g. the Stover scheme [Stover et al, 1976], might allow to distinguish between sorption on organic matter and precipitation as metal sulfides.

The complete disappearance of copper and decrease of the zinc content in the exchangeable and carbonate fraction following the L-cysteine addition with the influent of R1 (Fig. 7) coincides with an increase of the zinc and copper content in the organic/sulfide fraction. This suggests that the copper and zinc originally bound in the carbonate fraction were transformed to sulfides. If zinc and copper indeed were present as carbonates, which have distinctly higher solubility products of $7.4*10^{-14}$ and $1.4*10^{-11}$ for copper and zinc respectively [Quan *et al.*, 2003], such a transformation reaction indeed can be expected. However, this mechanism cannot explain the higher zinc content found in sludge of R1 compared that of the R2 sludge.

Molybdenum, added to the influent as an oxyanion, first has to be reduced before it can react with sulfide to form precipitates. Tucker et al. (1997) showed that molybdenum reduction by *D. desulfuricans* in the presence of sulfide resulted in the extracellular precipitation of the mineral molybdenite (MoS_2). This could explain the remarkable difference in molybdenum retention in the sludge of R1 and R2 during period I, because sulfide was not available to precipitate the molybdenum in R1 (Fig.8).

Surprisingly, accumulation of iron in the sludge of R1 only started after L-cysteine was added to the influent, while the favored fraction for iron accumulation was the carbonate fraction instead of the residual fraction as was found in the sludge of R2. This discrepancy is even more surprising as metals such as nickel, zinc and copper accumulated mainly in the organic/sulfide fraction in the absence of a sulfur source and biological activity. The difference in the preferred fraction may be attributed to the fact that iron is introduced to the reactor as a complex with the ligand L-cysteine, although this also holds for the other metals in the influent, such as nickel, copper and zinc. Dodd et al. [2000] observed a significant release of iron from the carbonate fraction in anaerobic canal bed mud using the same extraction protocol as was used in this research. They attributed this release to the abundance of vivianite ($Fe_3PO_4.8H_2O$) and not to the release from iron carbonates. Although vivianite may be formed in the anaerobic granular sludge bed, it cannot be excluded that iron carbonates were formed as well, as the influent contains a relatively high bicarbonate concentration (2.52 mM) compared to natural waters. Nevertheless, the possible carbonate and vivianite deposition cannot explain the onset of iron accumulation in the carbonate fraction upon the L-cysteine dosing to the influent. Therefore, further research is required to gain more insight in the mechanism of iron retention in the granular sludge. To enable distinction of different iron forms, X-ray adsorption spectroscopy (XAS) needs to be applied on the sludge.

CONCLUSIONS

- The absence of a sulfur source in the influent resulted in an instant nutritional sulfur deficiency. Supplementation of a sulfur source with the influent is therefore an essential requirement for growth of methanol converting biomass in granular sludge previously not exposed to methanol.
- Cobalt leaching was not clearly influenced by the different operational conditions with respect to the presence or the type of sulfur source (sulfate or L-cysteine).
- Cobalt leaches from the granular sludge in two-phases: a fast washout in which the exchangeable and carbonate fraction are depleted, followed by a slower depletion from the organic/sulfide fraction.
- L-cysteine addition to the influent of R1 caused a translocation of zinc and to a lesser extent copper bound in the carbonate fraction towards the organic/sulfide fraction of the sludge.
- The type of sulfur source also influenced the preferred fraction of iron accumulation, viz. the carbonate fraction in the presence of L-cysteine and the residual in the presence of sulfate.
- The onset of iron and molybdenum accumulation depended on the presence of a sulfur source.

REFERENCES

APHA (1985). Standard methods for the examination of water and wastewater. American Public Health Administration, Washington, D.C., USA.

Boone DR, Mah RA (1987). Effect of Calcium, Magnesium, pH, and extent of growth on the Moprphology *of Methanosarcina mazei* S-6. *Appl Environ Microbiol*, **53**, 1699-1700

Conway de Marco E, Macario AJL, Mok T, Beveridge TJ (1993). Immunochemistry and localization of the enzyme disaggregatase in *Methanosarcina mazei. J Bacteriol*, **175**, 3115-3150

Daniels L, Belay N, Rajagopal BS (1986). Assimilatory reduction of sulfate by methanogenic bacteria. *Appl Environ Microbiol*, **51**, 703-709

Davison W (1991). The solubility of iron sulphides in synthethic and natural waters at ambient temperatures. *Aquatic Sci*, **53**, 309-329

Dodd J, Large, DJ, Fortey NJ, Milodowski AE, Kemp SA (2000). petrographic investigation of two sequential extraction techniques applied to anaerobic canal bed mud. *Env Geograph and Health*, **22**, 281-296

Florencio L, Jenicek P, Field JA, Lettinga G (1993). Effect of cobalt on the anaerobic degradation of methanol. *J Fermen Bioeng*, **75**, 368-374

Gonzalez-Gil G, Kleerebezem R, van Aelst A, Zoutberg GR, Versprille AI, Lettinga G (1999). Toxicity effects from formaldehyde on methanol degrading on methanol degrading sludge and its anaerobic conversion in biobed expanded granular sludge (EGSB) reactors. *Wat Sci Technol* , **40**, 195-202

Gonzalez-Gil G, Lens PNL, van Aelst A, van As H, Versprille AI, Lettinga G (2001) Cluster structure of anaerobic aggregates of an expanded granular sludge bed reactor. *Appl Environ Microbiol*, **67**, 3683-3692

Gonzalez–Gil G, Jansen S, Zandvoort MH, van Leeuwen HP (2003). Effect of yeast extract on speciation and bioavailability in nickel and cobalt in anaerobic bioreactors. *Biotech Bioeng*, **82**, 134-142

Hullebusch van ED, Zandvoort MH, Lens PNL (2004). Nickel and cobalt sorption on anaerobic granular sludges: Kinetic and equilibrium studies *J Chem Technol Biotechnol*, **79**, 1219-1227

Hullebusch van ED, Peerbolte A, Zandvoort MH, Lens PNL (2005). Sorption of cobalt and nickel on anaerobic granular sludges: isotherms and sequential extraction. *Chemosphere*, **58**, 493-505

Jong T, Perry L (2003). Removal of sulfate and heavy metals by sulfate reducing bacteria in short-term bench scale upflow anaerobic packed bed reactors. *Wat Res*, **37**, 3379-3389

Kaksonen AH, Riekkola-Vanhanen ML, Puhakka JA (2003). Optimization of metal sulfide precipitation in fluidized-bed treatment of acidic wastewater. *Wat Res*, **37**, 255-266

Kelly CR, Schwitzenbaum M (1984). Anaerobic treatment and nutrient effects. *Agric Wastes*, **10**, 135-154

Martell AE, Smith RM, (1981). *Critical stability consants, vol. 4. inocrganic complexes*. Plenum press, New York

Mazumder TK, Nishio N, Fukazaki S, Nagai S (1986). Effect of sulfur-containing compounds on the growth of *Methanosarcina barkeri* in defined medium. *Apll Environ Microbiol*, **52**, 617-622

Modak DP, Singh KP, Chandra H, Ray PK (1992). Mobile and bound forms of trace metals in sediments of the lower Ganges. *Wat Res*, **26**, 1541-1548

Morse JW, Luther GW (1999). Chemical influences on trace metal-sulfide interactions in anoxic sediment. *Geochim et Cosmochim Acta*, **63**, 3373-3378

Mountfort DO, Asher RA (1979). Effect of inorganic sulfide on the growth and metabolism of *Methanosarcina barker*i strain DM. *Appl Environ Microbiol*, **37**, 670-675

Nishio N, Kakizono T, Silveira RG, Takemoto S, Nagai S (1992). Nutrient control by the gas evolution in methanogenesis of methanol by *Methanosarcina barkeri. J Ferment Bioieng*, **73**, 481-485

Paulo PL, Vallero MVG, Treviño RHM, Lettinga G, Lens PNL (2004). Thermophilic (55°C) conversion of methanol in methanogenic-UASB reactors: influence of sulphate on methanol degradation and competition. *Journal of Biotechnology. In Press.*

Quan ZX, La HJ, Gho YG, Hwang MH, Kim IS, Lee ST (2003). Treatment of metal-contaminated water and vertical distribution of metal precipitates in an upflow anaerobic bioreactor. *Environ Technol*, **24**, 369-376.

Santegoeds CM, Damgaard LR, Hesselink G, Zopfi J, Lens P, Muyzer G, Beer de D, Distribution of sulfate-reducing and methanogenic bacteria in anaerobic aggregates determined by microsensor and molecular analyses. *Appl Environ Microbiol*, **65**,198-4629

Scherer P, Sahm H (1979). Influence of sulfur-containing compounds on the growth of *Methansarcina barkeri* in a defined medium. *Eur J Appl Microbiol Biotechnol*, **12**, 38-35

Scherer P, Wolff G (1983). Composition of the major elements and trace elements of 10 methanogenic bacteria determined by inductive coupled plasma spectrometry. *Biol Trace Element Res*, 5, 149-163.

Sekiguchi Y, Kamagata Y, Nakamura K, Ohashi A, Harada H (1999). Fluoresence in situ hybridization using 16S rRNA-targeted oligonucleotides reveals localization of methanogens and selected uncultured bacteria in mesophilic and thermophilic sludge granules *Appl Environ Microbiol*, **65**, 1280-1288

Schmidt JE, Ahring BK (1993). Effects of magnesium on thermophilic acetate-degrading granules in upiflow anaerobic sludge blanket (UASB) reactors. *Enz Microb Technol*, **15**, 304-310

Speece RE, Parkin GF, Gallagher D (1983). Nickel stimulation of anaerobic digestion. *Wat Res*, **17**, 677-683

Stover RC, Sommers LE, Silveira DE (1976). Evaluation of metals in wastewater sludge. *J Water Pollut Con Fed*, **48**, 2165-2175

Tessier A, Campbell PGC, Bisson M (1979). Sequential extraction procedure for the speciation of particulate trace metals. *Anal Chem*, **51**, 844-851

Trüper HG, Schlegel HG (1964). Sulfur metabolism in *Thiordaceae* I. Qauntative measurment on growing cells of *Chromatium okenii*. *J Microbiol Ser*, **30**, 225-238

Tucker MD, Barton LL, Thomson BM (1997). Reduction and immobilization of molybdenum by *Desulfovibrio desulfuricans*. *J Env Qual*, **26**, 1146-1152

Vallero MVG, Treviño RHM, Paulo PL, Lettinga G, Lens PNL (2003). Effect of sulfate on methanol degradation in thermophilic (55°C) methanogenic UASB reactors. *Enz Microbial Technol*, **32**, 676-687

Veeken A, Removal of Heavy Metals from Biowaste (1998). PhD thesis, Department of Environmental Technology, Wageningen University, The Netherlands

Weijma J, Stams AJM, Hulshoff Pol LW, Lettinga G (2000). Thermophilic sulfate reduction and methanogenesis with methanol in a high-rate anaerobic reactor. *Biotechnol Bioeng*, **67**: 354-363.

Xun L, Boone DR, Mah RA (1988). Control of the life cycle of *Methanosarcina mazei* S-6 by manipulation of growth conditions. *Appl Environ Microbiol*, **54**, 2064-2068

Zandvoort MH, Geerts R, Lettinga G, Lens PNL (2002). Effect of long-term cobalt deprivation on methanol degradation in a methanogenic granular sludge reactor. *Biotechnol Progr*, **18**, 1233-1239

Zhang ZY, Maekawa T (1996). Effects of sulfur-containing compounds on the growth and the methane production of acclimated-mixed methanogens. *Biomass Bioenergy*, **10**, 45-56

Chapter 7

Influence of pH shocks on trace metal dynamics and performance of methanol fed granular sludge bioreactors

The influence of pH shocks on the trace metal dynamics and performance of methanol fed upflow anaerobic granular sludge bed (UASB) reactors was investigated. For this purpose, two UASB reactors were operated with metal pre-loaded granular sludge (1 mM Co, Ni and Fe; 30 °C; 96 h) at an organic loading rate (OLR) of 5 g COD.l reactor^{-1}.d^{-1}. One UASB reactor (R1) was inoculated with sludge that originated from a full scale reactor treating alcohol distillery wastewater while the other reactor (R2) was inoculated with sludge from a full scale reactor treating paper mill wastewater. A 30 h pH shock (pH 5) strongly affected the metal retention dynamics within the granular sludge bed in both reactors. Iron losses in soluble form with the effluent were considerable: 2.3 and 2.9% for R1 and R2, respectively, based on initial iron content in the reactors, while losses of cobalt and nickel in soluble form were limited. Sequential extraction of the metals from the sludge showed that cobalt, nickel, iron and sulfur were translocated from the residual to the organic/sulfide fraction during the pH shock in R2, increasing 34, 47, 109 and 41% in the organic/sulfide fraction respectively. Such a translocation was not observed for the R1 sludge during the first 30h pH shock, but a second 4 day pH shock induced significant losses of cobalt (18%), iron (29%) and sulfur (29%) from the organic/sulfide fraction, likely due iron sulfide dissolution and concomitant release of cobalt. After the 30h pH shock VFA accumulated in the R2 effluent, whereas both VFA and methanol accumulated in R1 after the 4 day pH. The formed VFA, mainly acetate, were not converted to methane due to the loss of the specific methanogenic activity of the sludge on this substrate. Zinc, copper and manganese supply did not have a clear effect on the acetate formation and methanol conversion, but zinc may have induced the onset of methanol degradation after day 152 in R1.

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INTRODUCTION

In full scale anaerobic wastewater treatment, trace elements are generally supplied with the influent to ensure good bioreactor performance. These metals accumulate partly in the anaerobic granular sludge bed, thus yielding a stock of trace metals, which sustains the trace metal requirements of the microbial population that develops in the bioreactor. The metals are distributed over the different phases of the granular matrix: biomass, extracellular polymeric substances (EPS) and inorganic precipitates, e.g. sulfides, phosphates and carbonates [van Hullebusch et al., 2003].

Little is known about the dynamics, i.e. storage vs. leaching, of various trace metals in anaerobic granular sludge and the effect of operational conditions and process disturbances on these dynamics. Such knowledge is essential to gain better understanding of metal retention and bioavailability, thus allowing more effective supplementation of trace elements to anaerobic bioreactors. Already small differences in reactor conditions can influence the retention and speciation of trace metals. For instance, the presence and/or type of sulfur source, even at concentrations as low as 13 $mg.l^{-1}$, already influence the metal retention and speciation [Chapter 6]. Also the pH can strongly influence the metal retention, as metal solubility increases at low pH values, independent of the mineralogical composition [Alloway, 1990]. Operational disturbances can result in severe pH drops of the reactor mixed liquor due to volatile fatty acid (VFA) accumulation when treating weakly buffered wastewaters [Lettinga et al., 1979]. Moreover, transient pH gradients within a single aggregate might occur as well [Lens et al., 1993]. These pH variations can therefore influence trace metal speciation in the granular matrix, resulting in e.g. translocation of metals in the sludge due to dissolution metal of precipitates, which can lead metal to losses with the effluent.

In this paper, methanol fed upflow anaerobic sludge bed (UASB) reactors inoculated with sludge pre-loaded with the methanogenic key-metals cobalt, nickel and iron were used to investigate the influence of short term (30 h and 4 days) pH drops from pH 7 to 5 on the metal retention dynamics and reactor performance. A pH of 5 was chosen as metals are significantly mobilised at that pH without inducing irreversible toxic effects on the sludge. The metal retention was assessed by monitoring the metal content of the effluent and sludge. The distribution of the metals over different operationally defined fractions of the sludges was also monitored as a function of time using a sequential extraction procedure. The metabolic properties of the sludge that developed in the UASB reactors were monitored as well by batch activity tests.

MATERIAL AND METHODS

UASB reactor operation

Two UASB reactors (working volume 0.75 l; inner diameter 5 cm) were operated at a hydraulic retention time (HRT) of 8 h and at a superficial upflow velocity of 1 $m.h^{-1}$ in a temperature-controlled (30 ± 2°C) room as described in [Chapter 4]. A pH controller maintained the pH at 7.0 (± 0.2) by the addition NaOH in the effluent recycle tube. 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 produced methane volume was measured by water displacement from mariot-flasks using an on-line balance system as described by Gonzalez et al. [1999]. Due to a problem with the data collection system, no gas production data are available from day 41 until day 49 and from day 163 onwards.

Basal medium

The reactors were fed a synthetic wastewater containing methanol and macro nutrients, as described previously [Chapter 3], without addition of HCO_3^{-1}. The influent contained 0.41 mM sulfate (13 mg $S.l^{-1}$) as sulfur source and no trace elements. Individual metals were added to the influent in pulse or continuous mode to one or both reactors from day 70 onwards, the conditions and time of dosage are presented in Table 1. To avoid precipitation in the storage vessels, the influent consisted of three different streams; viz. basal medium without K_2HPO_4; methanol with K_2HPO_4 and dilution water. The medium was prepared with demineralised water.

Source of biomass

The anaerobic granular sludge used to inoculate R1 originated from a full scale UASB reactor treating alcohol distillery wastewater (Nedalco, Bergen op Zoom, the Netherlands) and the sludge from R2 originated from a full scale UASB reactor treating paper mill wastewater (Industriewater Eerbeek B.V., Eerbeek, the Netherlands). The sludge was elutriated to remove the fines. The total suspended solids (TSS) and volatile suspended solids (VSS) content were, respectively, 8.3 (±0.2) % and 7.6 (±0.2) % for the R1 sludge and 22.6 (±0.2) % and 16.7 (±0.2) % for the R2 sludge. The sludge concentration after inoculation was 14 and 27 g $VSS.l^{-1}$ in R1 and R2, respectively. This resulted in sludge beds of similar heights, which allowed frequent sludge sampling for metal analyses.

Table 1. Times and conditions of metal additions to the UASB reactors.

Time		Metal	Amount	Mode	Reactor
Day	Code[1]		μmole.d^{-1}		
70	A	Cu	11.25	Pulse[2]	R2
73	B	Zn	11.25	Pulse	R2
75	C	Cu, Zn, Ni, Mn	11.25	Pulse	R1 and R2
84	D	Zn	11.25	Pulse	R1 and R2
96	E	Ni, Mn	22.50	Pulse	R1 and R2
102	F	Zn	33.75	Pulse	R2
123	G	Zn	33.75	Pulse	R1 and R2
130-151	H	Zn	1.12	Cont.[3]	R1 and R2
152-162	I	Zn	11.25	Cont.	R1 and R2
163-end	J	Cu, Zn, Ni, Mn, W, Mo, Se	11.25	Cont	R1 and R2

[1] Code used in the figures to express time of metal dosage; [2] pulses were dosed in a 1.25 h periods; [3] Continuous supply to the influent.

In order to study the metal retention dynamics in the two different sludges, the sludges of both reactors were pre-loaded with cobalt ($CoCl_2.6H_2O$), nickel ($NiCl_2.6H_2O$) and iron ($FeCl_2.2H_2O$) by contacting 400 g of sludge (wet weight) with 1600 ml of a solution containing 1 mM of each of these metals for 96 h at 30 °C in a 2 liter serum bottle in the absence of substrate. The characteristics and the metal content of the initial sludges before loading are presented in Table 2. The cobalt, nickel and iron content of the sludges after loading is presented in Table 5.

Experimental design

Four operational periods were defined and they are described in Table 3. The organic loading rate (OLR) of both reactors was maintained at 5 g COD.l reactor^{-1}.d^{-1} during the entire experiment, corresponding to a sludge loading rate (SLR) of 0.27 and 0.14 g COD.g VSS^{-1}.d^{-1}, for R1 and R2, respectively, based on the initial amount of VSS added to the reactors at the start of the experiment.

Table 2. Initial characteristics and total metal content of the two anaerobic granular sludges.

Parameters	Nedalco sludge	Eerbeek sludge
Total suspended solids (TSS)	8.3 (±0.2) %	22.6 (±0.2) %
Volatile suspended solids (VSS)	7.6 (±0.2) %	16.7 (±0.2) %
Carbonates (% of TSS)	0.8 (±0.2) %	0.4 (±0.2) %
Total sulfur	22.7 (±0.1) $mg.g^{-1}$ TSS	41.8 (±1.0) $mg.g^{-1}$ TSS
Total phosphorus	3.3 (±0.1) $mg.g^{-1}$ TSS	6.6 (±0.1) $mg.g^{-1}$ TSS
Total metal content		
Cobalt	18.7 (±0.6) $\mu g.g^{-1}$ TSS	59.4 (±2.0) $\mu g.g^{-1}$ TSS
Nickel	130.3 (±3.6) $\mu g.g^{-1}$ TSS	38.3 (±0.9) $\mu g.g^{-1}$ TSS
Copper	690 (±10) $\mu g.g^{-1}$ TSS	124.6 (±5.6) $\mu g.g^{-1}$ TSS
Zinc	760 (±20) $\mu g.g^{-1}$ TSS	195.7 (±7.0) $\mu g.g^{-1}$ TSS
Manganese	54.8 (±0.6) $\mu g.g^{-1}$ TSS	275 (±8.0) $\mu g.g^{-1}$ TSS
Iron [1]	20.8 (±0.2) $mg.g^{-1}$ TSS	39.8 (±0.8) $mg.g^{-1}$ TSS

[1] Note that iron content is expressed as $mg.g^{-1}$ TSS

Table 3. Operational periods defined for R1 and R2.

Period	R1 (days)	R2 (days)	Description
I	0-36	0-36	Reactor start-up
II	37-41	37-41	Imposed 30 h pH shock (pH 5); R1 at day 37 and R2 at day 39
II	42-69	42-69	Recovery from 30 h pH shock
IV	70-194	70-178	Supply of trace elements with the influent (see Table 1)
IVa	105-109	-	Imposed 4 days pH shock (pH 5)
IVb	110-194	-	Recovery from 4 days pH shock

Maximum specific methanogenic activity assays

Maximum specific methanogenic activity (SMA) of the sludge was assessed on methanol, acetate and H_2/CO_2 always in duplicate and at a temperature of 30 (± 2)°C using on-line gas production measurements [Chapter 3]. The SMA of the sludge on these substrates was assessed at day 0, 34, 37/39 (R1 and R2 respectively), 45 and at termination of the experiment (day 194/178 for R1 and R2, respectively). The SMA on acetate the R1 sludge was also measured on day 84 in order to asses whether the sludge was still capable to use this substrate. The SMA on H_2/CO_2 was not determined at termination of the experiment.

Metal analyses

The total metal content of the granular sludge was determined according to the method described previously [Chapter 3]. In order to assess the metal speciation in the granular sludge matrix, a four-step sequential extraction procedure was used as described Chapter 6, in which each next extraction step is more stringent. The nomenclature applied for the subsequent extraction steps is as follows, viz. the exchangeable fraction (1M NH_4CH_3COO), the carbonate fraction (1M CH_3COOH), organic/sulfide fraction (30% H_2O_2) and the residual fraction (3:1 HCl/HNO_3).

Chemical analyses

The metal concentrations in the effluent and the metal content of the sludge were determined using Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES; Varian Vista MPX, Palo Alto, USA) as described previously [Chapter 3]. The concentration of methanol and VFA were determined using gas liquid chromatography as described by Weijma et al. [2000]. The TSS and VSS concentrations were determined according to Standard Methods [American Public Health Association, 1985]. All chemicals were of analytical or biological grade and purchased from E. Merck AG (Darmstadt, Germany).

RESULTS

Start up and response to short-term pH shock (30 h)

Reactor performance

Methanol removal started only after day 26 in R1 and day 14 in R2 and a complete conversion of methanol to methane was only attained on day 35 in R1 and day 28 in R2. During the first imposed pH shock at the start of period II, on day 37 and 39 in R1 and R2, respectively, the pH was lowered from 7 to 5 for 30 hours (Fig.1A). This resulted in a fairly

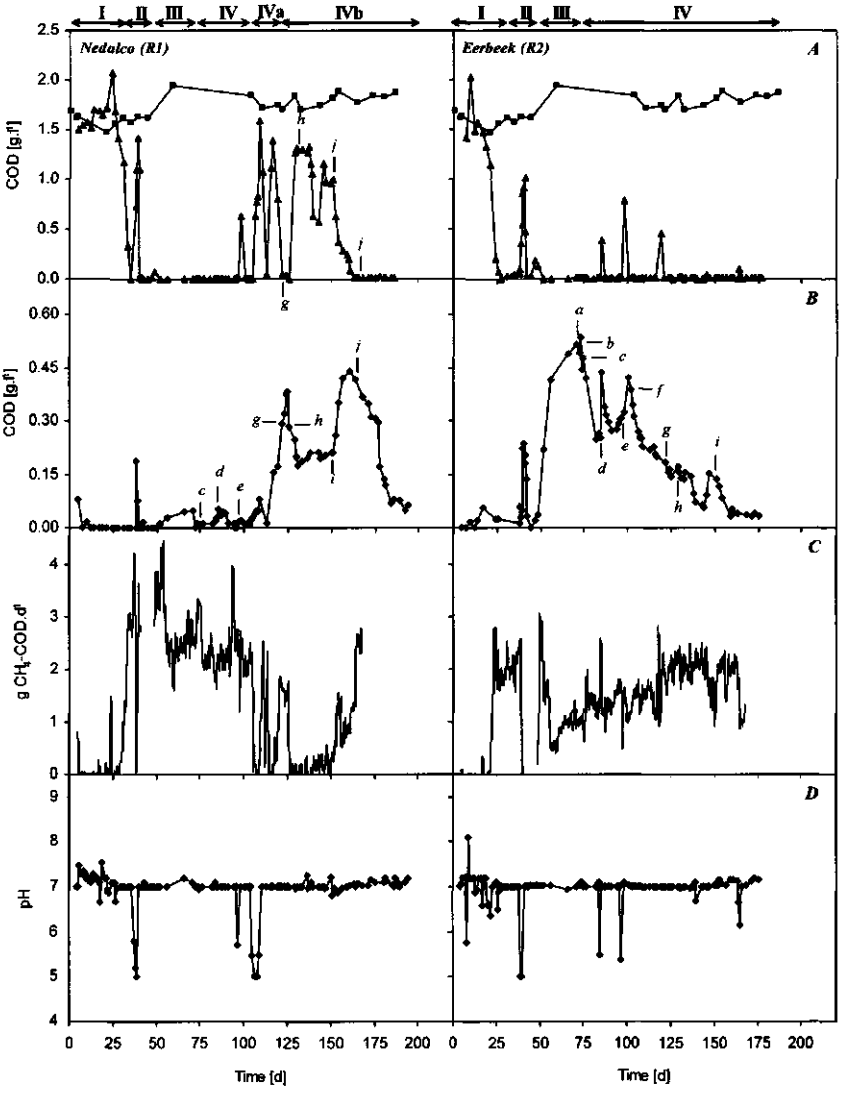


Figure 1 Evolution of the reactor performance of R1and R2 with time. (**A**) Influent (■) and effluent (▲) methanol concentration. (**B**) Effluent VFA (♦) concentration. (**C**) Methane production and (**D**) pH. (a) Copper pulse; (b) Zinc pulse; (c) copper, zinc, manganese and nickel pulse; (d) zinc pulse; (e) nickel and manganese pulse (2x initial dose); (f) zinc pulse (3x initial dose); (g) zinc pulse (3x initial dose); (h) zinc pulse (3x initial dose); (i) start continuous zinc dosing (0.5 μM); start continuous zinc dosing (5 μM); (j) Start continuous dosing all metals except iron (5 μM).

similar response of the reactors, viz. in both reactors the effluent methanol concentration instantly increased to 1418 mg COD.l^{-1} in R1 on day 39 and in R2 to 1009 mg COD.l^{-1} on day 41. At the same time, the VFA concentration in the effluent increased to 186 mg COD.l^{-1} on day 39 in R1 and 224 mg COD.l^{-1} on day 40 in R2 (Fig. 1B). After the pH was restored to 7 (Period III), the performance of both reactors recovered within approximately 3 days and no methanol and VFA were present in the effluent (Fig. 1 A, B).

Although not intentional, R2 was exposed to two other short (less than one day) pH shocks i.e. at day 85 (pH 5.5) and day 97 (pH 5.4). In both cases this resulted in a sharp increase of effluent methanol and VFA concentrations (Fig. 1). The accidental pH drop for less than 24 h on day 97 (pH 5.7) in R1 merely resulted in methanol accumulation in the effluent (Fig. 1 A, D).

Effluent metal concentration

Immediately after the start of the experiments losses, of cobalt, nickel and iron from the sludge occurred as indicated by their effluent concentrations (Fig. 2, period I). The cobalt and nickel effluent concentration decreased steadily to values lower than 1 μg.l^{-1} after 16 days of operation in both reactors. From day 16 onwards, a slight increase in both the cobalt and nickel effluent concentration occurred in both reactors, simultaneous with the onset of methanol removal and biogas formation. Iron was initially present in the effluent at relatively high concentrations (around 0.8 mg.l^{-1} on day 5) in both reactors, but it decreased gradually during period I to 42 μg.l^{-1} and 46 μg.l^{-1} for R1 and R2 on day 34 and 38, respectively.

During the pH shock on day 37 (R1) and day 39 (R2), very discrete peaks in the effluent cobalt, nickel and iron concentrations manifested, with in R1 maximal cobalt, nickel and iron concentrations of respectively, 15 μg.l^{-1}, 13 μg.l^{-1} and 3 mg.l^{-1} and in R2, 57 μg.l^{-1}, 19 μg.l^{-1} and 11 mg.l^{-1}, respectively. However, the losses of cobalt and nickel in soluble form were minimal during the first 30 h pH shock in R1, i.e. at the maximum 37 μg of cobalt and 27 μg nickel were released from the sludge, representing 0.13 and 0.08% of the cobalt and nickel, respectively, based on the total amount of these metals present in the sludge before the shock. In contrast, losses of iron were much higher (Fig. 2) with values of 4.81 and 22.48 mg lost for R1 and R2, respectively, corresponding to 2.3 and 2.9% of the total amount of iron present in the sludge before the pH shock (based on the initial sludge TSS content in the reactor).

Immediately following the pH shock the effluent concentration of these metals in both reactors quickly returned to values found prior to the shock, except for cobalt and nickel in R1, which remained elevated for more than 30 days after the pH shock (Fig. 2).

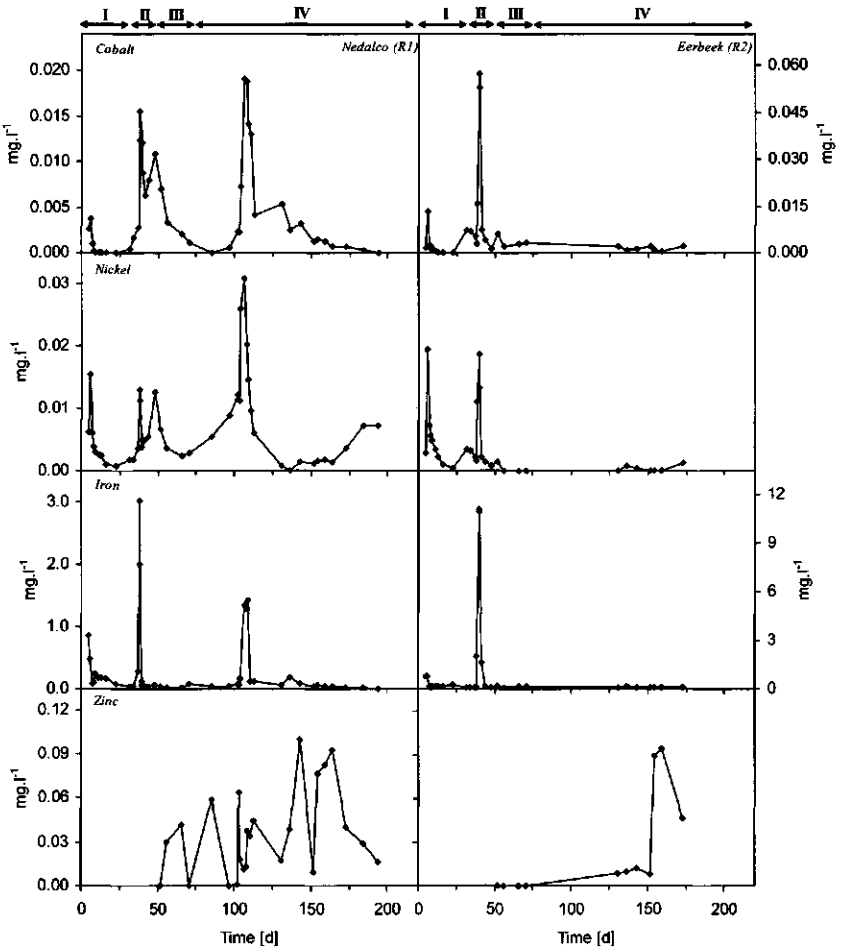


Figure 2 Evolution of the cobalt, nickel iron and zinc concentration in the effluent of R1 and R2.

Metal retention in the sludge

The pH shock did not have a significant effect on the total cobalt, nickel and iron content of the sludge (Fig. 3). During the pH shock (period II), higher amounts of cobalt and nickel were extracted from the exchangeable fraction, especially in case of the R1 sludge (Fig. 4). However, immediately after the pH shock (day 40), the amount extracted from this fraction was again similar to that before the 30h pH shock (Fig. 4).

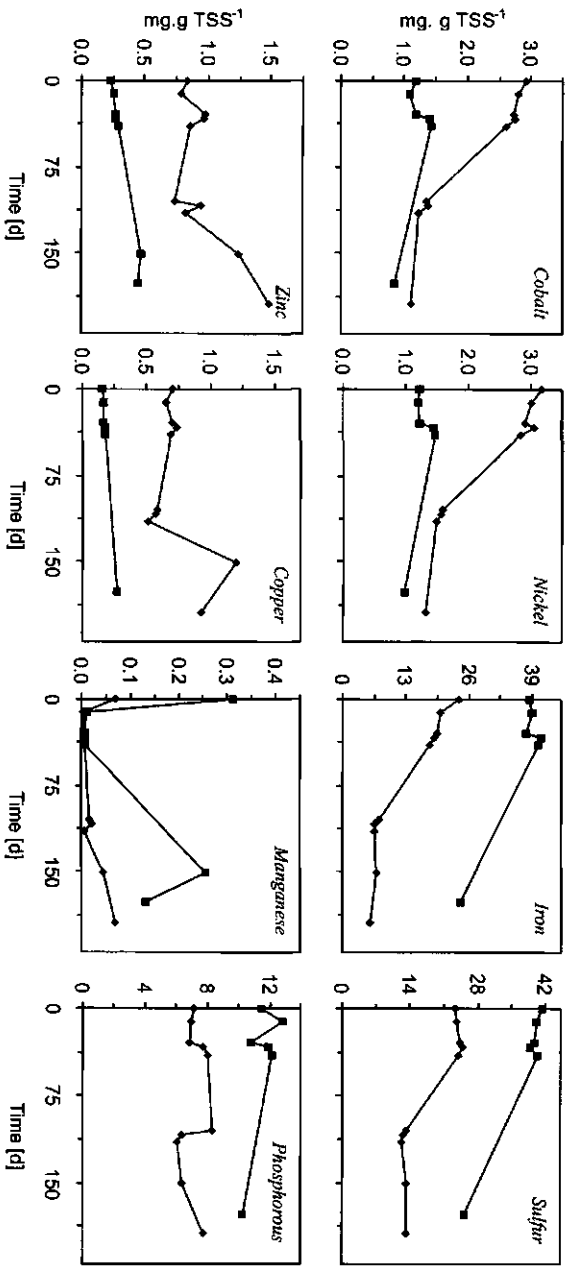


Figure 3 Evolution of the total cobalt, nickel, iron, sulfur, zinc, copper, manganese and phosphorous concentration of the R1 (♦) and R2 (■) sludge.

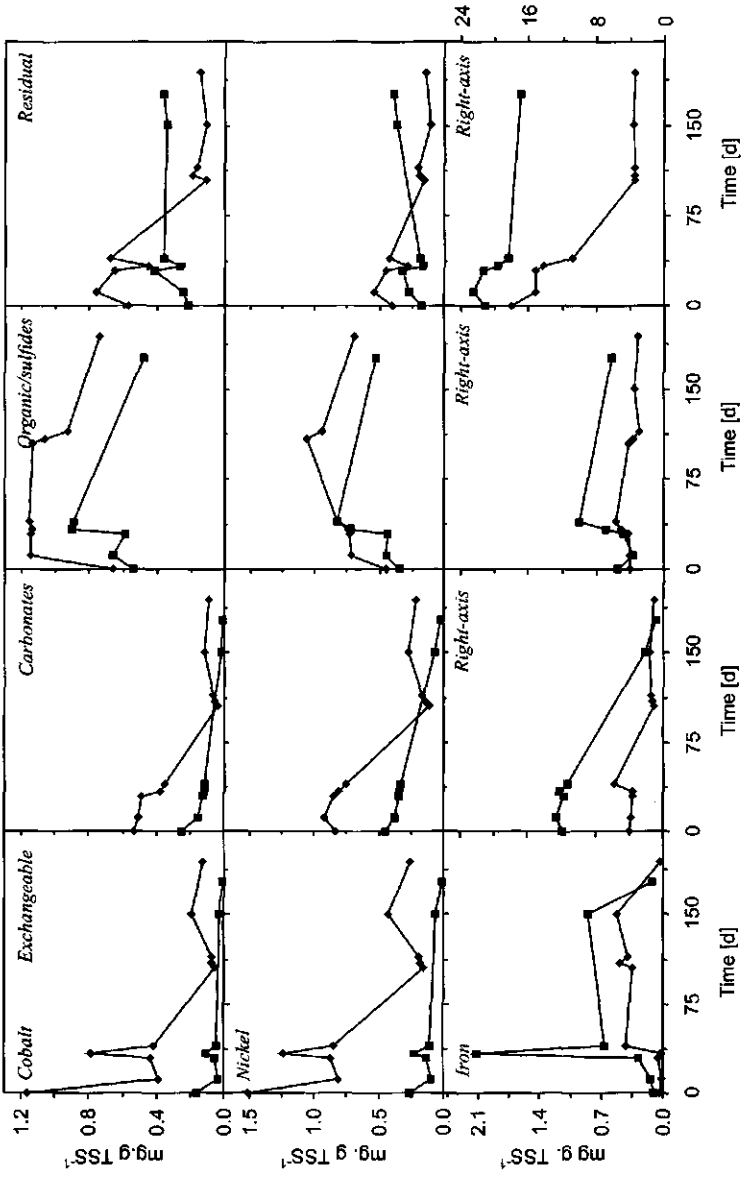


Figure 4 Evolution of the cobalt, nickel and iron content present in the extracted fractions of the R1 (♦) and R2 (■) the sludge.

The iron content in the exchangeable fraction of both sludges increased during the pH shock, particularly in the R2 sludge viz. from 0.27 mg.g TSS^{-1} before to 2.11 mg.g TSS^{-1} during the shock. The iron concentration in this fraction remained at a higher level until day 151 (Fig. 4) in the sludge of both reactors.

During the pH shock, the cobalt, nickel and iron content of the organic/sulfide fraction of the R2 sludge increased with 34, 47 and 109% respectively (Fig. 4). In contrast, the pH shock did not have a significant effect on this fraction of the R1 sludge (Fig. 4). While the iron content of the organic/sulfide fraction of the R2 sludge increased from 4.73 to 9.89 mg.g TSS^{-1} at the same time, the iron content in the residual fraction decreased from 21.22 to 18.38 mg.g TSS^{-1}. The sulfur content in the organic/sulfide fraction of the R2 sludge increased as well during the shock, i.e. from 9.79 to 16.49 mg.g TSS^{-1}, while the sulfur content in the residual fraction decreased from 28.81 to 23.41 mg.g TSS^{-1} (Fig. 5). In the R1 sludge, the iron content of the residual fraction decreased considerably with 4.41 mg.g TSS^{-1} (29%) lower than before the pH shock.

Acidogenesis in R2

From day 42 onwards, VFA started to accumulate in the effluent of R2 and reached a maximum concentration of 528 mg $COD.l^{-1}$ on day 73, mainly as acetate while all remaining methanol was still converted to methane (Fig. 7). In order to asses whether addition of trace metals, other than those which were initially loaded on the sludge, could reduce the acetate formation in R2, pulses of individual metals and combinations of metals were supplied with the influent during period IV from day 70 onwards (Table 1). From day 75 onwards, these metals were also supplied to R1 (Table 1), even though the performance of this reactor became only disturbed by a second pH shock on day 105 (Period IVa).

Following pulse doses a, b and c (Table 1) a sharp decrease in the effluent VFA concentration manifested, apparently stabilizing at a concentration of 250 mg $COD.l^{-1}$. A second pulse dose of zinc (d) coincided with a pH drop, which resulted in a VFA increase in the effluent and confounded a possible zinc dosage effect. The same holds for the next imposed nickel/manganese pulse, which also coincided with a pH drop at day 97 (e). Thereafter two pulse doses of zinc (f, g) were supplied followed by continuous addition of zinc (h, i), which both did not affect the rate of VFA decrease in the effluent (Fig. 7).

Before zinc was supplied with the influent, this element was hardly detectable in the R2 effluent (Fig. 2). The effect of its continuous dosing clearly manifested in the R2 effluent, on average effluent concentrations were 8 $\mu g.l^{-1}$ (0.12 μM) from day 130 to 152 when it was continuously supplied at a relatively low concentration (Table 1) and an average effluent concentration of 76 $\mu g.l^{-1}$ (1.16 μM) was found during the second continuous zinc dosing phase at high concentrations (day 152 to 173).

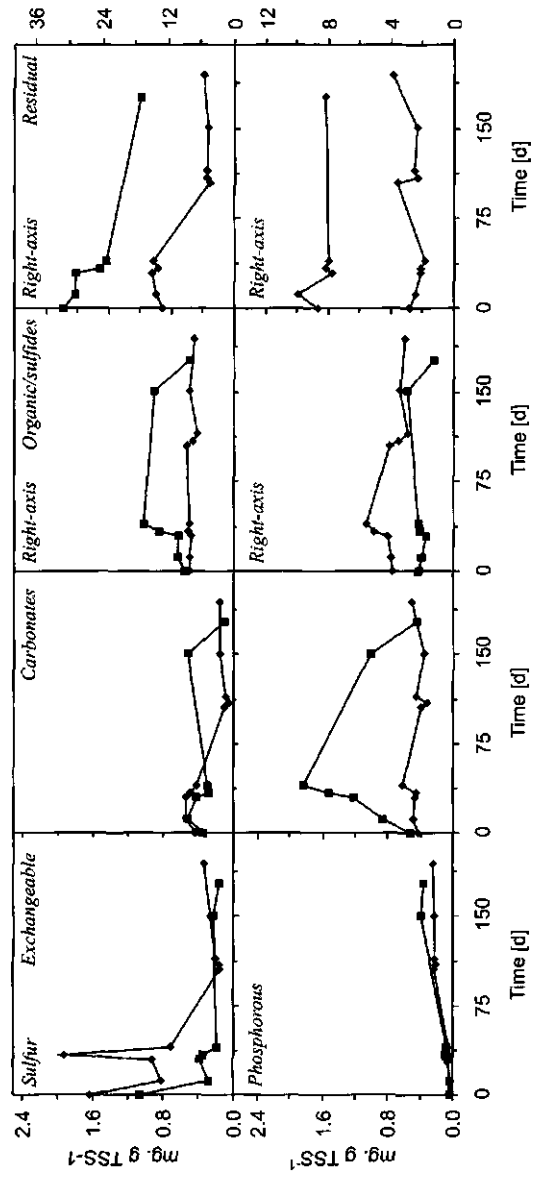


Figure 5 Evolution of the phosphorus and sulfur content present in the extracted fractions of the R1 (♦) and R2 (■) sludge.

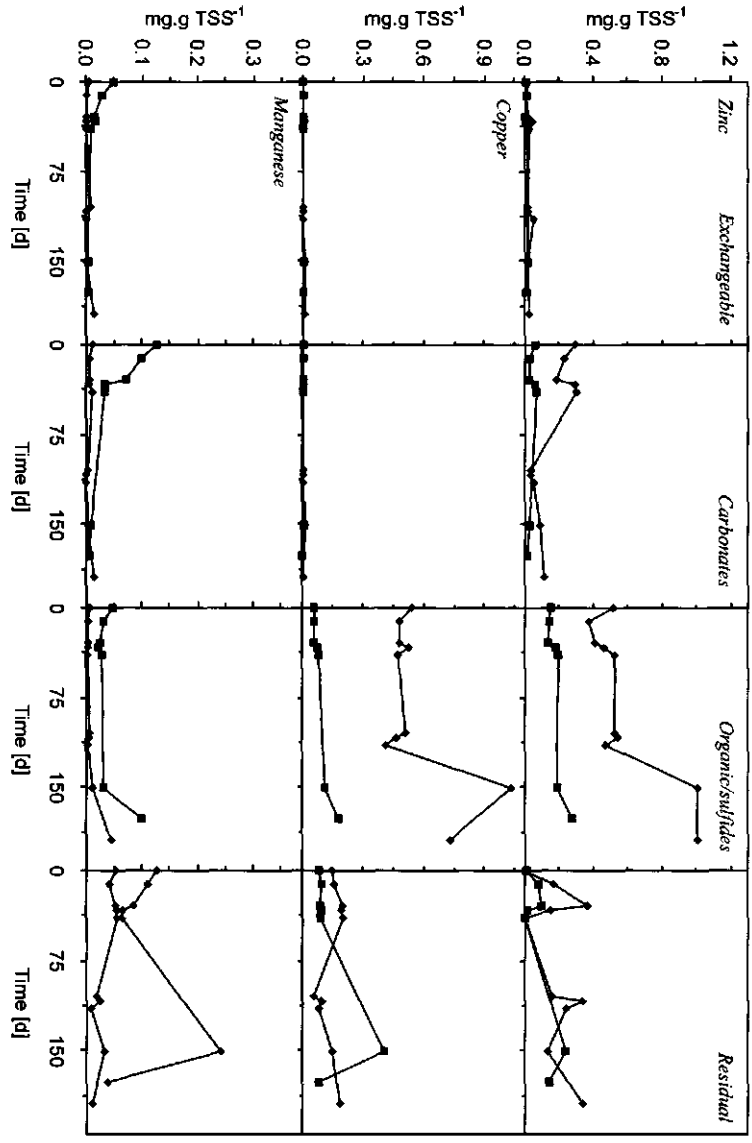


Figure 6 Evolution of the zinc, copper and manganese content present in the extracted fractions of the sludge in R1(♦) and R2 (■).

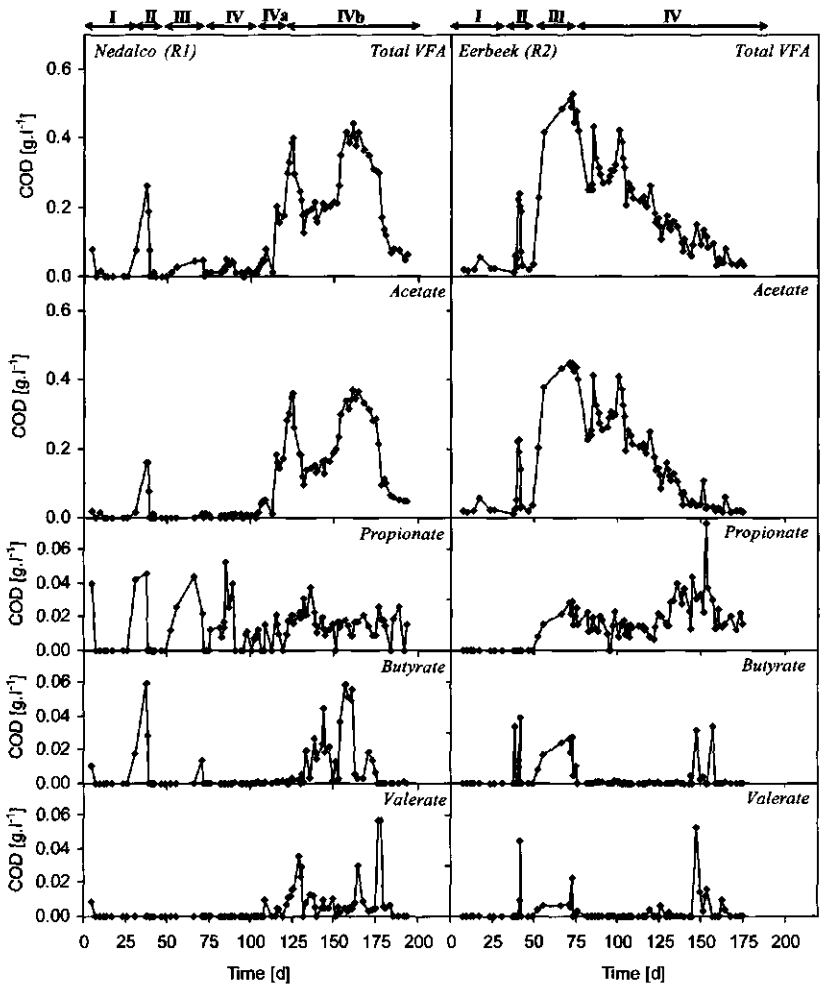


Figure 7 Evolution of the total and individual VFA concentration in the effluent.

Long term pH shock (4 days)

During a second 4 day pH shock (pH 5) imposed to R1 on day 105 (period IVa), the effluent methanol concentrations raised to 1579 mg. $COD.l^{-1}$ on day 109, while the VFA concentration only increased slightly to a value 49 mg $COD.l^{-1}$.

Similarly to the first 30h pH shock again discrete peaks manifested in the effluent concentration for cobalt, nickel and iron, i.e. 19 $\mu g.l^{-1}$, 31 $\mu g.l^{-1}$ and 1.41 $mg.l^{-1}$, respectively (Fig. 2) and concentrations of these metals remained elevated during the entire period of the shock. Losses of cobalt, nickel and iron in soluble form from the reactor during this pH shock

were 0.156 and 0.197 and 12.12 mg, respectively. At termination of the pH shock, the cobalt, nickel and especially the iron concentration returned rapidly to values similar or slightly higher than those measured before the shock (Fig. 2).

The cobalt content of the organic/sulfide fraction of the R1 sludge decreased with 0.20 mg.g TSS^{-1} (18%). The total iron content of the sludge decreased with 0.87 mg.g TSS^{-1} or 11%, losses form the organic/sulfide fraction were even higher 1.21 mg.g TSS^{-1} or 29%. The iron content of the exchangeable and carbonate fraction became slightly higher, which explains the higher losses from the organic/sulfide fraction compared to the loss of the total iron content. During this 4 day pH shock, the sulfur content of the sludge decreased as well, sulfur was merely lost (1.83 mg.g TSS^{-1}, 29%) from the organic/sulfide fraction.

Acidogenesis in R1

After the second 4 day pH shock, VFA accumulated in the R1 effluent, while in contrast to R2 also the methanogenesis was clearly disturbed as appeared from the higher concentration of methanol in the effluent from day 106 onwards (Fig. 1A). The effluent methanol concentration of R1 started to decrease from day 151 onwards, accompanied by the onset of methane formation (Fig. 1C), and methanol could not be detected anymore in the effluent on day 161. At the same time (day 151), the VFA formation started to increase from 201 mg $COD.l^{-1}$ on day 145 to 441 mg $COD.l^{-1}$ on day 161, but after day 161 the VFA concentration sharply decreased (Fig. 1B).

During the poor performance of R1 (Fig. 1 A, B), zinc was dosed to the system, either in pulses (Table 1; f and g) or continuous (Table 1; h and i). The decrease in effluent methanol concentrations with the simultaneous increase of the VFA concentration occurred when zinc was dosed continuously at higher concentrations. Once several metals were dosed (j), the VFA concentration in the effluent decreased sharply as well.

Zinc was present in the R1 effluent even before it was supplied to the system. The continuous dosing of zinc did not result in elevated effluent concentrations and the concentration averaged 37 $\mu g.l^{-1}$ (0.56 μM) during period III and IV.

Evolution of the specific methanogenic activity

In the SMA test conducted with the inoculum sludge (day 0), a significant methane formation only started after ± 150 h of incubation (Fig. 8). This suggests methanol utilizing methanogenic populations were absent (or inactive) in both types of sludge. The assessed SMA at day 0, with a feed of only 4 g $COD.l^{-1}$, amounted to 740 and 294 mg CH_4-COD.g $VSS^{-1}.d^{-1}$ for the R1 and R2 sludge, respectively (Table 4). The imposed short term pH shock did not affect the SMA on methanol of both sludges. The SMA's at termination of the

experiment of both R1 and R2 sludge were remarkably high, with values of 6619 and 2165 mg.CH_4-COD.g $VSS^{-1}.d^{-1}$, respectively.

Table 4. Evolution of the SMA with methanol, acetate and H_2/CO_2 as the substrate of both sludges.

Time	R1			R2		
	Methanol	Acetate	H_2/CO_2	Methanol	Acetate	H_2/CO_2
	mg CH_4-COD.g $VSS^{-1}.d^{-1}$			mg CH_4-COD.g $VSS^{-1}.d^{-1}$		
0	740 / 268[1]	671 / 639[1]	577	294 / 243	242 / 237[1]	330
34	1411	998	513	700	359	185
37/39	1594	663	290	832	366	170
45	1496	690	382	792	336	189
84	n.d	n.d.	n.d.	n.d.	0	n.d.
194/178	6619	0	n.d.	2165	0	n.d.

[1] SMA before pre-loading with cobalt, iron and nickel; n.d. not determined

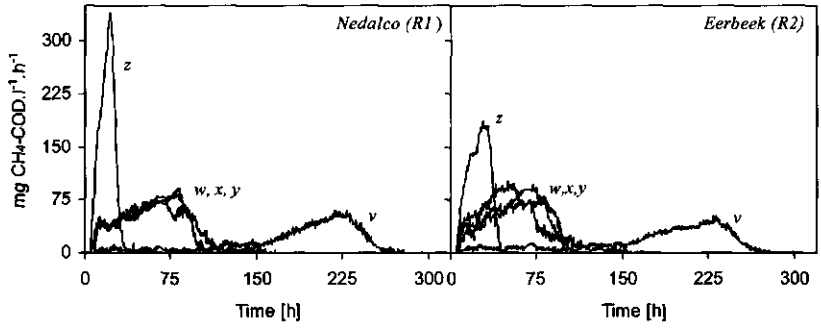


Figure 8 Methane production rate curves of methanogenic activity tests with methanol as the substrate. (v) initial rate (day 0), (w, x, y) rate curves before, during and after the first pH shock (day 34, day 37/39 and day 45), (e) the methane formation rate at termination of the experiment (day 194/178, respectively).

Methane formation in the SMA assay on acetate with the inoculum started after a lag phase of only ±7 h (data not shown), indicating that an active acetate utilizing methanogenic population was present in both sludges. The SMA with acetate of the R1 sludge on day 34

was 51% higher than the inoculum, but it decreased to the values found for the inoculum during and after the pH shock (Table 4). No clear effect of the pH shock was observed for the R2 sludge. The different effect of the pH shock on the acetotrophic methanogens of the R1 and R2 sludges becomes even more clear from the methane production rate curves (Fig. 9): the maximum methane production rates during and after the pH shocks for the R1 sludge are lower and appear at a later stage of the assay. In contrast, the methane production rate curves assessed for the sludge in R2 remain almost the same (Fig. 9). Surprisingly, no SMA on acetate could be determined for the R2 sludge anymore on day 84. At termination of the experiment, no SMA on acetate was present in the sludge of both reactors anymore.

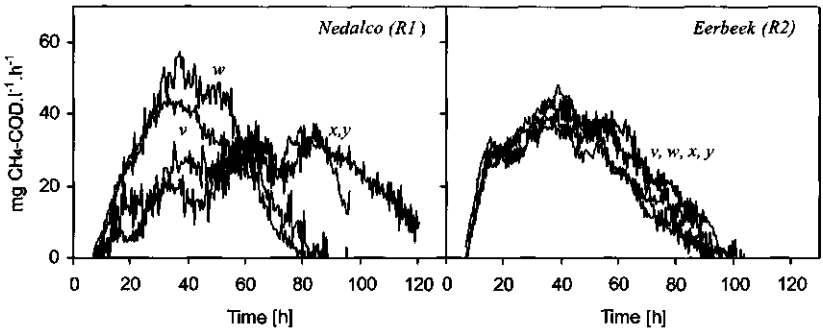


Figure 9 Methane production rate curves of the methanogenic activity tests with acetate as the substrate. (v) initial rate (day 0), (w, x, y) rate curves before, during and after the first pH shock (day 34, day 37/39 and day 45, respectively).

The SMA on H_2/CO_2 of the R1 sludge decreased from 513 mg.CH_4-COD.g $VSS^{-1}.d^{-1}$, before the pH shock to 290 mg CH_4-COD.g $VSS^{-1}.d^{-1}$ during the pH shock (Fig. 10, Table 4), but then the SMA increased again to 382 mg CH_4-COD.g $VSS^{-1}.d^{-1}$ 7 days after the pH shock. However, the pressure in the headspace of the bottles decreased at a similar rate during and after the pH shock (Fig. 10). The assessed higher SMA can be attributed to the higher methane percentage (81%) in the headspace found at termination of the experiment after the pH shock, compared to the 62% methane found during the pH shock. Prior to the pH shock the methane percentage in the headspace was 82 and 86%, respectively, during the start up and at day 37. For the R2 sludge, a less pronounced effect of the pH shock on the SMA with H_2/CO_2 as the substrate was found (Table 4). A similar reduction of the methane percentage in the headspace of the assay bottles was observed: 62% during, 90% before and 83% after the pH shock, respectively. The sludge sample during the pH shock had a longer lag phase before a faster pressure decrease occurred (Fig. 10).

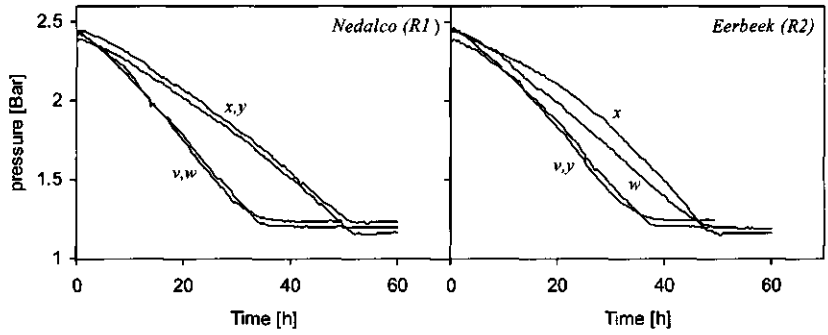


Figure 10 Pressure decrease during methanogenic activity tests with H_2/CO_2 as the substrate. (v) day 0, (w, x, y) rate curves before, during and after the first pH shock (day 34, day 37/39 and day 45, respectively).

Evolution of the metal retention in the sludge during the reactor run

The total amount of cobalt (2.930 mg.g TSS^{-1} and 1.182 mg.g TSS^{-1} for the R1 and R2 sludge, respectively) and nickel (3.187 mg.g TSS^{-1} and 1.235 mg.g TSS^{-1} for the R1 and R2 sludge, respectively) of both inoculum sludges differed considerably (Table 5). A significant amount of cobalt (1.166 mg.g TSS^{-1}; 40%) and nickel (1.511 mg.g TSS^{-1}; 47%), was present in the exchangeable fraction of the R1 sludge, while the relative contribution of this fraction was small in the R2 sludge, 0.161 mg.g TSS^{-1} (14%) and 0.265 mg.g TSS^{-1} (21%) for cobalt and nickel, respectively (Table 5).

Absolute cobalt and nickel losses were considerably higher from the R1 sludge, mainly because of depletions of the exchangeable and carbonate fraction, which initially contained high amounts of these metals (Table 5, Fig. 4). The pattern of the cobalt and nickel losses from the different fractions during the reactor run, however, was similar for the R1 and R2 sludge. Depletion of the exchangeable and carbonate fraction occurred in both the R1 and R2 sludge and the cobalt and nickel content of the organic/sulfide fraction was maintained in both sludges or even increased during operation (Table 5, Fig. 3).

The total iron content of the R1 and R2 sludge at the termination of the experiment were, respectively, 76 and 37% lower than the initial content. The relatively large iron losses of the R1 sludge can mainly be attributed to depletion of the residual fraction (81%), which initially contained 76% of the total iron content (Table 5, Fig. 3). For the R2 sludge, the main loss of iron was from the carbonate fraction, which contained 31% of the iron and was for 92% depleted. Also significant losses from the residual fraction occurred (Table 5).

Table 5. Cobalt, nickel and iron content at the start (day 0) and end (day 194 and 177 of operation, for R1 and R2, respectively) of the experiment and percentage of the metals lost (or accumulated) in total and in individual extracted fractions.

	R1			R2		
	day 0	**day 194**	**Loss/acc.**	**day 0**	**day 177**	**Loss/acc.**
	mg.g TSS^{-1}	mg.g TSS^{-1}	%	mg.g TSS^{-1}	mg.g TSS^{-1}	%
Cobalt						
Total	2.930	1.101	- 63	1.182	0.847	-28
Exchangeable	1.166	0.122	- 90	0.161	0.008	-95
Carbonates	0.536	0.093	- 83	0.256	0.007	-97
Organic/sulfide	0.657	0.738	+13	0.545	0.475	-13
residual	0.571	0.148	-74	0.220	0.357	+62
Nickel						
Total	3.187	1.315	-59	1.235	0.970	-21
Exchangeable	1.511	0.259	-83	0.265	0.017	-94
Carbonates	0.834	0.209	-75	0.453	0.027	-94
Organic/sulfide	0.445	0.702	+58	0.346	0.533	+54
residual	0.397	0.145	-63	0.172	0.393	+128
Iron						
Total	24.006	5.783	-76	38.350	24.094	-37
Exchangeable	0.035	0.037	+5	0.096	0.125	+31
Carbonates	4.037	1.127	-72	11.875	0.962	-92
Organic/sulfide	3.931	3.113	-21	5.359	6.093	+14
residual	18.141	3.461	-81	21.058	16.914	-20

The total zinc content of the R1 sludge remained approximately the same until day 115. In the inoculum of R1, some (0.29 mg.g TSS^{-1}) zinc was present in the carbonate fraction, which was depleted during operation (Fig. 6). On day 151 however, due to the continuous supply of zinc with the effluent (1,12 µmole.day^{-1}), the zinc content of the sludge had increased from 0.82 to 1.24 mg.g TSS^{-1} and increased further to 1.48 mg.g TSS^{-1} on day

194 (after continuous dosing at 11.25 μmole.d^{-1} from day 151 onwards). The zinc supplied with the effluent mainly accumulated in the organic/sulfide fraction (Fig. 6). In R2, zinc also accumulated in the sludge from 0.29 to 0.45 mg.g TSS^{-1} upon dosing (also mainly in the organic/sulfide fraction).

DISCUSSION

Metal retention

This research showed that both the pH of the reactor mixed liquor and the sludge source strongly affect the metal retention dynamics in UASB reactors. The losses resulting from the pH shocks of metal pre-loaded sludge (cobalt and nickel) were limited, except for iron, which was significantly solubilised from the sludge. A significant effect of the short term pH shock was the translocation of metals between the different operationally defined fractions (Fig. 4 and 5). Particularly in the R2 sludge, the iron and sulfur content increased in the organic/sulfide fraction with 5.15 and 6.53 mg.g TSS^{-1} (molar ratio Fe:S, 1:2.2), respectively (Fig. 4 and 5). At the same time, the iron and sulfur content in the residual fraction decreased with 2.83 and 5.4 mg.g TSS^{-1}, respectively. Apparently, a transformation of the stability of the iron sulfides present in the solid phase occurred during the pH shock. Anderko and Shuler [1997] showed indeed that the pH conditions may cause such a transformation of crystalline into more amorphous iron sulfide solids. This means that iron and sulfide will be already extracted from the sludge by less stringent reagents, i.e. they are extracted in an earlier extraction step of the sequential extraction procedure (Fig. 5).

Cobalt and nickel were concomitantly translocated from the residual fraction to the organic/sulfide fraction during the pH shock. Morse and Luther [1999] showed that cobalt and nickel tend to adsorb on, or co-precipitate with iron sulfide, which may explain why the nickel and cobalt content of the organic/sulfide fraction of the R2 sludge also increased during the pH shock. Watson et al. [1995] showed that iron sulfide is a very efficient sorbant (high binding energy) for heavy metals such as cobalt and nickel.

In the sludge of R1, the cobalt and iron concentration during the first pH shock did not increase in the organic/sulfide fraction, while also no effect on the sulfur concentration in both the organic/sulfide and the residual fractions was observed. In contrast, during the 4 day pH shock a decrease of the cobalt, iron and sulfur content occurred from the organic/sulfide fraction. This indicates that cobalt and iron sulfides present in this organic/sulfide fraction dissolve and are then lost with the effluent during a longer pH shock. Various readily soluble minerals can be expected to be present [Maes et al., 2003], e.g. amorphous FeS and/or crystalline FeS (such as mackinawite and greigite), which are the precursors of pyrite [Billon et al., 2001]. According to Maes et al. [2003], dissolution of iron starts around a pH of 6 in a sulfidic canal sediment, an anoxic environment with similar physico-chemical characteristics

to those in anaerobic granular sludge. This pH lies between the theoretical solubility of iron carbonate (siderite) and iron sulfide (mackinawite), cobalt dissolution can also occur directly as the dissolution of cobalt sulfides is complete at pH 5 [van der Lee, 2000]. In order to explain the differences in metal retention, more insight in the sulfur speciation within the sludge is required with respect, to for instance, the amorphous and crystalline state of the sulfides present in the sludge. These can be determined by coupling the determination of acid volatile sulfide (AVS) with sequential extraction [Jong and Parry, 2004] or X-ray adsorption near edge structures spectroscopy (XANES) [van Hullebusch et al., 2003].

The loss of cobalt, nickel and iron from the carbonate fraction (Table 6) during reactor operation is remarkable, but this has been observed previously for cobalt after pre-loading Nedalco sludge [Chapter 6]. When nickel (5 μM) and iron (50 μM) were dosed with the influent, a constant nickel content in the carbonate fraction prevailed during reactor operation, while the iron content of this fraction even increased [Chapter 6]. Sorption studies using the same sludges [van Hullebusch et al., 2005] showed that the exchangeable and carbonate fraction have a lower affinity for cobalt and nickel compared to the organic/sulfide fraction, which may explain why these fractions were depleted first.

Previous experiments conducted with cobalt pre-loaded R1 sludge showed a similar biphasic release of cobalt from the sludge during reactor operation (Fig. 3), i.e. a relatively fast depletion of the exchangeable and carbonate fraction then followed by a slower depletion of the organic/sulfide fraction [Chapter 6]. The R2 inoculum sludge did not accumulate cobalt well in the exchangeable and carbonate fraction (Fig. 4), which explains its relatively better cobalt retention (Table 5) and the absence of such biphasic cobalt loss (Fig. 3).

The total amount of cobalt and nickel lost from R1, based on its average effluent concentration from day 37 until day 103, were 0.89 and 1.21 mg cobalt and nickel, respectively. Based on the initial sludge content of the reactor, 13.3 and 13.2 mg cobalt and nickel was lost from the R1 sludge during this period, comprising a loss of approximately, 6.7 and 9.0% of the cobalt and nickel in a soluble form. This indicates that the main part of the cobalt and nickel is lost from the sludge in particulate form e.g. as precipitates or associated to biomass.

Reactor start up

The start up of both reactors proceeded considerably slower than observed in previous experiments with the same inoculum. The inoculum sludge from both R1 and R2, not pre-loaded with cobalt, nickel and iron completely removed methanol after 17 and 12 days, respectively (unpublished results), while it only needed 10 days for the Nedalco sludge (R1) when pre-loaded with a 1 mM cobalt solution for 24h [Chapter 5]. Apparently, the pre-loading of the sludge with a 1 mM cobalt, iron and nickel solution for 4 days initially

inhibited the growth or activation of the methanol degrading methanogens. Also the relatively long lag-phase of ±150 h needed for methane formation on methanol in the SMA assays (day 0) is an indication that these organisms are inhibited (Fig. 8). Remarkable is also the close similarity found for the lag-phases for both innoculum sludges and for the shape of the rate curves with methanol (Fig 8). Apparently, the growth/activation of the methanol degrading methanogens in both sludges, which had previously not been exposed to methanol, proceeds at a similar rate. The acetotrophic methanogens already present in the sludge remained unaffected by the metal loading procedure: because the SMA before and after loading was similar (Table 4) while also a lag phase was almost absent in the SMA assays after loading.

Reactor acidification

For the formation of acetate from methanol, CO_2 is required as a cosubstrate (Equation 1). Because no bicarbonate was supplied with the influent merely the CO_2 generated by the direct formation of methane from methanol (Equation 2) will be available for acetogenesis.

$$4CH_3OH + 2HCO_3^- \rightarrow 3CH_3COO^- + H^+ + 4H_2O \quad (1)$$

$$4CH_3OH \rightarrow 3CH_4 + HCO_3^- + H^+ + H_2O \quad (2)$$

Based on the stoichiometry of these conversion reactions at the maximum one third of the methanol supplied can be converted to acetate. This implies that, when methanogenesis is not the limiting factor, at the maximum 600 mg COD.l^{-1} of acetate can be formed from the 1800 mg COD.l^{-1} of methanol supplied. This explains the limited extent of the acidification proceeding in both reactors, as well as the observation that in R1 higher acetate concentrations in the effluent only prevail when methanogensis from methanol occurs (Fig. 1; day 121 to 126 and day 151 to 161). The amount of acetate formed in R1 (±165 mg.l^{-1}) in the absence of methane formation (Fig. 1; day 117 to 119 and day 132 to 151) therefore can only be attributed to the presence of some CO_2 in the system.

The question why the acetate formed in both reactors is not converted to methane remains to be elucidated, the more because just 4 days before the onset of acetate accumulation in R2 (day 49), the SMA on acetate of the R2 sludge was still considerable (336 mg.g $VSS^{-1}.d^{-1}$) and a negative effect of the pH-shock on the SMA was not observed (Table 4, Fig. 9). Consequently, in principle the acetate formed could have been converted to methane. On the other hand, a serious loss of SMA on acetate was observed for the R2 sludge at day 84 and for the R1 sludge at the termination of the experiment (but this most probably already occurred in an earlier stage). Loss of the SMA on acetate has been observed previously after

prolonged (260 days) UASB reactor operation with methanol as the substrate [Chapter 4]. Apparently, the loss of methanogenic activity on acetate can occur rather abruptly. *Methanosarcina sp.*, the methanogen responsible for the direct conversion of methanol, in principle is also capable to use acetate, but looses this ability after prolonged cultivation on methanol [Smith and Mah 1978; Boone et al., 1987].

Effect of metal dosage on reactor performance

Upon continuous zinc dosage at 11.25 μmole.d^{-1} (day 152), the elevated methanol concentration in the R1 effluent, which occurred after the second pH shock, dropped rapidly (Fig. 1A). This may indicate that the recovery of the methanol degradation can be attributed to the zinc addition. Because zinc is involved in methanogenesis as it is part of coenzyme M, which catalyzes the first step in the methanogenic pathway of methanol conversion, it is indeed an essential trace element [Sauer and Thauer, 2000]. The high amount of zinc in the sludge (0.821 mg.g TSS^{-1}) present at the start of the zinc supply (Fig. 3), is either not bioavailable or incidentally the onset of methanogenesis coincided with the continuous supply of zinc.

From day 163 onwards, when a mixture of metals was supplied continuously (Table 1), the VFA concentration in the effluent of R1 decreased. Except for the addition of these metals, another reason for this decrease might be the outcompetition of the acetogens by the methanogens, because of the big differences in apparent substrate affinity coefficients (K_s) for acetogens (777 mg $COD.l^{-1}$) and methanogens (12 mg $COD.l^{-1}$). This favors the growth of methanogens at low methanol concentrations [Florencio et al., 1994] and such low methanol concentrations prevailed in R1 on day 161, when the VFA decrease started.

The decrease of the effluent acetate concentration in R2 proceeded slower compared to R1, and a clear impact of the zinc supply at different concentrations is not apparent. The acetate concentration of the R2 effluent decreased slowly from day 105 until day 140 at a more or less constant rate of 6 $mg.l^{-1}$ per day. Likely this drop in R2 effluent VFA concentrations is also related to the outcompetition of the acetogens by methanogens and not to the methanogenesis of the formed acetate as a result of the zinc supply, because the SMA on acetate after 84 days and at the end of the experiment was nihil.

CONCLUSIONS

- The retention of cobalt, nickel and iron in granular sludge pre-loaded with these elements during reactor operation depends on the sludge characteristics/source.
- In both types of sludge studied the cobalt, nickel and iron content in the carbonate fraction decreased significantly during reactor operation.
- A translocation of cobalt, nickel, iron and sulfur occurs from the residual to the organic/sulfide fraction as a result of a 30 h pH shock (pH 5) in the R2 (Eerbeek) sludge.
- No clear translocation of metals was observed for the R1 (Nedalco) sludge during the first 30 h pH shock, but a second 4 day pH shock (pH 5) resulted in significant losses of cobalt (18%), iron (29%) and sulfur (29%) from the organic/sulfide fraction of the sludge.
- Significant losses of iron in soluble form resulted from the 30 h pH shock (pH 5), i.e. 2.3% and 2.9% for R1 and R2, respectively, based on the initial iron content in the reactors. The losses of cobalt and nickel in soluble form were rather limited.
- During prolonged reactor operation both sludges loose their ability to use acetate for methanogenesis.

REFERENCES

Alloway BJ (1990). Heavy metals in soils, 1st ed. (pp 7-27). Chapman and Hall

American Public Health Association (1985). *Standard methods for examination of water and wastewater*, 16th ed. American Public Health Association, Washington D.C.

Anderko A, Shuler PJ (1997). A computational approach to predicting the formation of iron sulfide species using stability diagrams. *Comp Geosci*, **23**, 7-658

Boone DR, Menaia JAGF, Boone JE, Mah RA (1987). Effects of hydrogen pressure during growth and effects of pregrowth with hydrogen on acetate degradation by *Methanosarcina* species. *Appl Env Microbiol*, **53**, 83-87

Billon G, Oudane B, Laureyns J, Boughriet A (2001). Chemistry of metal sulfides in anoxic sediments. *Phys Chem*, **3**, 3586-3592

Florencio L, Field JA, Lettinga G (1994). The importance of Cobalt for the individual trophic groups in an anaerobic methanol degrading consortium. *Appl Environ Microbiol*, **60**, 227-234

Gonzalez-Gil G, Kleerebezem R, van Aelst A, Zoutberg, Versprille AI, Lettinga G (1999). Toxicity effects from formaldehyde on methanol degrading on methanol degrading sludge and its anaerobic conversion in biobed expanded granular sludge (EGSB) reactors. *Wat Sci Technol,* **40**, 195-202

Hullebusch van ED, Zandvoort MH, Lens PNL (2003). Metal immobilization by biofilms: Mechanisms and analytical tools. *Re/Views in Env Sci & Bio/tech,* **2**, 9-33

Hullebusch van ED, Peerbolte A, Zandvoort MH, Lens PNL (2005). Sorption of cobalt and nickel on anaerobic granular sludges: isotherms and sequential extraction. *Chemosphere*, **58**, 493-505

Jong T, Parry DL (2004). Heavy metal speciation in solid-phase materials from a bacterial sulfate reducing bioreactor using sequential extraction procedure combined with acid volatile sulfide analyses. *J Env Monit,* **6**, 278-285

Lee van der J. CHESS (2000). updated for version 2.5, Technical report, LHM/RD/13, école des Mines de Paris, Fontainbleau, France

Lens PNL, Beer de D, Cronenberg CCH, Houwen FP, Ottengraf SPP, Verstraete WH (1993). Heterogeneous distribution of microbial activity in methanogenic aggregates: pH and Glucose microprophiles, *Appl Environ Microbiol*, **59**, 3803-3815

Lettinga G, van der Geest AT, Hobma S, van der Laan J (1979). Anaerobic treatment of methanolic wastes. *Wat Res*, **13**, 725-737

Maes A, Vanthuyne M, Cauwenberg P, Engels B (2003). Metal partitioning in a sulfidic canal sediment: metal solubility as a function of pH combined with EDTA extraction in anoxic conditions. *Sci Total Environ,* **312**, 181-193

Morse JW, Luther GW (1999). Chemical influences on trace element sulfide interactions in anoxic sediments. *Geochim Cosmochim Acta,* **63**, 3373-3378

Sauer K, Thauer R (2000). Methyl-coenzyme M formation in methanogenic archaea; involvement of zinc in coenzyme M activation. *Eur J Biochem*, **267**, 2498-2504

Smith MR, Mah RA (1978). Growth and methanogenesis by *Methanosarcina* strain 227 on acetate and methanol. *Appl Environ Microbiol*, **36**, 870-879

Watson JHP, Ellwood DC, Deng Q, Mikhalovsky S, Hayter CE, Evans, J, (1995). Heavy metal adsorption on bacterially produced FeS. *Miner Eng*, **8**, 1097-1108

Weijma J, Stams AJM, Hulshoff Pol LW, Lettinga G (2000). Thermophilic sulfate reduction and methanogenesis with methanol in a high-rate anaerobic reactor. *Biotechnol Bioeng*, **67**, 354-363

Chapter 8

Summary and general discussion

OBJECTIVES

The main objective of this thesis was to elucidate the trace metal dynamics in methanol fed anaerobic granular sludge bed reactors and how these influence the reactor performance. Insight in the metal dynamics is required from a practical point of view in order to foresee limitations for essential trace elements, viz. to know when dosing of these elements is required in full-scale anaerobic bioreactor applications. Further such knowledge will allow rational dosing of these metals, e.g. to ensure maximal substrate conversion rates and to prevent disturbances in reactor performance using a minimal amount of metals. Therefore, this research focused on the retention, accumulation and release of trace metals in anaerobic granular sludge and the factors affecting these processes. Figures 1, 2 and 3 depict the aspects of the trace metal dynamics in anaerobic bioreactors that were addressed in this research.

SUMMARY AND GENERAL DISCUSSION

Metal limitation

The content of individual metals in the sludge represents an important factor for the stable operation of anaerobic bioreactors, while it also may sometimes already indicate for which metal limitations can occur. Screening of a number of sludges from full-scale reactors treating different pollutants for their metal responses using as substrate either methanol, acetate or H_2/CO_2 [Chapter 2] demonstrated that metal limitations in full-scale reactors are already present, although mainly on the substrate methanol, as evidenced by the significant increases in the SMA upon 5 μM cobalt addition (Fig. 2, Chapter 2). The four sludge samples tested in Chapter 2 contained a total cobalt content in the range of 27 to 51 $\mu g.g\ TSS^{-1}$, but this apparently is not directly bioavailable to the SMA on methanol. Initial limitations for other metals exist as well, e.g. for nickel in the Eerbeek sludge [Zandvoort et al., in preparation].

If not already existing in the seed sludge, trace metal limitations can be easily induced in the (granular) sludge upon feeding it for prolonged periods of time (1-3 months) in a UASB reactor (Table 1). In methanol fed UASB reactors inoculated with Nedalco granular sludge and operated at 30 °C, pH 7 and at sludge loading rates (SLR) stepwise increased from 0.3 to 0.9 g COD.g $VSS^{-1}.d^{-1}$, trace metal limitations developed in the absence of cobalt (± 30 days; Chapter 3), nickel (± 60 days; Chapter 4) or iron (± 100 days; Chapter 4). When using another type of sludge, Eerbeek granular sludge [Zandvoort et al., in preparation], with an initial cobalt content of 59 μg. g TSS^{-1}, 2.5 times higher than the Nedalco sludge (and the highest initial content measured in this research), cobalt limitation could be induced within 50 days of operation at 30°C, although a high SMA on methanol could still be obtained.

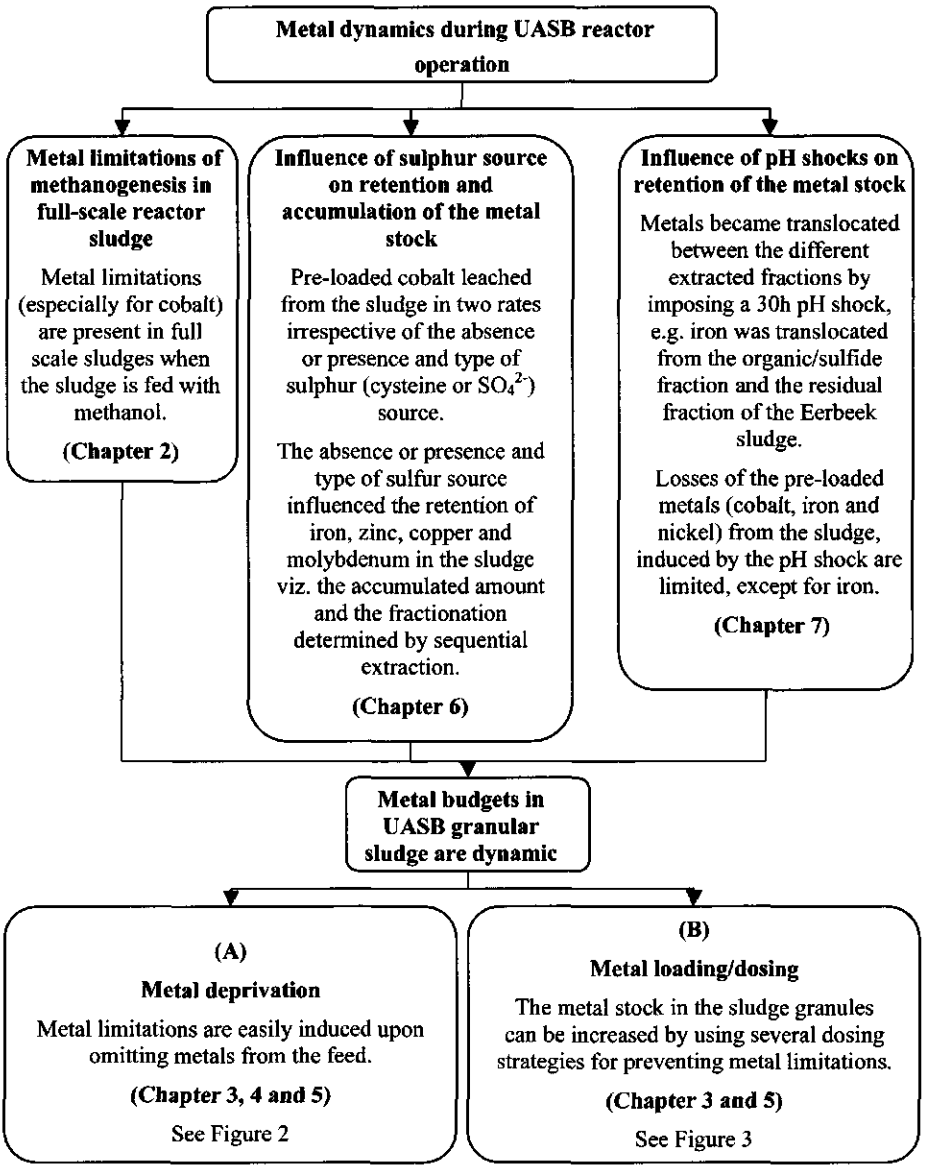


Figure 1 Overview of the thesis structure and the aspects of metal dynamics in UASB reactor operation that were addressed.

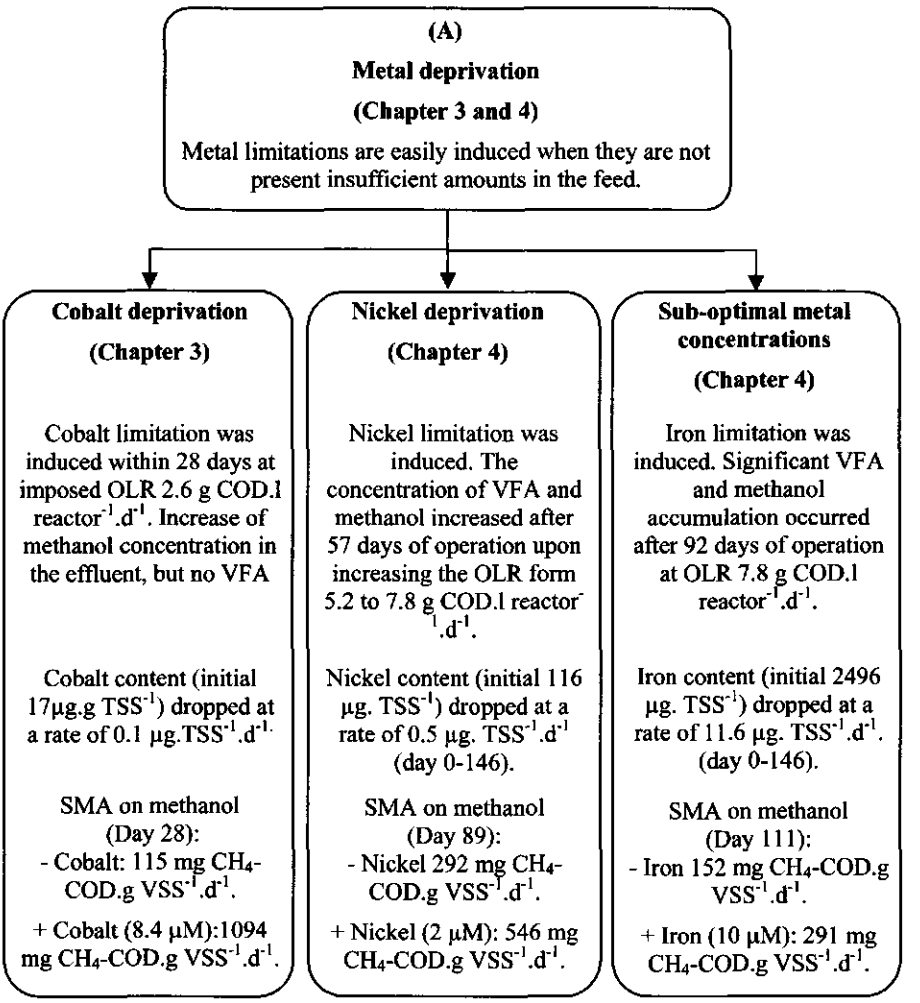


Figure 2 Overview of the results of this thesis concerning metal deprivation. Abbreviations: volatile fatty acid (VFA), chemical oxygen demand (COD), organic loading rate (OLR), Specific methanogenic activity (SMA)

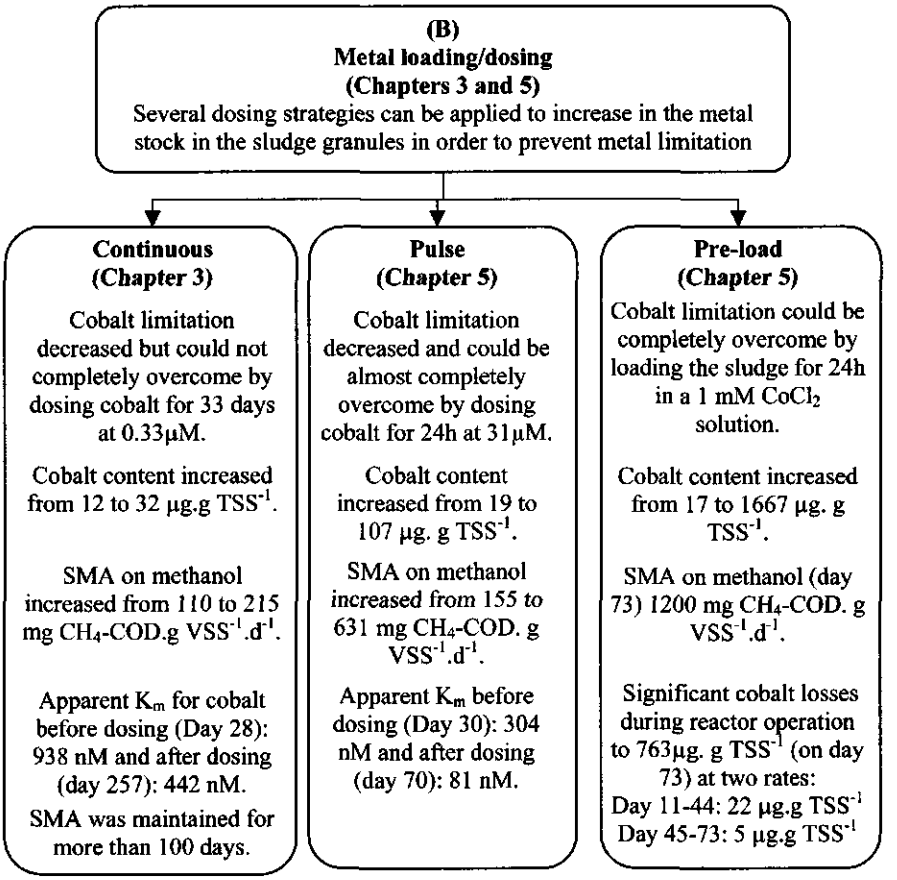


Figure 3 Overview of the results of this thesis concerning cobalt leaching and dosing. Abbreviations: see Figure 2.

Metal limitations can also develop on other substrates than methanol. Omitting the supply of trace metals from the feed of a UASB reactor (30°C; pH 7) inoculated with Nedalco granular sludge and fed with a mixture of acetate, propionate and butyrate, resulted in a sludge with a 30% lower SMA compared the sludge in a reactor with metal in the feed. Moreover, propionate was not degraded in the metal deprived reactor [Osuna et al., 2002].The operation of a thermophilic UASB reactor with Eerbeek granular sludge fed with glucose at 55°C, OLR of 5 g COD.l reactor^{-1}.d^{-1} and operated at a low pH of 4 and 5 also lead to metal limitation. Full trace element supply in batch activity assays increased the hydrogen formation rate from glucose [Rowlette, 2005].

Table 1. Overview of the assessed cobalt content and SMA of granular sludges from lab scale UASB-bioreactors

Inoculum			**(After) start-up**					**End of reactor operation**					
Cobalt Content μg.g TSS^{-1}	Substrate	OLR[a]	Inf. Cobalt μM	Day	Cobalt μg.g TSS^{-1}	SMA mg CH_4 COD. g $VSS.d^{-1}$ - Cobalt	SMA mg CH_4 COD. g $VSS.d^{-1}$ + Cobalt[b]	Day	Cobalt μg.g TSS^{-1}	SMA mg CH_4-COD. g $VSS.d^{-1}$ -Cobalt	SMA mg CH_4-COD. g $VSS.d^{-1}$ + Cobalt[b]	Objective	Ref
17	Methanol	7.8	0	28	n.d	110	1094 (8.4)	255	12	215[c]	507 (8.4)	Cobalt deprivation	[1]
17	Methanol	7.8	0.8	28	n.d.	1549	1517 (8.4)	259	237	n.d.	193 (8.4)	Sub-opt metal conditions[d]	[2]
17	acetate,propionate , butyrate (3:1:1)	10	0	n.d.	n.d.	n.d.	n.d.	140	10	175	175 (6.3)	Deprivation All Metals[e]	[3]
17	acetate,propionate , butyrate (3:1:1)	10	6.3	n.d.	n.d.	n.d.	n.d	140	2890	260	260 (6.3)	Deprivation All Metals[e]	[3]
19	Methanol	15	0	0	19	487	521 (5.0)	48	n.d.	402	1927 (5.0)	Deprivation All metals	[4]
59	Methanol	10	0	0	59	237	245 (5.0)	44	51	907	1010 (5.0)[f]	Deprivation All metals	[4]
59	Methanol	20	0	0	59	243	245 (5.0)	55	50	1687	2515 (5.0)[g]	Cobalt deprivation	[4]
17	Methanol	20	0 (31)[h]	30	20	155	540 (5.0)	77	57	631	828	Pulse dose cobalt	[5]
1667	Methanol	20	0	30	1169	906	843 (5.0)	77	763	1200	1126	Pre loading Co	[5]
1990	Methanol	5	0	29	1370	80	n.d.	118	470	1119	n.d.	S-source vs. Met. Retention	[6]
1990	Methanol	5	0	29	1540	744	n.d.	118	610	1382	n.d.	S-source vs. Met. Retention	[6]

[a] Organic loading rate (g COD.l $reactor^{-1}.d^{-1}$); [b] Cobalt concentration (μM) added to the batches is presented between brackets; [c] After continuous dosing of 0,33 μM cobalt to the influent from day 54 to 85 operation; [d] all metals were supplied to the reactor but at sub-optimal concentration; [e] all metals (Co, Ni, Fe, Mn, Cu, Zn, Mo, Se) were left out of the influent and sludge was tested for its response on cobalt alone and all metals; [f] inoculum source Eerbeek sludge; activity in the absence of all other trace elements, in the presence of all trace elements acitivity was 1543 mg CH_4-COD. g VSS^{-1};[g] inoculum source Eerbeek sludge [h] pulse dosing of 24h at day 57 days; n.d. not determined; references, [1] Chapter 3, [2] Chapter 4, [3] Osuna et al., 2003; [4] Zandvoort et al., (in preparation). [5] Chapter 5; [6] Chapter 6.

Metal dynamics in granular sludge

Leaching

When the supply of sufficient trace elements to the influent is omitted, the metal content of the sludge will drop due to a process of leaching from the sludge granules during the operation of UASB reactors [Chapter 3 and 4]. Losses from the sludge (especially for cobalt, nickel and iron) are proportional with their initial content in the sludge [Chapter 3]. Part of the observed decline in metal content may be attributed to dilution of the biomass as a result of bacterial growth, but – as demonstrated by the results presented in Chapter 3-, iron and nickel losses greatly exceed the dilution factor resulting from biomass growth, despite the 30% increase of the sludge bed volume.

The metal retention properties of granular sludges from different full-scale reactors can differ distinctly [Chapter 7; Osuna et al., 2004] and these differences in metal retention properties will also contribute to a different metal retention behavior during reactor operation. So for instance pre-loading the Nedalco with cobalt results in a Co-leaching rate at an initial fast rate of ±22 μg. g $TSS^{-1}.d^{-1}$ from the exchangeable and carbonate fraction, and later at a substantially lower rate of ±9 μg. g $TSS^{-1}d^{-1}$ from the organic/sulfide fraction [Chapter 6] after the depletion of the latter fractions.

Such a two phase depletion of cobalt did not manifest for the pre-loaded Eerbeek sludge. Only 28% of cobalt was lost during 194 days of reactor operation, while it was 63% for the pre-loaded Nedalco sludge [Chapter 7]. It should be noted here that the Co-content of the Eerbeek sludge with a value of 1.18 mg.g TSS^{-1} was much lower than the Nedalco sludge with a value of 2.93 mg.g TSS^{-1}. Moreover also the distribution of over the fractions differed completely for both sludges, i.e. contrary to the Nedalco sludge, the Eerbeek sludge hardly had accumulated cobalt in the loosely bound exchangeable and carbonate fractions. Sorption experiments revealed that the affinity for the sorption of cobalt and nickel was the highest in the organic/sulfide fraction in both sludges [van Hullebusch et al., 2005], which explains the observed differences in leaching dynamics of the sludges. This implies that the metals dosed with the influent preferably accumulate in this high affinity fraction, as indeed was confirmed in-situ dosed cobalt experiments [Chapter 5].

Metal forms in granular sludge - sequential extraction

Metals are distributed over different distinguished phases of the granular matrix, i.e. biomass, extra cellular polymeric substances (EPS) and inorganic precipitates e.g. sulfides, phosphates and carbonates [van Hullebusch et al., 2003]. The total metal content of the sludge (granules) obviously hardly can provide relevant information about how the metals are bound to the sludge matrix, especially not how strongly they are bound. The sequential extraction

procedure used in this study represents a valuable tool for gaining insight in the way metals are retained in the sludge, because this method provides information on the metal distribution over operationally defined fractions. The losses of the trace metals from the sludge and their accumulation in the various fractions of the sludge has been monitored during the reactor operation [Chapter 6]. The method clearly demonstrated the occurrence of translocations of metals due to imposed pH shocks, viz. from the residual fraction to the organic/sulfide fraction [Chapter 7]. Apparently significant changes in the way the metals are bound/retained in the sludge can occur, and consequently this also is the case for their retention and bio-availability.

This research shows [Chapter 6 and 7] that the carbonate, but especially the organic/sulfide, fractions are the important scavenging phases for the trace metals in anaerobic granular sludge. Very similar observations were made by van Hullebusch et al. [2004], they also found a close relationship between the sulfur released in the organic/sulfide extraction and the cobalt, copper, nickel and zinc content of granules. Sulfide, ubiquitously present in bioreactor systems and in granular sludge, clearly is an important factor in the metal retention in bioreactors, not merely because it is an important scavenger [Chapter 6], but especially also because losses from this fraction during reactor operation are very limited compared to those from other fractions [Chapter 6 and 7]. Therefore, this organic/sulfide fraction plays a key role (together with the residual fraction) in the long term retention of the trace metals in the granular sludge.

Effect of sulfur compounds on metal retention

The retention of cobalt in the pre-loaded sludge is not significantly influenced by the presence sulfur, or the type of sulfur compound fed to the UASB reactor [Chapter 6]. Already concentrations of a sulfur source as low as 13 $mg.l^{-1}$ significantly influenced the metal retention/accumulation in the sludge bed [Chapter 6]. In the absence of a sulfur source, zinc preferentially accumulated in the carbonate fraction, but once L-cysteine was added to the feed the zinc content of the carbonate fraction dropped considerably while it increased in the organic /sulfide fraction. This strongly suggests that the zinc present in the carbonate fraction was transformed to sulfides, in this way dramatically altering the mode it was retained within the granules. Iron (and molybdenum) even did not accumulate in the absence of a sulfur source, and here the sulfur source clearly determined the preferred fraction in which iron accumulated, viz. the carbonate fraction when sulfur was supplied as cysteine and the residual fraction when sulfate was the sulfur source.

Effect of pH on metal retention

In general, metal solubility increases at lower pH values, independent of the mineralogical composition of the compound [Alloway, 1990]. Hence, the pH of the reactor mixed liquor might represent an operational parameter that can influence the metal retention in anaerobic sludge granules. This research shows that short term pH shocks to values of pH 5 (30h and 4 days) strongly can affect the speciation of the metals in granular sludge pre-loaded with cobalt, nickel and iron [Chapter 7]. Remarkably, the losses of metals with the effluent due to the imposed pH shock remained rather limited, except for iron. Moreover, the origin of the sludge influences the impact of the pH shock as well. In the Eerbeek sludge, the 30h pH shock caused a translocation of iron, cobalt, nickel and sulfur from the residual to the organic sulfide fraction, likely due to the dissolution and reprecipitation of these metal sulfides. Such translocation was absent in the Nedalco sludge in case of the 30h pH shock, but the 4 day pH shock resulted in significant losses of cobalt (18%), iron (29%) and sulfur (29%) from the organic sulfide fraction, which most likely can be due to cobalt and iron sulfide dissolution. The impact of these losses and transformations for the biomass in anaerobic granules is not yet clear: obviously losses of metals are highly undesirable, but on the other hand a certain dissolution/mobilization in the granular bed or within a single granule due to pH gradients, may actually improve the metal bioavailability.

Metal dosing

Quantity

Clearly there still does not exist a "magic" procedure for supplying trace metal to UASB reactors. However, it seems reasonable to develop a procedure that tunes metal dosing in practice to the metal content of the sludge and influent. Wastewater streams generally already contain trace elements and knowledge of the metal content of the influent will help in optimizing the metal dosage, viz. some metals may not needed in the trace metal cocktail supplied to the influent. Knowledge of the total metal content of the inoculum sludge does not suffice to predict limitations [Chapter 2], the more so because leaching of metals from the sludge during reactor operation [Chapter 3 and 4] eventually may lead to limitations. Therefore, the total metal content of the sludge should be determined at regular intervals (e.g. monthly) during reactor operation in order to asses the extent and rate of metal loss. This should preferably be combined with a characterization of the metabolic characteristics of the sludge and its response to metal addition in SMA assays. Indicative values for the rate of metal accumulation can be derived from this research, e.g. a continuous cobalt and nickel supply at 0.84 μM and 0.4 μM, respectively lead to an increase of the sludge metal content at a rate of approximately 0.82 $\mu g.g\ TSS^{-1}.d^{-1}$ and 0.4 $\mu g.g\ TSS^{-1}.d^{-1}$ for cobalt and nickel respectively [Chapter 4].

Extrapolating the results from this research to full scale practices, such as the Nedalco anaerobic wastewater (AnWT) treatment system where the UASB-reactor contains on the average 10.000 kg of sludge solids with a cobalt content of 20 μg.g TSS^{-1}, means that the reactor contains 200 g of cobalt in the bioreactor. According to the results in Chapter 3, cobalt will be lost at a rate of 0.1 μg.g $TSS^{-1}.d^{-1}$, consequently the daily loss from the reactor amounts to 1 g cobalt. Increasing the cobalt content of the Nedalco sludge up to a value of 60 μg.g TSS^{-1}, i.e. the Co-content of the Eerbeek sludge, which was found capable to maintain a significantly higher SMA with methanol as the substrate [Zandvoort et al., in preparation] would require the addition of 400 g of cobalt, provided it is retained by the sludge.

Cobalt dosing strategies

A rational supply of trace elements to the sludge is required in order to achieve maximal substrate conversion rates, stable reactor performance and a minimum of metal losses with the effluent. From the three different dosing strategies studied for methanol fed UASB reactors in this thesis (Fig. 3), the continuous dosing procedure of cobalt at low concentrations is favorable with respect to the amount of cobalt required and the minimal losses with the effluent [Chapter 3]. It was suggested previously that dosing at low concentrations would be favorable [Gonzalez-Gil et al., 1999] as it provides relatively high free metal concentrations directly available for uptake by the biomass, thus preventing that metal dissolution becomes the rate limiting step for growth of the microorganisms [Gonzalez-Gil et al., 1999]. However, in our present research a relatively limited impact on the specific methanogenic activity was found, while also the cobalt limitations could not be completely overcome by dosing cobalt at 0.33 μM for 33 days [Chapter 3]. After termination the cobalt dosing, the activity and methanol removal capacity could be maintained for more than 100 days. The sludge bed increased only 30% in volume, far less than the reactors operated under the same conditions, but with cobalt in the influent [Chapter 4]. This suggests that metal dosing even can also be used as a means of controlling excess sludge production.

Pulse dosing (in situ loading) of cobalt, comprising an intermediate dosing strategy between the pre-loading and continuous dosing, is very effective in overcoming almost immediately acute cobalt limitations [Chapter 5]. However, with this procedure the losses of cobalt from the sludge were found to be considerably higher as compared to the continuous dosing strategy. Furthermore it should be noted here, that a distinct formation of VFA occurred after pulse dosing of cobalt contrary to the continuous dosing strategies.

Pre-loading, although found to be effective in overcoming cobalt limitations and resulted in a relatively high SMA of the sludge, this procedure was ineffective with respect to reducing cobalt (metal) losses [Chapter 5 and 6]. Especially, immediately after start-up these losses were significant (Fig 3; Chapter 5).

Trace metals versus macro-element dosing

Iron can not really be classified as a trace element, because it is present in relatively high amounts in the granules e.g. 75.7 mg.g TSS^{-1} in Hoogeveen sludge [Chapter 2] and it is normally dosed at relatively high concentrations. The iron content of 20.8 mg.g TSS^{-1} of the Nedalco sludge [Chapter 7] implies that the Nedalco full-scale reactor contains 200 kg iron.

The iron retention appeared to be highly sulfur and sulfur source dependent [Chapter 6]. It is not clear whether dosing of the other trace metals together with the iron may result in a negative sink of the trace metals due to co-precipitation with or sorption onto the iron sulfide phase. It would make them less (not directly) bio-available. Besides the observation that microorganisms require much higher iron concentrations for their growth, it is likely that iron sulfide precipitates are important for the granular sludge structure. It may therefore be practical to dose the other trace elements separately from the iron. The "real' trace metals could be supplied in 'bound form' to ligands, because this will prevent their precipitation as highly insoluble compounds, and thus making sure that they are (more) directly bioavailable. A sequential dosing procedure of the individual trace metals can also be an attractive option, e.g. of nickel and cobalt, because for these elements competitive effects have been observed with respect to their bio-uptake [Jansen et al., 2004a].

Anaerobic methanol conversion

The mesophilic conversion of methanol and its strong cobalt dependency make it an excellent model system to study processes related to e.g. trace metal uptake, bioavailability and metal dosing impact on the SMA of methanogenic granular sludges of anaerobic sludge reactors. However, on the other hand prolonged bioreactor operation with solely methanol as substrate frequently results in performance instability, viz. acidification, and this will complicate studies aimed at assessment of long term effects of trace of metal deprivation.

Overloading is an important trigger for the build-up of VFA [e.g Chapter 4 and 5], but also pH shocks seem to induce VFA build-up [Chapter 7]. Moreover, also changes in the metabolic properties of the sludge appear to be an important factor for the VFA-formation and build-up, because in the presence of a "healthy" acetotrophic methanogenic population, the formed acetate would not accumulate in the effluent.

By operating the reactor with solely methanol as the substrate, the sludge enriches with *Methanosarcina sp.,* the organisms responsible for the direct conversion of methanol to methane. *Methanosarcina strain 227* grown on methanol as the sole substrate is unable to use acetate in the presence of methanol, viz. methanol is the preferred substrate and is converted first, and in some cases it even was found not capable of degrading acetate at all [Smith and Mah, 1978]. This indicates that when acetate is present or formed in the presence of high

methanol concentrations, *Methanosarcina* species likely are unable to contribute to the conversion of acetate. The presence of an acetotrophic methanogenic population of e.g. *Methanosaeta sp.* may therefore be important in preventing build-up of VFA in the system of methanol fed anaerobic bioreactors. In the experiments described in Chapter 6, a relatively high value of the SMA on acetate of 608 mg CH_4-COD.g $VSS^{-1}.d^{-1}$ was found after 118 days of operation. A significant raise in the effluent VFA concentration was absent after a 4-day reactor disturbance viz. an increase of the methanol concentration to values exceeding 1000 mg $COD.l^{-1}$, which normally is a trigger for VFA accumulation [Florencio et al., 1995]. This indicates the importance of a high SMA on acetate for preventing reactor acidification even in the presence of high cobalt concentrations, which comprises another prerequisite for acetate accumulation [Florencio et al., 1995].

FUTURE PERSPECTIVES

This thesis merely explored the behavior of metals in anaerobic granular sludge and its effect on the reactor performance. However this work creates a lot of new perspectives for extremely useful future research, and certainly not merely in the field of AnWT. The complexity of the different processes involved in metal retention in anaerobic bioreactors/biofilms as well as the metal bioavailability clearly requires a well organized multidisciplinary approach of further long term investigations. A number of suggestions will be presented below.

Metal retention

The mechanisms involved in the metal immobilization in the sludge granules should be elucidated in more detail with respect to e.g. chemical speciation, (bio-)sorption characteristics, spatial distribution and mass transfer. A comprehensive review of the mechanisms involved in metal immobilization within bioaggregates (films and granules) and the analytical tools that can be used to study this immobilization in bacterial aggregates/biofilms was recently published [van Hullebusch et al., 2003]. Figure 4 presents a scheme of these analytical tools and how they could be combined to improve the insight in metal dynamics and bioavailability in granular biofilms, which ultimately will lead to an significantly improved reactor performance.

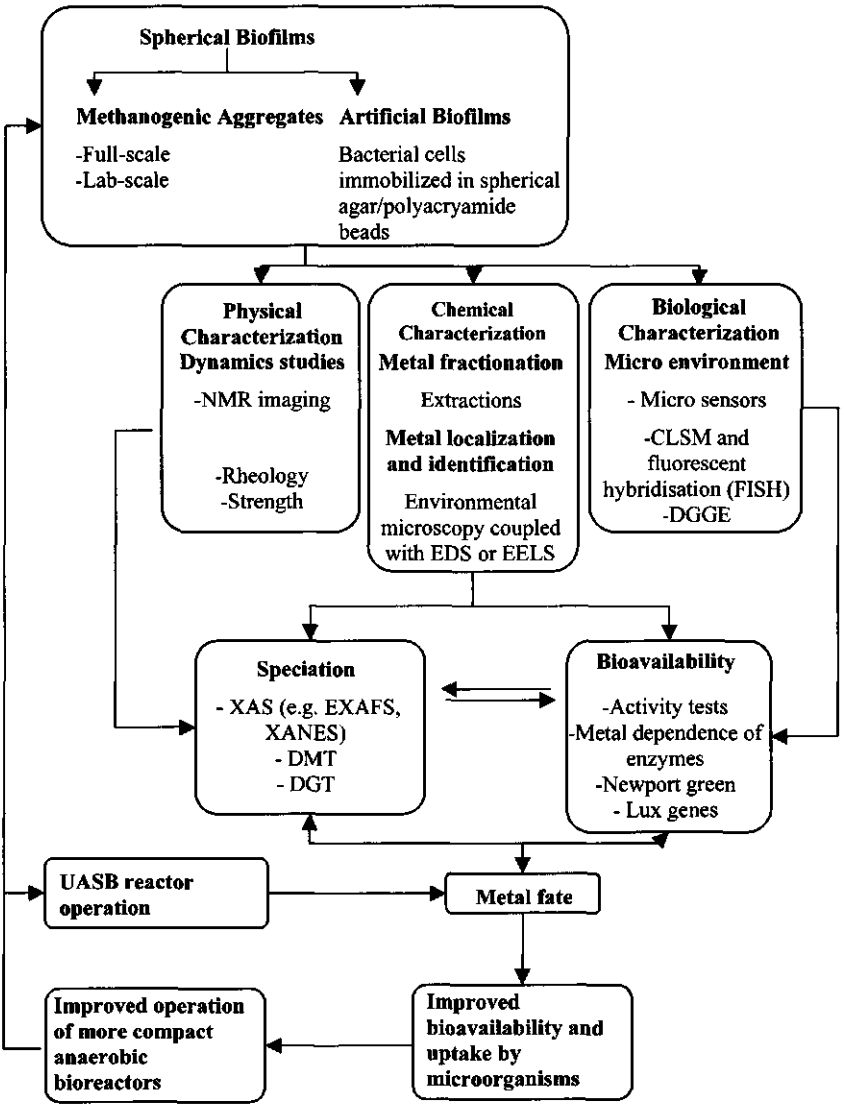


Figure 4 Analytical tools to study metal dynamics in granular biofilms. Nuclear magnetic resonance (NMR), Energy-dispersive X-ray spectroscopy (EDS), Electron energy loss spectroscopy (EELS), Confocal laser scanning microscopy (CLSM), Denaturing gradient gel electrophoresis (DGGE), Fluorescent in situ hybridization (FISH), X-ray adsorption spectroscopy (XAS), Extended X-ray adsorption fine structure (EXAFS), X-ray adsorption near edge structure spectroscopy (XANES), Diffusive gradient thin films (DGT), Donnan membrane technique (DMT).

This research shows that the organic/sulfide fraction comprises an important scavenger for metals and is important for their long-term retention in the granules. This fraction has also the highest affinity for cobalt and nickel sorption [van Hullebusch et al., 2004]. Likely most of the metals in this fraction are bound as metal sulfides. However, the modified Tessier sequential extraction procedure used in the research does not allow the separation of the organic bound sulfide and metal sulfide precipitates. Also the sulfur speciation in granular sludge requires more attention with respect to for instance the amorphous state and crystalline state of the sulfides, because it determines their solubility, mobility and bioavailability. This could be achieved by coupling acid volatile sulfide (AVS) extraction, which is considered the biological active/available sulfide fraction with sequential extraction [Jong and Parry, 2003] or X-ray adsorption near edge structure spectroscopy (XANES) [Prange and Modrow, 2002].

Liquid phase

Besides the retention and speciation of metals in the granular matrix, which is important for the metal retention/dosing efficiency, attention needs to be afforded to metal speciation and metal dynamics in the reactor liquid. For instance, the observed blackening of the reactor liquid and effluent (e.g. in chapter 6) shows that the metal sulfide precipitates are not merely found in the granular matrix, but also in the reactor liquid, as a result of which the free metal in solution becomes very low. The free metal in solution is likely the form available for bio-uptake [Jansen et al., 2004b]. According to Jansen et al. [2004a] even the extremely low free metal concentrations encountered in the sulfide containing bioreactor media might still suffice to maintain reasonable metal uptake fluxes. The more so, because of the dissolved metal-sulfide species would create a large dissolved metal buffer, which is important in preventing metal depletion [Jansen et al., 2004c]. The dynamics of the free metal concentrations and/or labile complexes should be monitored in lab-scale reactors as a function of the reactor conditions or even as a function of the sludge bed height, because it is important for metal dynamics and bioavailability in the bioreactors. Promising techniques to study these dynamics in bioreactors are diffusive gradient thin films (DGT) proposed by Zhang and Davison [2000]; this procedure can be used to asses the concentration of labile metal complexes. Also promising is the Donnan membrane technique (DMT) proposed by Temminghoff et al. [2000] because it allows assessment of the free metal content in the reactor liquid.

Chemical form of dosed metal/bioavailability

The metals dosed to anaerobic bioreactors should be (made) available for all the viable biomass present in the granule. When trace metals are dosed as free metal ions, they likely will precipitate with the ubiquitously present sulfide, which makes them much less or even not (anymore) bio-available. The supply of metals bound to organic ligands might provide a

buffer of dissolved metals and prevent them from direct precipitation as sulfides, even though the free metal concentration then still will be determined by precipitation equilibria. Gonzalez-Gil et al. [2003] found in a *Methanosarcina* enrichment culture that yeast extract, although a quite undefined mixture of organic ligands (amino acids and peptides), considerably improved the metal bio-avialbility in the presence of sulfide. Binding of cobalt and nickel to the ligand EDTA also appeared to be efficient in keeping the metals in solution and available for growth of a pure culture *Methanosarcina barkeri* grown in the presence of sulfide [Jansen et al., 2004a].

Therefore, it looks attractive to supply the metals bound to ligands because it improves the metal bioavailability and consequently the metal dosing efficiency. However, it should be noted here, that enrichment cultures differ substantially from the situation in the matrix of an anaerobic sludge granule. Likely in bioreactors, diffusion, dissolution and depletion are the most important sources of metal limitation. The use of ligands will therefore probably be most effective for providing trace metals on the bioreactor/granular level [Jansen et al., 2004c]. However, the interference of the sludge matrix on metal transport, the metal response and bio-availablity so far has been hardly investigated. Lens and van As [2003] used nuclear magnetic resonance (NMR) to study metal transport inside the granular matrix. This technique could also be used to assess the effect of the metal form e.g. ligand type on metal transport. In batch assays the impact of metal bound to ligands with defined metal binding properties on the SMA of e.g. granular and crushed sludge can create insight in the metal transport limitations within the granular sludge matrix and the effect on the biological activity.

An example of such SMA experiments exploring the effect of complexes of cobalt with different ligands on the SMA, using both crushed and intact granules showed clear differences in the response (Fig. 5). In all cases, cobalt was added at a concentration of 0.5 μM and the ligands citrate, NTA and EDTA were used. The calculated free metal concentrations in the medium were $6.91*10^{-2}$, $9.9*10^{-7}$ and $1.1*10^{-7}$ μM for citrate, NTA and EDTA, respectively. EDTA is considered not biodegradable, while citrate and NTA are. With both granular and crushed sludge, the highest SMA was observed for the ligand NTA and the increase is exponential. In contrast, for EDTA in both cases the lowest and similar SMA's were found. The SMA increased linearly, probably because the EDTA maintained a constant cobalt concentration in the batch medium (Jansen et al., 2004a). Citrate is initially bio-available but a plateau value is reached for the SMA, indicating the occurrence of a radical change in the cobalt speciation (i.e. low free cobalt available due biodegradation of citrate). A similar plateau was reached using crushed sludge but a higher SMA was observed compared to the granular sludge. The lag phase in the SMA-assay with crushed sludge was considerably shorter for the assay amended with citrate compared to EDTA and NTA amended assays. It would be very worthwhile to develop this experimental set-up further, because it creates possibilities for quantifying the metal transport processes and bio-availability in sludge

granules. Moreover, the results can also be used to model metal transport processes/limitations in granular sludge, e.g. by expanding the model developed by Jansen et al. (2004a,c) which describes the cobalt and nickel uptake by *Methanosarcina sp.*

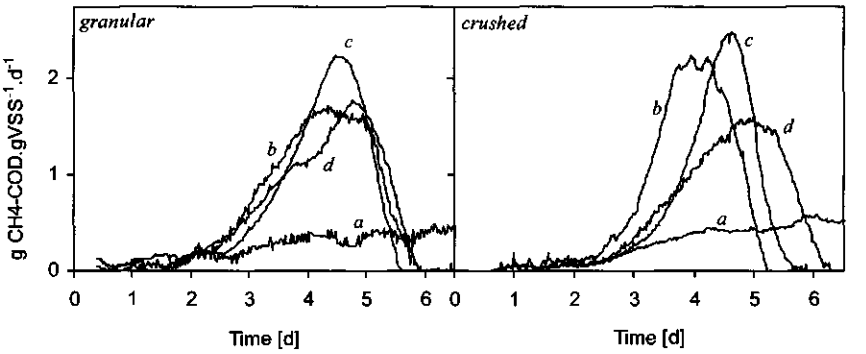


Figure 5 Effect of cobalt complexes with different ligands on the SMA of granular and crushed sludge. (*a*) blanc, (*b*) Citrate, (*c*) NTA, (*d*) EDTA.

Population dynamics

The impact of metal dosing regimes on the microbial population in granular sludge has not been explored yet. However, very likely the concentration or form in which metals are dosed to a bioreactor will affect the composition of the microbial population or may induce selective stress on a particular population. Use of molecular ecology techniques such as denaturing gradient gel electrophoresis (DGGE) or fluorescent in situ hybridisation (FISH) could be used to monitor the changes in biomass compositions resulting from the imposed metal dosing regime. Such knowledge may be used to steer bioreactors with mixed cultures to desired microbial populations and conversions, e.g. H_2/CO_2 conversion to methane or acetate, competition between sulfate reducing bacteria and methanogens.

REFERENCES

Alloway BJ (1990). Heavy metals in soils, 1st ed. (pp 7-27). Chapman and Hall

Florencio L, Field JA, Lettinga G (1995) Substate competition between methanognes and acetogens during the degradation of methamol in UASB reactors. *Wat Res*, **29**, 915-922

Gonzalez-Gil G, Jansen S, Zandvoort MH, Leeuwen van HP (2003). Effect of yeast extract on speciation and bioavailability of nickel and cobalt in anaerobic bioreactors. *Biotechnol Bioeng*, **82**, 134-142

Hullebusch van ED, Zandvoort MH, and Lens PNL, (2003), Metal immobilisation by biofilms: Mechanisms and analytical tools, *Re/Views in Env Sci Bio/Technol,* **2**, 9-33

Hullebusch van ED, Sudarno U, Zandvoort MH, Lens PNL (2005) Comparison of three sequential extraction procedures to describe metal fractionation in anaerobic granular sludges, *Talanta*, **65**, 549-558

Hullebusch van ED, Zandvoort MH and Lens PNL, (2004), Nickel and Cobalt sorption on anaerobic granular sludges: kinetic and equilibrium studies, *J Chem Technol Biotechnol*, **79**, 1219-1227

Jansen S, Jiang B, Buykx S, Stams AJM, Leeuwen HP (2004a) Uptake of Co and Ni by *Methanosarcina barkeri* and influence on growth on methanol: a systematic study and model description (submitted)

Jansen S, Gonzalez-Gil G, Leeuwen van HP (2004b) The impact of Co (II) and Ni (II) speciation in sulfidic media on methanogensis. (submitted)

Jansen S, Gonzalez-Gil G, Leeuwen van HP (2004c) Modeling uptake of Co and Ni by methanogenic bacteria and effects on growth in bioreactor media: the influence of speciation, precipitation and mass transfer. (submitted)

Jong T, Parry DL (2004) Heavy metal speciation in solid-phase materials from a bacterial sulfate reducing bioreactor using sequential extraction procedure combined with acid volatile sulfide analyses. *J Environ Monit*, **6**, 278-285

Lens PNL,van As H (2003). Use of ^{1}H NMR to study transport processes in biofilms. In: Biofilms in Medicine, Industry and Environmental Biotechnology: analysis, compostion and control. Lens P, Mahony T, Moran T, Stoodley P & O'Flaherty V. (Ed.). International Water Association, London, U.K. pp. 285-307

Osuna MB, Zandvoort MH, Iza JM, Lettinga G and Lens PNL (2002) Effect of metal addition on volatile fatty acid conversions in anaerobic granular sludge reactors. *Environ Technol*, **24**, 573-587

Osuna, MB, Hullebusch van ED, Zandvoort MH, Iza J, Lens PNL (2004) Effect of cobalt sorption on metal fractionation in anaerobic granular sludge. *J Env Qual,* **33**: 1256-1270

Prange A, Modrow (2002) X-ray adsorption spectroscopy and its application in biological, agricultural and environmental research *Re/Views in Environ Sci & Bio/Technol,* **1**, 259-276

Rowlette P (2005). The effect of trace metals on sulfate reduction under acidifying conditions (pH 5), MSc Thesis. Sub-department of environmental technology. Wageningen University, in Dutch

Smith MR, Mah RA (1978) Growth and methanogenesis by Methanosarcina strain 227 on acetate and methanol. *Appl Environ Microb*, **36**, 870-879

Temminghof EJM, Plette ACC, Eck van R, Riemsdijk van WH (2000) Determination of the chemical speciation of trace metals in aqueous systems by the Wageningen donnan membrane technique. *Anal Chim Acta*, **417**, 149-157

Zandvoort MH, Van Hullebusch ED, Peerbolte A, Golubnic S, Lettinga G (in preparation) Dual metal limitation (nickel and cobalt) limitation in a methanol fed methanogenic granular sludge reactor.

Zhang H and Davison W. (2000). Direct In Situ Measurements of Labile Inorganic and Organically Bound Metal Species in Synthetic Solutions and Natural Waters Using DGT. *Anal Chem*, **72**, 4447-4457

Hoofdstuk 8

Samenvatting en discussie

DOEL

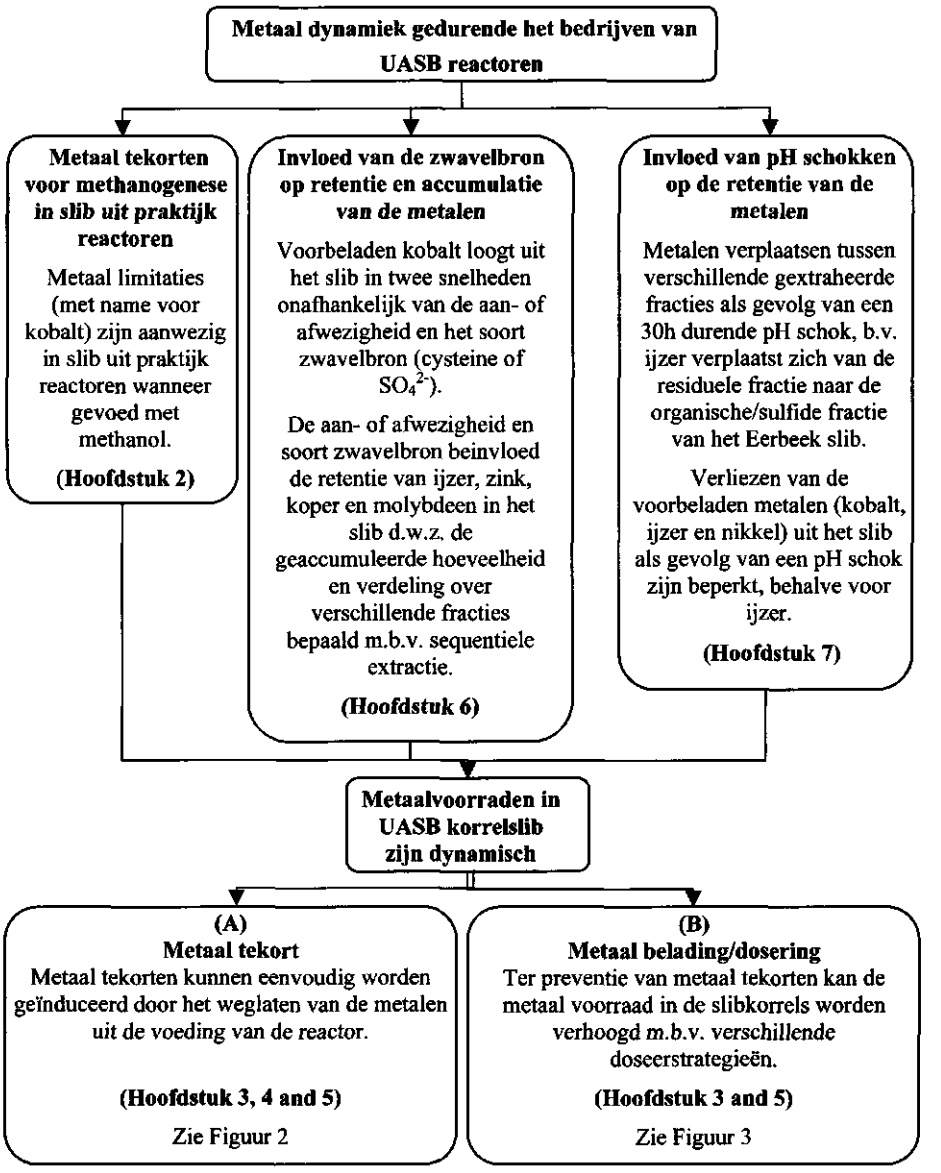
Het hoofddoel van dit proefschrift is het beschrijven van de dynamiek van de sporenmetalen in methanol gevoede anaërobe korrelslib reactoren en hoe deze dynamiek het functioneren van de reactor beïnvloed. Vanuit praktisch oogpunt is het vereist inzicht te verkrijgen in de metaal dynamiek, dit om limitaties van essentiële metalen te kunnen voorzien en om te kunnen bepalen wanneer dosering van de metalen in de praktijk nodig is. Verder zal dergelijke kennis leiden tot een meer rationele doseringswijze van metalen in praktijk situaties, d.w.z. het bereiken van optimale substraat omzettingssnelheden en preventie van reactor verstoringen met een minimum aan metalen. Daarom richt dit onderzoek zich op de retentie, accumulatie en verlies van sporenmetalen in anaëroob korrelslib en de factoren die deze processen beïnvloeden. Figuren 1, 2 en 3 illustreren de verschillende aspecten van de metaal dynamiek in anaërobe bioreactoren die worden besproken in dit onderzoek.

SAMENVATTING EN DISCUSSIE

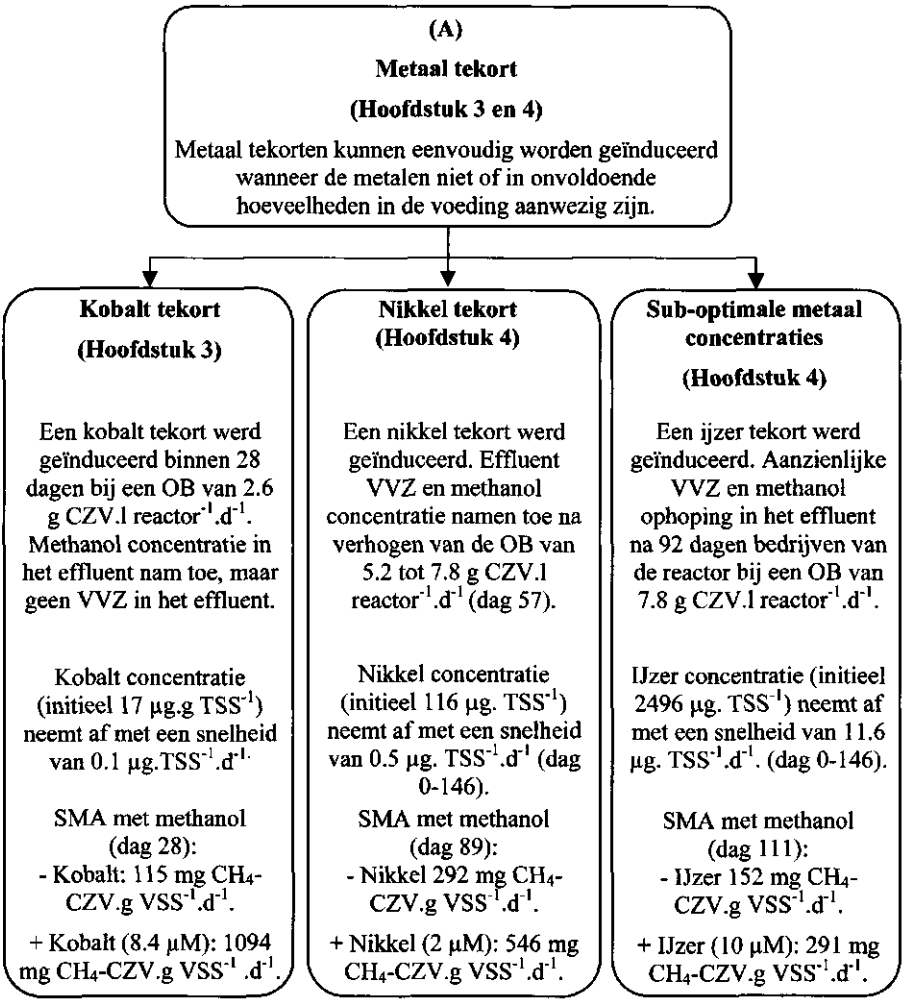
Metaal tekorten

Het gehalte aan individuele sporenmetalen in het slib is een belangrijke parameter voor het stabiel opereren van anaërobe bioreactoren, in sommige gevallen kan dit gehalte reeds indiceren voor welk metaal een tekort zal ontstaan. Het screenen van een aantal slibsoorten uit praktijkschaal reactoren (welke verschillende afvalwaters behandelen) voor de respons op het toedienen van metaal, gebruik makende van verschillende substraten methanol, acetaat of H_2/CO_2 [Hoofdstuk 2], laat zien dat metaal tekorten in praktijk reactoren reeds aanwezig kunnen zijn. Hoewel dit voornamelijk het geval is met het substraat methanol. Toediening van 5 μM kobalt resulteerde in dit geval in een aanzienlijke toename van de specifieke methanogene activiteit (SMA) (Fig. 2, Hoofdstuk 2). Het totaal gehalte aan kobalt in de vier geteste slibsoorten in Hoofdstuk 2 varieerde van 27 tot 51 $\mu g.g\ TSS^{-1}$, dit kobalt is kennelijk niet direct biologisch beschikbaar voor de micro-organismen en het verhogen van de SMA met methanol. Initiële tekorten in het slib voor andere metalen komen ook voor, bijvoorbeeld voor nikkel in Eerbeek slib [Zandvoort et al., in voorbereiding].

Wanneer metaal tekorten initieel niet aanwezig zijn in het slib, kunnen deze eenvoudig worden geïnduceerd in het (korrel)slib door het in een UASB reactor voor langere tijd (1-3 maanden) te voeden zonder het toedienen van metalen (Tabel 1). In methanol gevoede UASB reactoren geënt met Nedalco korrelslib, opererend bij een temperatuur van 30 °C, pH 7 en een stapsgewijs toenemende slibbelasting van 0.3 naar 0.9 g $COD.g\ VSS^{-1}.d^{-1}$, ontwikkelden sporenmetaal tekorten in de afwezigheid van kobalt (±30 dagen; Hoofdstuk 3), nikkel (±90 dagen; Hoofdstuk 4) of ijzer (± 150 dagen; Hoofdstuk 4).

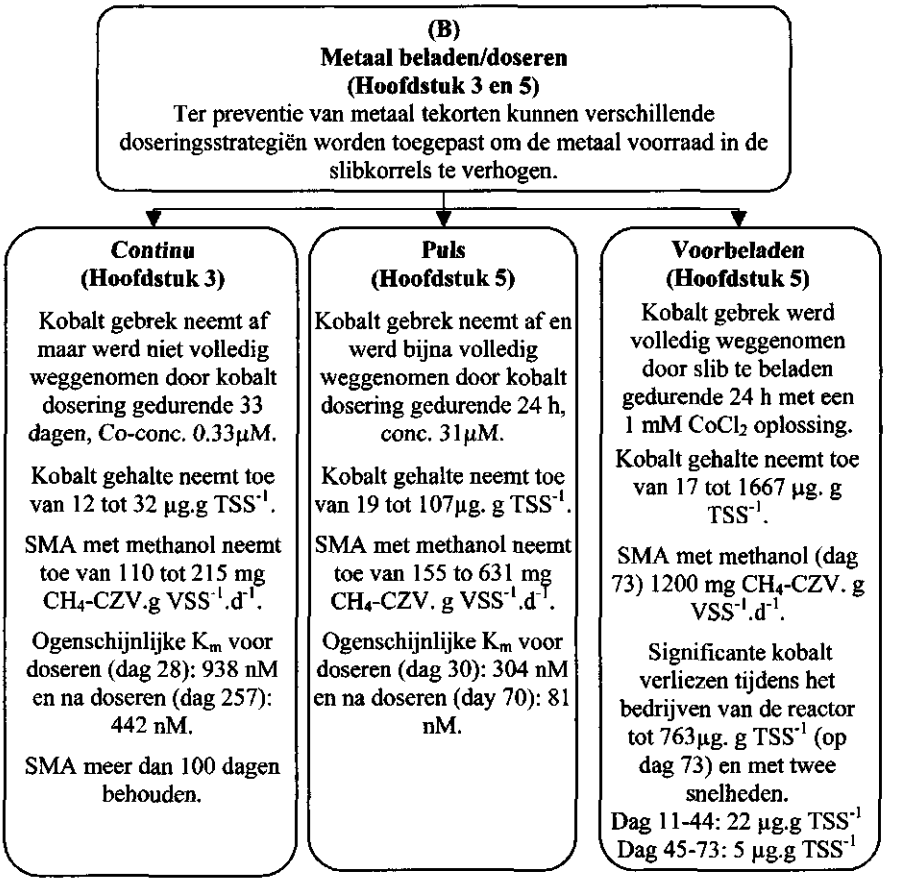


Figuur 1 Overzicht van de structuur van dit proefschrift en de aspecten van metaal dynamiek in UASB reactoren die hierin worden behandeld.



Figuur 2 Overzicht van de resultaten in dit proefschrift betreffende het metaal depriveren van korrelslib. Afkortingen: vluchtige vetzuren (VVZ), chemisch zuurstof verbruik (CZV), organische belasting (OB), specifieke methanogene activiteit (SMA)

Gebruik makende van een ander slibsoort, Eerbeek korrelslib [Zandvoort et al., in voorbereiding], met een initieel kobalt gehalte van 59 µg. g TSS^{-1}, 2.5 keer het gehalte van het Nedalco slib (en het hoogste initiële gehalte gemeten in dit onderzoek), kon binnen 50 dagen een gebrek aan kobalt worden geïnduceerd. Hierbij moet worden opgemerkt dat zonder het toevoegen van kobalt reeds een hoge SMA werd bereikt.



Figuur 3 Overzicht van de resultaten in dit proefschrift m.b.t. kobalt uitloging en dosering. Afkortingen: Specifieke methanogene activiteit (SMA).

Tekorten voor metalen kunnen ook ontstaan met andere substraten dan methanol. Het weglaten van de sporenmetalen uit het influent van een UASB reactor (30°C; pH 7) geënt met Nedalco korrelslib en gevoed met een cocktail van acetaat, propionaat en butyraat, resulteerde in een slib met een 30% lagere SMA vergeleken met een slib in een parallel geopereerde reactor waar alle metalen werden toegediend aan de voeding. Tevens werd propionaat niet omgezet in de metaal gelimiteerde reactor [Osuna et al., 2002]. Het opereren van thermofiele UASB reactoren geënt met Eerbeek korrelslib, gevoed met glucose (T=55°C, OLR of 5 g COD.l reactor^{-1}.d^{-1}) en bedreven bij lage pH's van 4 en 5 leidt ook tot metaal tekorten. Toediening van de volledige sporenmetalen cocktail aan het slib in activiteitstesten verhoogde de snelheid waarmee waterstof werd gevormd vanuit glucose [Rowlette, 2005].

Tabel 1. Overzicht van de kobalt gehaltes en SMA van korrelslib in labschaal UASB bioreactoren

Entslib			**(Na) start-up**					**Einde van het experiment**					
Kobalt Gehalte µg.g TSS^{-1}	Substraat	OB[a]	Inf. kobalt µM	Dag	Kobalt µg.g TSS^{-1}	SMA mg CH_4 CZV. g $VSS.d^{-1}$ - Kobalt	SMA mg CH_4 CZV. g $VSS.d^{-1}$ + Kobalt[b]	Dag	Kobalt µg.g TSS^{-1}	SMA mg CH_4-CZV. g $VSS.d^{-1}$ -Kobalt	SMA mg CH_4-CZV. g $VSS.d^{-1}$ + Kobalt[b]	Doel	Ref
17	Methanol	7.8	0	28	n.d	110	1094 (8.4)	255	12	215[c]	507 (8.4)	Kobalt tekort	[1]
17	Methanol	7.8	0.8	28	n.d.	1549	1517 (8.4)	259	237	n.d.	193 (8.4)	Sub-opt, metaal condities[d]	[2]
17	acetaat,propionaat , butyraat (3:1:1)	10	0	n.d.	n.d.	n.d.	n.d.	140	10	175	175 (6.3)	Tekort alle metalen[e]	[3]
17	acetaat,propionaat , butyraat (3:1:1)	10	6.3	n.d.	n.d.	n.d.	n.d	140	2890	260	260 (6.3)	Tekort alle metalen[e]	[3]
19	Methanol	15	0	0	19	487	521 (5.0)	48	n.d.	402	1927 (5.0)	Tekort alle metalen	[4]
59	Methanol	10	0	0	59	237	245 (5.0)	44	51	907	1010 (5.0)[f]	Tekort alle metalen	[4]
59	Methanol	20	0	0	59	243	245 (5.0)	55	50	1687	2515 (5.0)[g]	Kobalt tekort	[4]
17	Methanol	20	0 (31)[h]	30	20	155	540 (5.0)	77	57	631	828	Pulse doseren Kobalt	[5]
1667	Methanol	20	0	30	1169	906	843 (5.0)	77	763	1200	1126	Voorbeladen Kobalt	[5]
1990	Methanol	5	0	29	1370	80	n.d.	118	470	1119	n.d.	S-bron vs. Metaal retentie	[6]
1990	Methanol	5	0	29	1540	744	n.d.	118	610	1382	n.d.	S-bron vs. Metaal retentie	[6]

[a] Organische belasting (g CZV.l $reactor^{-1}.d^{-1}$); [b] Kobalt concentratie (µM) toegevoegd aan batches weergegeven tussen haakjes; [c] Na het van dag 54 tot 85 continu doseren van 0,33 µM kobalt met het influent; [d] Alle metalen toegevoegd aan de reactor in suboptimale concentraties; [e] Alle metalen (Co, Ni, Fe, Mn, Cu, Zn, Mo, Se) weggelaten uit influent en slib is getest op de respons op additie van enkel kobalt en additie van alle metalen; [f] Entslib bron Eerbeek slib; activiteit in de afwezigheid van alle andere sporenmetalen, in de aanwezigheid van alle sporenmetalen was de activiteit 1543 mg CH_4-CZV. g VSS^{-1}; [g] Entslib bron Eerbeek sludge [h] Pulse doseren gedurende 24h op dag 57; n.d. not determined; referenties, [1] Hoofdstuk 3, [2] Hoofdstuk 4, [3] Osuna et al., 2003; [4] Zandvoort et al., (in voorbereiding). [5] Hoofdstuk 5; [6] Hoofdstuk 6.

Metaal dynamiek in korrelslib

Uitlogen

Wanneer onvoldoende sporenmetalen aan het influent worden toegediend zal tijdens het bedrijven van de reactor het gehalte aan sporenmetalen in het slib afnemen door uitloging. [Hoofdstuk 3 en 4]. De verliezen uit het slib zijn (voornamelijk voor kobalt, nikkel en ijzer) proportioneel met hun initiële gehalte in het slib [Hoofdstuk 3]. Een deel van de waargenomen afname kan worden toegeschreven aan "verdunning" als gevolg van groei van nieuwe biomassa, maar - zoals aangetoond door de resultaten in Hoofdstuk 3 -, overschrijden de verliezen van nikkel en ijzer, ondanks de 30% toename van het slibbedvolume, duidelijk de verdunningsfactor als gevolg van de groei van nieuwe biomassa.

De metaal retentie eigenschappen van korrelslib uit verschillende praktijkschaal reactoren kunnen aanmerkelijk verschillen [Hoofdstuk 7; Osuna et al., 2004]. Deze verschillende eigenschappen zullen ook resulteren in verschillende metaal retentie eigenschappen gedurende het bedrijven van de reactor. Bijvoorbeeld het vooraf beladen van Nedalco slib met kobalt resulteert in een relatief snelle initiële kobalt afname van ± 22 μg.g $TSS^{-1}.d^{-1}$ vanuit uitwisselbare en carbonaat fractie. Nadat deze fracties zijn uitgeput loogt kobalt uit de organische/sulfide fractie van het slib met een substantieel lagere snelheid van ± 9 μg. g $TSS^{-1}d^{-1}$ [Hoofdstuk 6].

Een dergelijke twee fase uitloging deed zich niet voor in voorbeladen Eerbeek slib. Na 194 dagen bedrijven van de reactor was het kobalt verlies slechts 28%, terwijl een kobalt verlies van 63% werd gevonden voor het voorbeladen Nedalco slib [Hoofdstuk 7]. Echter moet worden opgemerkt dat het kobalt gehalte van het Eerbeek slib met 1.18 mg.g TSS^{-1} veel lager is dan het gehalte van het Nedalco slib, welke een kobaltgehalte van 2.93 mg.g TSS^{-1} had. Daarbij verschilde de verdeling over de verschillende fracties voor beide slibsoorten aanzienlijk, d.w.z. in tegenstelling tot in het Nedalco slib accumuleerde kobalt in het Eerbeek slib nauwelijks in de minder sterk gebonden uitwisselbare en carbonaat fracties. Sorptie experimenten hebben laten zien dat de sorptie affiniteit voor kobalt and nikkel het hoogst is voor organische/sulfide fracties van beide slibben [van Hullebusch et al., 2004], hetgeen de waargenomen verschillen in uitloog dynamiek van de beide slibsoorten verklaart. Dit indiceert dat de metalen gedoseerd met het influent bij voorkeur accumuleren in de fractie met de hoogste sorptie affiniteit, dit is inderdaad bevestigd in experimenten waarbij kobalt in-situ werd gedoseerd [Hoofdstuk 5].

Metaal in korrelslib – sequentiële extractie

In korrelslib zijn de metalen verdeeld over de verschillende bestanddelen van de slibmatrix, biomassa, extracellular polymeric substances (EPS) en anorganische neerslagen

zoals sulfiden, fosfaten en carbonaten [van Hullebusch et al., 2003]. Vanzelfsprekend kan het totaal gehalte aan metalen van het slib nauwelijks relevante informatie verschaffen m.b.t hoe de metalen zijn gebonden in de slibmatrix en met name over hoe sterk ze zijn gebonden. De sequentiële extractie methode zoals gebruikt in dit onderzoek is waardevol voor het verkrijgen van inzicht in de manier waarop metalen zijn gebonden in het slib, dit omdat de methode informatie verschaft over de verdeling van de metalen over operationeel gedefinieerde fracties. De verliezen en accumulatie van sporenmetalen in de verschillende fracties van het korrelslib in UASB reactoren is in hoofdstuk 6 gevolgd als een functie van de tijd. Tevens toonde het gebruik van de sequentiële extractie methode aan dat een verplaatsing van metalen tussen de verschillende fracties optreedt als gevolg van opgelegde pH schokken, d.w.z. metalen verplaatsten van de residuele fractie naar de organische/sulfide fractie [Hoofdstuk 7]. Blijkbaar kunnen significante veranderingen optreden in de manier waarop de metalen zijn gebonden in het slib als gevolg van de reactorcondities, hetgeen uiteraard ook gevolgen heeft voor de retentie en de biologische beschikbaarheid van de metalen.

Dit onderzoek laat zien [Hoofdstuk 6 en 7] dat de carbonaat, en met name de organische/sulfide fractie, belangrijke fracties zijn voor het vasthouden van metalen in anaëroob korrelslib. Hetzelfde werd gevonden door van Hullebusch et al. [2005], tevens vonden zij een duidelijk verband tussen de hoeveelheid zwavel die vrijkomt tijdens de organische/sulfide fractie extractie en de hoeveelheid kobalt, koper nikkel en zink in deze fractie van het slib. Sulfide is altijd aanwezig in anaërobe bioreactor systemen, daarom is het een belangrijke factor in de retentie van metalen in bioreactoren, niet alleen omdat het van belang is voor het invangen van metalen in het slib [Hoofdstuk 6], maar vooral omdat verliezen uit deze fractie gering zijn ten opzichte van de andere fracties [Hoofdstuk 6 en 7]. De organische/sulfide fractie is daarom (samen met de residuele fractie) van essentieel belang voor de retentie van metalen in korrelslib op langere termijn.

Effect van de zwavelvorm op de metaal retentie

De retentie van kobalt in het voorbeladen slib wordt niet significant beïnvloed door de aanwezigheid en/of de vorm van de zwavelbron in het influent van de UASB reactor [Hoofdstuk 6]. Echter lage influent concentraties (13 $mg.l^{-1}$) van een zwavelbron hebben reeds een significant effect op de metaal retentie/accumulatie in het slibbed [Hoofdstuk 6]. In de afwezigheid van een zwavelbron accumuleerde zink bij voorkeur in de carbonaat fractie en nadat L-cysteine werd toegevoegd aan het influent nam de zink concentratie in de carbonaat fractie aanzienlijk af terwijl de concentratie toenam in de organische/sulfide fractie. Dit indiceert dat de zink aanwezig in de carbonaat fractie is omgezet in zinksulfiden en dat de manier waarop zink in het slib wordt vastgehouden dus aanzienlijk is veranderd. IJzer (en molybdeen) accumuleerden niet in het slib in de afwezigheid van een zwavelbron. Het type

zwavelbron bepaalde duidelijk de geprefereerde fractie voor ijzer accumulatie d.w.z. ijzer accumuleerde in de carbonaat fractie wanneer zwavel werd toegediend als cysteine en in de residuele fractie wanneer het wordt toegediend als sulfaat.

Effect van de pH op de metaal retentie

In het algemeen neemt onafhankelijk van de mineralogische samenstelling de oplosbaarheid van metalen toe bij lagere pH waarden [Alloway, 1990]. De pH van de reactorvloeistof zou daarom een belangrijke operationele parameter kunnen zijn welke de metaal retentie in anaërobe slibkorrels kan beïnvloeden. Uit dit onderzoek blijkt dat korte pH schokken tot pH 5 (30 h en 4 dagen) de metaalspeciatie in korrelslib, voorbeladen met kobalt, nikkel en ijzer, inderdaad sterk beïnvloed [Hoofdstuk 7]. Het is echter opmerkelijk dat de metaal verliezen met het effluent als gevolg van de pH schok, behalve voor ijzer, beperkt blijven. De oorsprong van het slib beïnvloed tevens het effect van de pH schok. De 30 h durende pH schok veroorzaakte in het Eerbeek slib een verplaatsing van ijzer, kobalt, nikkel en zwavel van de residuele naar de organische/sulfide fractie, waarschijnlijk veroorzaakt door de dissolutie en het opnieuw neerslaan van metaalsulfiden. Een dergelijke verplaatsing werd niet waargenomen tijdens de 30 h durende pH schok in het Nedalco slib, echter de 4 dagen durende pH schok resulteerde in significante verliezen van kobalt (18%), ijzer (29%) en zwavel (29%) uit de organische/sulfide fractie. Deze verliezen worden waarschijnlijk veroorzaakt door de dissolutie van kobalt- en ijzersulfiden. De effecten van dergelijke verliezen en veranderingen in de metaal retentie op de biomassa in de anaërobe slibkorrels is nog niet duidelijk. Vanzelfsprekend zijn metaalverliezen zeer ongewenst, daarentegen zou een zekere mate van dissolutie/mobilisatie van metalen in het korrelslibbed of in een slibkorrel als gevolg van pH gradiënten de biologische beschikbaarheid van de metalen kunnen laten toenemen.

Metaaldosering

Hoeveelheid

Het is duidelijk dat er nog steeds geen "magische" procedure voor het toedienen van sporenmetalen aan UASB reactoren bestaat. Het lijkt echter redelijk dat een procedure wordt ontwikkeld die de metaal dosering in de praktijk afstemt op het gehalte aan metalen aanwezig in het slib en het influent. In het algemeen bevatten afvalwaterstromen reeds een aantal metalen, kennis van de metaalsamenstelling van het afvalwater zal kunnen bijdragen aan de optimalisatie van de metaaldosering, sommige metalen kunnen eventueel achterwege worden gelaten in de metaal cocktail die wordt toegediend aan het influent. Kennis m.b.t. tot het metaalgehalte van het entslib is niet voldoende voor het voorspellen van metaal tekorten [Hoofdstuk 2], eens te meer omdat het uitlogen van metalen uit het slib gedurende het

bedrijven van de reactor [Hoofdstuk 3 and 4] uiteindelijk kan leiden tot tekorten. Daarom zou om de snelheid van de metaal verliezen te bepalen het totaal gehalte aan metalen in het slib tijdens het bedrijven van de reactor regelmatig moeten worden gemeten (bijvoorbeeld maandelijks). Dit zou bijvoorkeur moeten worden gecombineerd met de bepaling van de metabole eigenschappen van het slib en het effect van metaal additie op de SMA. Indicatieve waarden voor de snelheid waarmee de metalen accumuleren in het slib kunnen worden afgeleid uit dit onderzoek, bijvoorbeeld continue dosering van respectievelijk 0.84 μM en 0.4 μM kobalt en nikkel leidde tot een toename van de metaalgehalte in het slib met een snelheid van ongeveer 0.82 μg.g $TSS^{-1}.d^{-1}$ en 0.4 μg.g $TSS^{-1}.d^{-1}$ voor respectievelijk kobalt en nikkel [Hoofdstuk 4].

Wanneer de resultaten van dit onderzoek worden geëxtrapoleerd naar de praktijkschaal, zoals bijvoorbeeld de Nedalco anaërobe afvalwaterzuivering waar de UASB reactor gemiddeld 10.000 kg slib bevat met kobaltgehalte van 20 μg.g TSS^{-1}, betekend dit dat de reactor in totaal 200 g kobalt bevat. Volgens de resultaten uit Hoofdstuk 3, zal het kobalt gehalte van het slib afnemen met een snelheid van 0.1 μg.g $TSS^{-1}.d^{-1}$, resulterend in een dagelijks verlies van 1 g kobalt uit de reactor. Om het kobalt gehalte van het Nedalco slib toe te laten nemen tot 60 μg.g TSS^{-1}, d.w.z. het kobalt gehalte van het Eerbeek slib welke een aanzienlijk hogere SMA met methanol kon bereiken [Zandvoort et al., in voorbereiding], zou 400 g kobalt nodig zijn aannemend dat al het kobalt word vastgehouden in het slib.

Kobalt doseerstrategieën

Een rationele doseringstrategie voor sporenmetalen is nodig om een stabiel opererende bioreactor met maximale substraatomzettingssnelheden te verkrijgen, waarbij de verliezen van de metalen met het effluent worden geminimaliseerd. Van de drie in dit proefschrift bestudeerde metaal doseerstrategieën aan methanol gevoede bioreactoren (Fig. 3), wordt het continue doseren van kobalt geprefereerd vanwege de lage hoeveelheid benodigd kobalt en de minimale verliezen met het effluent [Hoofdstuk 3]. Dat continue dosering bij lage concentratie gunstig zou zijn is reeds eerder gesuggereerd [Gonzalez-Gil et al., 1999]. Continue dosering resulteert in relatief hoge vrije metaalconcentraties welke direct beschikbaar zijn voor opname door de biomassa, op deze manier wordt voorkomen dat de dissolutie de beperkende factor wordt voor de groei van micro-organismen [Gonzalez-Gil et al., 1999]. Echter in ons onderzoek werd een relatief beperkt effect op de specifieke methanogene activiteit gevonden als gevolg van een 33 dagen durende continue dosering van kobalt met een concentratie van 0.33 μM, tevens kon het in het korrelsib aanwezige kobalt tekort niet volledig worden weggenomen [Hoofdstuk 3]. Na het beëindigen van de kobalt dosering kon de methanogene activiteit en de methanol verwijderingcapaciteit meer dan 100 dagen worden behouden. Er werd een geringe toename in het slibbed volume waargenomen van 30%, veel minder dan in

de reactoren bedreven onder dezelfde condities met kobalt in het influent [Hoofdstuk 4]. Dit indiceert dat metaaldosering ook kan worden gebruikt om de overproductie van slib te beperken.

Puls doseren (in-situ beladen) van kobalt kan worden gezien als een doseerstrategie tussen het voorbeladen en continue doseren. Deze strategie is zeer effectief in het bijna direct wegnemen van acute kobalt tekorten [Hoofdstuk 5]. Echter met deze procedure waren de verliezen van kobalt uit het slib aanzienlijk hoger vergeleken met de continue doseerstrategie. Tevens moet worden opgemerkt dat, in tegenstelling tot de continue doseerstrategie, er een duidelijke toename van de vluchtige vetzuur (VVZ) concentratie in het effluent werd waargenomen na puls doseren van kobalt.

Voorbeladen, hoewel effectief in het wegnemen van kobalt tekorten en resulterend in een relatief hoge SMA van het slib, was ineffectief in het beperken van kobalt (metaal) verliezen [Hoofdstuk 5 and 6]. Met name onmiddellijk na het opstarten van de reactor waren deze verliezen aanzienlijk (Fig. 3; Hoofdstuk 5).

Sporenmetalen versus macronutriënten dosering

IJzer kan niet echt worden geclassificeerd als een sporenmetaal, dit omdat het metaal in relatief hoge concentraties aanwezig is in de slibkorrels, bijvoorbeeld 75.7 mg.g TSS^{-1} in het Hoogeveen slib [Hoofdstuk 2], tevens wordt ijzer normaliter gedoseerd in relatief hoge concentraties. Het ijzer gehalte van 20.8 mg.g TSS^{-1} in het Nedalco slib [Hoofdstuk 7] betekend dat de Nedalco reactor 200 kg ijzer bevat.

De retentie van ijzer bleek erg afhankelijk te zijn van de aanwezigheid van zwavel en van de zwavelbron [Hoofdstuk 6]. Het is niet duidelijk of de dosering van andere metalen samen met ijzer resulteert in een negatieve "sink" voor de sporenmetalen als gevolg van co-precipitatie met ijzer en/of als gevolg van sorptie aan de ijzersulfiden. Dit zou de sporenmetalen minder (niet direct) biologisch beschikbaar kunnen maken. Naast de constatering dat de micro-organismen voor hun groei hogere concentraties aan ijzer nodig hebben is het aannemelijk dat de ijzersulfide neerslagen belangrijk zijn voor korrelslibstructuur. Het zou daarom verstandig kunnen zijn om ijzer apart van de andere sporenmetalen te doseren. De "echte" sporenmetalen kunnen dan gebonden aan een ligand worden gedoseerd, dit voorkomt dat deze metalen neerslaan, en op deze manier blijven ze (meer) direct biologisch beschikbaar. Een sequentiële doseerprocedure van de individuele sporenmetalen is mogelijk ook een attractieve manier van doseren, dit omdat bijvoorbeeld m.b.t. de biologische opname van kobalt en nikkel een competitief effect is waargenomen [Jansen et al., 2004a].

Anaërobe omzetting van methanol

De sterke kobalt afhankelijkheid maakt de mesofiele omzetting van methanol een zeer goed modelsysteem om de processen gerelateerd aan bijvoorbeeld de metaalopname, biologische beschikbaarheid en de impact van metaaldosering op de SMA van korrelslib in anaërobe bioreactoren te bestuderen. Echter langdurig bedrijven van een reactor met enkel methanol als substraat leidt regelmatig tot reactor instabiliteit, d.w.z. verzuring, dit compliceert studies m.b.t. de lange termijn effecten van sporenmetaal deprivatie.

Overbelasting is een belangrijke factor voor het initiëren van VVZ accumulatie in het effluent [b.v. Hoofdstuk 4 en 5], maar ook pH schokken lijken een dergelijke VVZ accumulatie te induceren [Hoofdstuk 7]. Daarbij lijken metabole veranderingen in het slib een belangrijke factor te zijn voor de ophoping van VVZ, dit omdat wanneer een "gezonde" acetotrofe methanogene populatie aanwezig is het gevormde acetaat niet zou ophopen in het effluent.

Door de reactor met enkel methanol als substraat te bedrijven wordt het slib verrijkt met *Methanosarcina sp.*, het organisme dat verantwoordelijk is voor de directe omzetting van methanol naar methaan. *Methanosarcina strain 227* gekweekt met methanol als het enige substraat is niet in staat acetaat te gebruiken in de aanwezigheid van methanol, methanol is het geprefereerde substraat en word als eerste omgezet. In sommige gevallen was *Methanosarcina* zelfs helemaal niet in staat om acetaat om te zetten [Smith and Mah, 1978]. Dit indiceert dat *Methanosarcina sp.* niet zullen bijdragen aan de omzetting van acetaat wanneer dit aanwezig is in het influent of wanneer het wordt gevormd in de aanwezigheid van hoge methanol concentraties. Het is daarom aannemelijk dat de aanwezigheid van een acetotrofe methanogene populatie van bijvoorbeeld *Methanosaeta sp.* een belangrijke factor is waarmee de accumulatie van VVZ in methanol gevoede bioreactoren zou kunnen worden voorkomen. In de experimenten beschreven in Hoofdstuk 6, werd na het 118 dagen bedrijven van de reactor een relatief hoge SMA met acetaat van 608 mg CH_4-CZV.g $VSS^{-1}.d^{-1}$ gemeten. Een significante toename in de VVZ concentratie in het effluent bleef achterwege na een 4 dagen durende reactor verstoring d.w.z. een toename van de methanol concentratie in het effluent tot waarden hoger dan 1000 mg $CZV.l^{-1}$, hetgeen normaal gesproken VVZ accumulatie induceert [Florencio et al., 1995]. Dit indiceert dat een hoge SMA met acetaat van belang is voor het voorkomen van reactorverzuring, zelfs in de aanwezigheid van hoge kobalt concentraties, een ander vereiste is voor het ontstaan van VVZ accumulatie [Florencio et al., 1995].

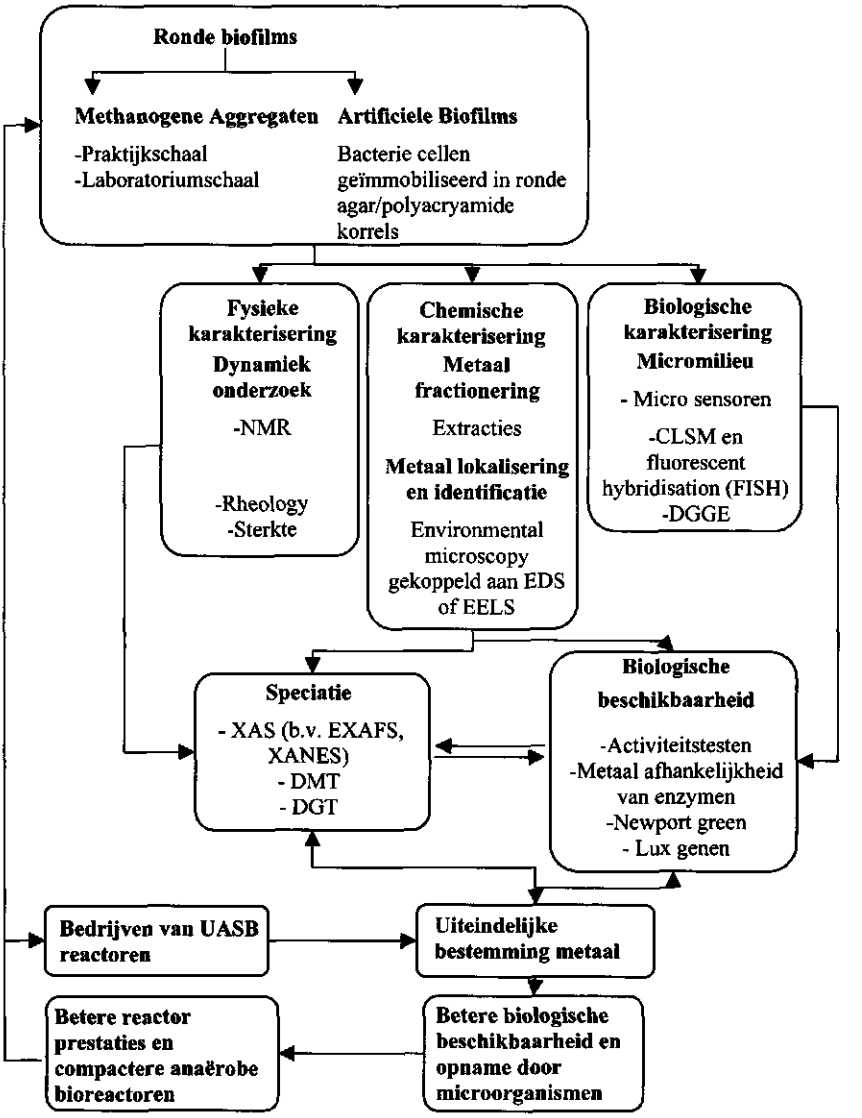
PERSPECTIEVEN

Dit proefschrift heeft enkel het gedrag van metalen in anaëroob korrelslib/bioreactoren en de invloed hiervan op de methanol omzetting verkend. Dit onderzoek creëert echter nieuwe perspectieven voor bijzonder relevant vervolgonderzoek en zeker niet alleen m.b.t. anaërobe waterzuivering. Vanwege de complexiteit van de verschillende processen betrokken bij de retentie en biologische beschikbaarheid van metalen in bioreactoren/biofilms is een goed georganiseerd langdurig multidisciplinair onderzoek vereist. Een aantal suggesties voor vervolgonderzoek worden hieronder gepresenteerd.

Retentie van metalen

De mechanismen betrokken bij de metaalimmobilisatie in de slibkorrels, m.b.t. bijvoorbeeld de chemische speciatie, (bio)-sorptie, ruimtelijke verdeling en massatransport, moeten in detail worden opgehelderd. Recent is een uitgebreid overzichtsartikel over de mechanismen betrokken bij de metaalimmobilisatie in biologische aggregaten (biofilms en korrels) en de analytische technieken die kunnen worden gebruikt om deze immobilisatie te bestuderen gepubliceerd [van Hullebusch et al., 2003]. In Figuur 4 worden deze analytische technieken gepresenteerd en wordt aangegeven hoe deze kunnen worden gecombineerd om het inzicht in de metaal dynamiek en beschikbaarheid in biofilms te vergroten, hetgeen uiteindelijk in de praktijk zal leiden tot betere reactor prestaties.

Dit onderzoek laat zien dat de organische/sulfide fractie belangrijk is voor het invangen en de lange termijn retentie van metalen in het korrelslib. Deze fractie heeft ook de hoogste affiniteit voor de sorptie van kobalt en nikkel [van Hullebusch et al., 2004]. Waarschijnlijk zijn de meeste metalen in deze fractie gebonden als metaalsulfide. Met de gewijzigde Tessier sequentiële extractie procedure zoals gebruikt in dit onderzoek is het echter niet mogelijk de organisch gebonden sulfide te onderscheiden van metaalsulfide neerslagen. De zwavelspeciatie in het korrelslib vereist daarom meer aandacht bijvoorbeeld m.b.t. de amorfe en kristallijne staat van de metaalsulfiden, omdat deze de oplosbaarheid, mobiliteit en biologische beschikbaarheid van de sulfiden bepaalt. Dit kan worden gedaan door de zogenaamde "acid volatile sulfide" (AVS) extractie (deze AVS fractie kan worden beschouwd als de biologisch actieve/beschikbare fractie), te koppelen met sequentiële extractie [Jong and Parry, 2003] of d.m.v. X-ray adsorption near edge structure spectroscopy (XANES) [Prange and Modrow, 2002].



Figuur 4 Analytische technieken voor het bestuderen van de metaal dynamiek in korrelslib. Nuclear magnetic resonance (NMR), Energy-dispersive X-ray spectroscopy (EDS), Electron energy loss spectroscopy (EELS), Confocal laser scanning microscopy (CLSM), Denaturing gradient gel electrophoresis (DGGE), Fluorescent in situ hybridization (FISH), X-ray adsorption spectroscopy (XAS), Extended X-ray adsorption fine structure (EXAFS), X-ray adsorption near edge structure spectroscopy (XANES), Diffusive gradient thin films (DGT), Donnan membrane technique (DMT).

Vloeistoffase

Naast de retentie en speciatie van metalen in de korrelmatrix moet ook aandacht worden besteed aan de metaalspeciatie en dynamiek in de reactorvloeistof. Het zwart worden van de reactorvloeistof en effluent (zoals b.v. in Hoofdstuk 6) laat zien dat metaalsulfide neerslagen niet alleen in de korrelslibmatrix aanwezig zijn maar ook in de reactor vloeistof, als gevolg hiervan wordt de vrije metaalconcentratie in de reactorvloeistof erg laag. Het vrije metaal is waarschijnlijk de vorm waarin het beschikbaar is voor biologische opname [Jansen et al., 2004b]. Volgens Jansen et al. [2004a] kunnen de extreme lage vrije metaalconcentraties die voorkomen in de reactormedia echter nog steeds voldoende zijn om redelijke metaalopname fluxen te bewerkstelligen. Daarbij kan de vorming van opgeloste metaalsulfiden tevens bijdragen aan het voorkomen van metaaldepletie omdat deze een grote opgelost metaal buffer creëren [Jansen et al., 2004c]. In labschaal reactoren moet de dynamiek van de vrije metaalconcentraties en de labiele complexen worden gevolgd als een functie van de reactorcondities of zelfs als functie van de slibbedhoogte, dit omdat het een belangrijke parameter is voor de metaal dynamiek en biologische beschikbaarheid in bioreactoren. Een veel belovende techniek om deze dynamiek te bestuderen is "diffusive gradient thin films" (DGT) Zhang and Davison [2000], deze techniek kan worden gebruikt om de concentratie van labiele metaalcomplexen te bepalen. Een andere interessante techniek is de Donnan membrane technique (DMT) Temminghoff et al. [2000] hiermee is het mogelijk de vrije metaalconcentratie in de reactorvloeistof te bepalen.

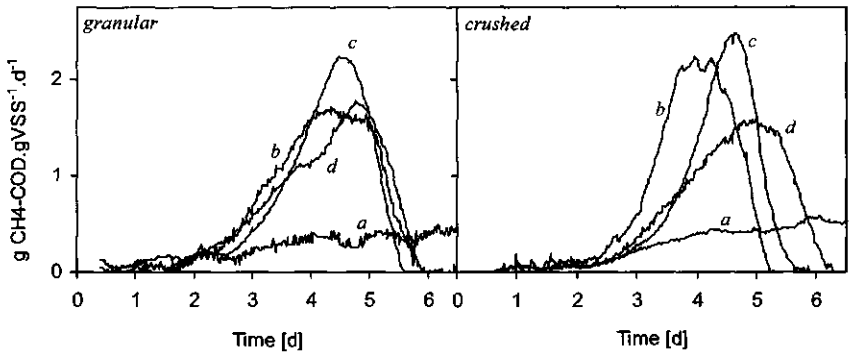
Chemische vorm gedoseerd metaal/biologische beschikbaarheid

Het aan de anaërobe bioreactoren gedoseerde metaal moet beschikbaar zijn voor alle biomassa aanwezig in de korrel. Wanneer de metalen worden gedoseerd als vrije metaal ionen zullen ze waarschijnlijk neerslaan met het altijd aanwezige sulfide, dit maakt de metalen minder of helemaal niet meer biologisch beschikbaar. Door het toedienen van de metalen gebonden aan organische liganden kan een buffer van opgelost metaal worden gevormd en op deze manier wordt voorkomen dat de metalen direct neerslaan als sulfiden, hierbij moet worden opgemerkt dat ook dan de vrije metaalconcentratie wordt bepaald door de neerslag evenwichten. Gonzalez-Gil et al. [2003] vond met een *Methanosarcina* ophopingcultuur dat gistextract, een ongedefinieerd mengsel van organische liganden (aminozuren en eiwitten), de biologische beschikbaarheid van de metalen in de aanwezigheid van sulfide aanzienlijk verbeterde. Ook het binden van kobalt en nikkel aan de ligand EDTA bleek effectief te zijn voor het in oplossing en biologisch beschikbaar houden van deze metalen voor de groei van een reincultuur van *Methanosarcina barkeri* in de aanwezigheid van sulfide [Jansen et al., 2004a]

Het toedienen van metalen gebonden aan liganden lijkt daarom een attractieve manier om de biologische beschikbaarheid van metalen te vergroten, hetgeen vervolgens zal

resulteren in een efficiëntere metaaldosering. Hierbij moet echter worden opgemerkt dat de condities in ophopingsculturen aanmerkelijk verschillen van de condities in de matrix van een slibkorrel. Waarschijnlijk zijn in een bioreactor diffusie, dissolutie en depletie de belangrijkste factoren voor metaal tekorten. Het is daarom aannemelijk dat gebruik van liganden het meest effectief is om sporenmetalen op bioreactor-/slibkorrelniveau toe te dienen [Jansen et al., 2004c]. Echter de invloed van de slibmatrix op het metaal transport en de biologische beschikbaarheid is tot dusverre nauwelijks bestudeerd. Lens en van As [2003] hebben met behulp van "nuclear magnetic resonance" (NMR) de metaal transport in de slibkorrel bestudeerd. Deze techniek zou ook kunnen worden gebruikt om het effect van de vorm van het metaal, bijvoorbeeld metaal gebonden aan liganden, op het metaaltransport in de slibkorrel te bestuderen. In batchtests kan het effect van metalen gebonden aan liganden met gedefinieerde metaal bindingseigenschappen op de SMA, van bijvoorbeeld intact versus gedispergeerd korrelslib, worden bepaald. Dit kan inzicht verschaffen in de metaal transport limitaties in de korrelmatrix en het effect hiervan op de biologische activiteit.

In een dergelijk SMA experiment waarin het effect van verschillende kobalt complexen op de SMA wordt verkent, met zowel intact als gedispergeerd korrelslib, werden duidelijke verschillen in respons geobserveerd (Fig. 5). Het effect van de binding van kobalt aan de liganden citraat, NTA en EDTA is getest, het kobalt werd in alle batches toegevoegd met een concentratie van 0.5 μM. De berekende vrije metaalconcentraties in de batch media waren respectievelijk, $6.91*10^{-2}$, $9.9*10^{-7}$ en $1.1*10^{-7}$ μM voor citraat, NTA en EDTA. Citraat en NTA zijn biologisch afbreekbaar, dit is niet het geval voor EDTA. Met zowel korrelslib als gedispergeerd slib werden de hoogste SMA's bepaald in de batches met de ligand NTA en de toename in de activiteit was exponentieel. Voor EDTA werd in beide gevallen de laagste SMA bepaald. De SMA nam lineair toe waarschijnlijk omdat de EDTA zorgt voor een constante kobalt concentratie in het batch medium [Jansen et al., 2004a]. Het citraatcomplex is initieel biologisch beschikbaar maar uiteindelijk wordt een plateau bereikt en neemt de SMA niet verder toe dit indiceert een radicale verandering in de kobalt speciatie (b.v. een lage vrije kobalt concentratie door de biologische afbraak van citraat). Een zelfde plateau wordt bereikt met het gedispergeerd slib maar uiteindelijk word een hogere SMA waargenomen vergeleken met het korrelslib. In het geval van gedispergeerd slib was de lagfase in de batches met citraat aanzienlijk korter vergeleken met de EDTA en NTA batches. Het is zeer zinvol om deze experimentele opzet verder te ontwikkelen, het creëert de mogelijkheid om de metaal transport en biologische beschikbaarheid in slibkorrels te kwantificeren. Tevens kunnen de resultaten worden gebruikt om metaal transportprocessen/beperkingen in korrelslib te modelleren, bijvoorbeeld door het door Jansen et al. [2004a,c] ontwikkelde model, welke de opname van kobalt en nikkel door *Methanosarcina sp.* beschrijft, te gebruiken en verder uit te breiden



Figuur 5 Effect van kobaltcomplexen met verschillende liganden op de SMA van korrel- en gedispergeerd slib. (*a*) blanco, (*b*) Citrate, (*c*) NTA, (*d*) EDTA.

Populatie dynamiek

Het effect van metaal doseringsstrategieën op de microbiologische populaties in het korrelslib is nog niet onderzocht. Het is echter zeer aannemelijk dat door de concentratie en/of de vorm waarin metalen worden gedoseerd een selectieve druk ontstaat waardoor uiteindelijk de samenstelling van de microbiologische populatie wordt beïnvloed. Moleculair ecologische technieken zoals "denaturing gradient gel electrophoresis" (DGGE) of "fluorescent in situ hybridisation" (FISH) zouden kunnen worden gebruikt om de veranderingen in de biomassa als gevolg van metaal doseringsregimes te monitoren. Dergelijke kennis zou dan kunnen worden toegepast om bioreactoren met gemengde culturen te sturen naar de gewenste microbiologische populaties en omzettingen, b.v. de omzetting van H_2/CO_2 naar methaan of acetaat en b.v. sturing van de competitie tussen sulfaatreducerende bacteriën en methanogenen.

REFERENTIES

Alloway BJ (1990). Heavy metals in soils, 1st ed. (pp 7-27). Chapman and Hall

Florencio L, Field JA, Lettinga G (1995) Substrate competition between methanogens and acetogens during the degradation of methanol in UASB reactors. *Wat Res*, **29**, 915-922

Gonzalez-Gil G, Jansen S, Zandvoort MH, Leeuwen van HP (2003). Effect of yeast extract on speciation and bioavailability of nickel and cobalt in anaerobic bioreactors. *Biotechnol Bioeng*, **82**,134-142

Hullebusch van ED, Zandvoort MH, and Lens PNL, (2003). Metal immobilisation by biofilms: Mechanisms and analytical tools, *Re/Views in Env Sci Bio/Technol,* **2**, 9-33

Hullebusch van ED, Sudarno U, Zandvoort MH, Lens PNL (2005).Comparison of three sequential extraction procedures to describe metal fractionation in anaerobic granular sludges, *Talanta*, **65**, 549-558

Hullebusch van ED, Zandvoort MH and Lens PNL, (2004). Nickel and cobalt sorption on anaerobic granular sludges: kinetic and equilibrium studies, *J Chem Technol Biotechnol*, **79**, 1219-1227

Jansen S, Jiang B, Buykx S, Stams AJM, Leeuwen van HP (2004a). Uptake of Co and Ni by *Methanosarcina barkeri* and influence on growth on methanol: a systematic study and model description (submitted)

Jansen S, Gonzalez-Gil G, Leeuwen van HP (2004b). The impact of Co (II) and Ni (II) speciation in sulfidic media on methanogensis. (submitted)

Jansen S, Gonzalez-Gil G, Leeuwen van HP (2004c). Modeling uptake of Co and Ni by methanogenic bacteria and effects on growth in bioreactor media: the influence of speciation, precipitation and mass transfer. (submitted)

Jong T, Parry DL (2004). Heavy metal speciation in solid-phase materials from a bacterial sulfate reducing bioreactor using sequential extraction procedure combined with acid volatile sulfide analyses. *J Environ Monit*, **6**, 278-285

Lens PNL,van As H (2003). Use of ^{1}H NMR to study transport processes in biofilms. In: Biofilms in Medicine, Industry and Environmental Biotechnology: analysis, compostion and control. Lens P, Mahony T, Moran T, Stoodley P & O'Flaherty V. (Ed.). International Water Association, London, U.K. pp. 285-307

Osuna MB, Zandvoort MH, Iza JM, Lettinga G and Lens PNL (2002). Effect of metal addition on volatile fatty acid conversions in anaerobic granular sludge reactors. *Environ Technol*, **24**, 573-587

Osuna, MB, Hullebusch van ED, Zandvoort MH, Iza J, Lens PNL (2004). Effect of kobalt sorption on metal fractionation in anaerobic granular sludge. *J Environ Qual,* **33**: 1256-1270

Prange A, Modrow (2002). X-ray adsorption spectroscopy and its application in biological, agricultural and environmental research *Re/Views in Environ Sci & Bio/Technol,* **1**, 259-276

Rowlette P (2005). The effect of trace metals on sulfate reduction under acidifying conditions (pH 5), MSc Thesis. Sub-department of environmental technology. Wageningen University, in Dutch

Smith MR, Mah RA (1978). Growth and methanogenesis by Methanosarcina strain 227 on acetate and methanol. *Appl Environ Microb*, **36,** 870-879

Temminghof EJM, Plette ACC, Eck van R, Riemsdijk van WH (2000). Determination of the chemical speciation of trace metals in aqueous systems by the Wageningen donnan membrane technique. *Anal Chim Acta*, **417**, 149-157

Zandvoort MH, Van Hullebusch ED, Peerbolte A, Golubnic S, Lettinga G (in preparation). Dual metal limitation (nickel and cobalt) limitation in a methanol fed methanogenic granular sludge reactor.

Zhang H and Davison W. (2000). Direct in situ measurements of labile inorganic and organically bound metal species in synthetic solutions and natural waters using DGT. *Anal Chem*, **72**, 4447-4457

LIST OF ABBREVIATIONS

COD	chemical oxygen demand
VSS	volatile suspended solids
TSS	total suspended solids
VFA	volatile fatty acids
CH_4	methane
UASB	upflow anaerobic sludge blanket
HRT	hydraulic retention time
OLR	organic loading rate
SLR	sludge loading rate
SMA	specific methanogenic activity
K_s	affinity constant

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Wanneer het proefschrift bijna af is, en je bijna vijf jaren "heavy metal" in je hoofd hebt gehad, is het moeilijk om alles in het juiste perspectief te plaatsen. Wel realiseer ik me nu al terdege dat de vele vrienden en goede collega's, die ik heb ontmoet tijdens mijn promotieonderzoek, van mijn AIO-schap een zeer waardevolle periode in mijn leven hebben gemaakt, die ik voor geen *goud* had willen missen.

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Marcel Zandvoort was born in Assen on October 7th, 1971. He obtained his BSc degree in environmental sciences in 1994 at the Prof. H.C. van Hall institute in Groningen. In 1998 he received his Master of Science degree granted by the Wageningen University (Sub-department of Environmental Technology), with the specialization treatment and reuse of wastewater. The topic of the master thesis was on the treatment of domestic sewage at low temperatures in anaerobic hybrid reactors. In January 2000 he started his PhD at the Sub-department of Environmental Technology of the Wageningen University. At this moment the author works as a postdoc at the sub-department of Ecological Microbiology of the Radboud University Nijmegen, where he conducts research on the topic of biological CS_2/H_2S removal from airstreams.

LIST OF PUBLICATIONS

Zandvoort MH, Geerts R, Lettinga G, Lens PNL (2002). Effect of long-term cobalt deprivation on methanol degradation in a methanogenic granular sludge reactor. *Biotechnol Progr*, **18**, 1233-1239

Zandvoort MH, Osuna MB, Geerts R, Lettinga G, Lens PNL (2002). Effect of nickel deprivation on methanol degradation in a methanogenic granular sludge reactor. *J Ind Microbiol Biotechnol*, **29**, 268-274

Zandvoort MH, Geerts R, Lettinga G, Lens PNL (2003). Methanogenic versus acetogenic degradation of methanol in granular sludge reactors at sub-optimal metal concentrations; role of iron, nickel and cobalt. *Enz Microbial Technol*, **33**, 190-198

Zandvoort MH, Gieteling J, Lettinga G, Lens PNL (2004). Stimulation of methanol degradation in UASB reactors: in situ versus pre-loading cobalt on anaerobic granular sludge. *Biotechnol Bioeng*, **87**, 897-904.

Zandvoort MH, van Hullebusch E, Gieteling J, Lettinga G, Lens PNL (2005, in press). Effect of sulfur source on the performance and metal retention of methanol fed UASB reactors, *Biotechnol Progr*

Zandvoort MH, Van Hullebusch ED, Peerbolte A, Golubnic S, Lettinga G (2005, in press). Influence of pH shocks on trace metal dynamics and performance of methanol fed granular sludge bioreactors, *Biodegradation*

Zandvoort MH, van Hullebusch E, Gieteling J, Lettinga G, Lens PNL (2005). Granular sludge in full scale anaerobic bioreactors: trace elements content and deficiencies, submitted to *Enz Microbial Technol*

Zandvoort MH, van Hullebusch ED, Peerbolte A, Golubnic S, Lettinga G, Lens PNL (in prep.) Dual metal limitation (nickel and cobalt) limitation in a methanol fed methanogenic granular sludge reactor

Elmitwalli TA, Zandvoort MH, Zeeman G, Bruning H, Lettinga G (1999). Low temperature treatment of domestic sewage in upflow anaerobic sludge blanket and anaerobic hybrid reactors. *Wat Sci Tech*, **39**, 177-185

Lens P, Vallero M, Esposito G, Zandvoort M (2002). Perspectives of sulfate reducing bioreactors in environmental biotechnology. *Re/view Envion Sci Bio/Technol*, **1**, 311-325

Osuna MB, Zandvoort MH, Iza M, Lettinga G, Lens PNL (2003). Effects of trace element addtion on fatty acid conversions in anaerobic granular sludge reactors. *Environ Technol*, **24**, 573-587

Osuna MB, van Hullebusch E, Zandvoort MH, Iza J, Lens PNL (2004). Effect of cobalt sorption on metal speciation in Anaerobic Granular Sludge, *J of Environ Qual*, **33**, 1256-1270

Hullebusch van ED, Zandvoort MH, Lens PNL (2003). Metal immobilisation in biofilms: mechanisms and analytical tools. *Re/view Envion Sci Bio/Technol*, **2**, 9-33

Hullebusch van E, Zandvoort MH, Lens PNL (2004). Nickel and cobalt sorption on anaerobic granular sludges: kinetic and equilibrium studies. *J Chem Technol Biotechnol*, **79**, 1219-1227

Hullebusch van ED, Peerbolte A, Zandvoort MH, Lens PNL (2005). Sorption of cobalt and nickel on anaerobic granular sludges: isotherms and sequential extraction. *Chemosphere*, **58**, 493-505

Hullebusch van ED, Utomo S, Zandvoort MH, Lens PNL (2005). Comparison of three extraction procedures to describe metal fractionation in anaerobic granular sludges. *Talanta*, **65**, 549-558

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Cover: Derived from the methane formation rate curves present in Figure 5 of the summary and general discussion.

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