Speciation and bioavailability of cobalt and nickel in anaerobic wastewater treatment

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Speciation and bioavailability of cobalt and nickel in anaerobic wastewater treatment

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Introduction

BACKGROUND

Anaerobic wastewater treatment

Clean water is a vital and valuable global commodity. According to an assessment in 2000 (WHO/UNICEF, 2000), 1.1 billion people lack access to appropriate water supply and 2.4 billion to proper sanitation. The number of people in need of proper water supply will only increase, with the world population expected to grow with 1.5 billion people from 2000 to 2015. Meanwhile, the amount of water consumed by inhabitants of rapidly developing countries is expected to increase. To illustrate this: a child born in the developed world consumes thirty to fifty times as much water resources as one in the undeveloped world (UNESCO, 2004). The high and increasing global water need is difficult to fulfill because clean freshwater resources are limited and vulnerable to pollution. It is evident, therefore, that for sustainable development, both in economically developed as well as in developing countries, wastewater treatment and reuse is a prerequisite. In 2000, the United Nations adopted the Millenium Declaration to ensure environmental sustainability. One of the targets was: 'to halve, by 2015, the proportion of people without sustainable access to safe drinking water' (WSSCC, 2000). One of the actions formulated to achieve this goal, as stated in 2002, was: (to) 'promote affordable and socially and culturally acceptable technologies and practices' (WSSD, 2002).

A useful technology in this respect is anaerobic wastewater treatment. This technology is based on conversion of chemical compounds by microorganisms in natural anoxic environments, such as sediments or the intestinal tract of animals. In the absence of oxygen or other inorganic electron acceptors, large organic substrates can be degraded to methane (CH_4) and carbon dioxide (CO_2) in a stepwise manner by different groups of microorganisms, as shown schematically in fig. 1.

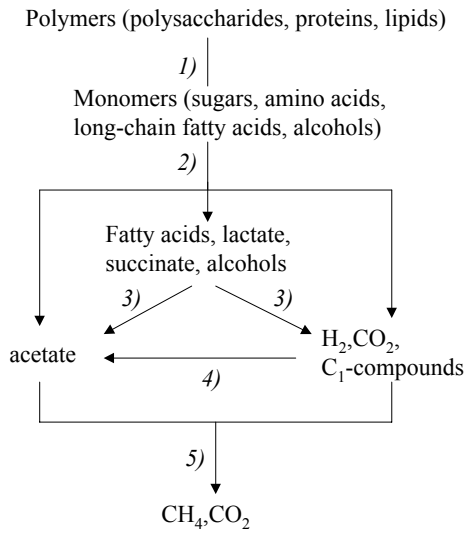

Fig. 1.
Summary of the main anaerobic conversions leading from complex organic substrates to methane
1) Hydrolysis
2) Fermentation by primary fermentive bacteria
3) Fermentation by syntrophic bacteria
4) Acetogenesis
5) Methanogenesis

After Gujer & Zehnder, 1983

In the first step, polymeric substrates such as polysaccharides, proteins and lipids are hydrolyzed into monomers such as fatty acids, lactate, succinate and alcohols. Subsequently, these monomers can be fermented into acetate, carbon dioxide, hydrogen (H_2) and reduced products such as alcohols, lactate and volatile fatty acids (VFAs). Acetogenic bacteria can degrade these reduced compounds into acetate, carbon dioxide, hydrogen and formate. Finally, methanogenic archaebacteria can convert carbon dioxide plus hydrogen and a number of one-carbon (C_1) compounds (such as methanol) into methane and carbon dioxide. Anaerobic wastewater treatment makes use of these conversions by optimizing the conditions for each of the microorganisms involved.

The use of anaerobic wastewater treatment is attractive for a couple of reasons (Lettinga et al., 1999; Frankin, 2001):

- It is a relatively simple technology;
- It is applicable everywhere and at any scale;
- Instead of consuming energy, these systems can actually produce methane (a fuel) and other useful products such as fertilizer;
- Due to the low growth yield, the nutrient requirements are relatively low;
- The amount of excess sludge is lower than in case of aerobic treatment.

Although anaerobic wastewater treatment has been known for a long time (e.g.: in septic tanks), important technological developments have taken place since the 1970's and application on a commercial scale has started well in the 1980's and increased ever since. According to an investigation in 2001, the technology was used in over 65 countries and the amount of treatment plants known was 1400, while the actual number was estimated to be around 2000 (Frankin, 2001).

The UASB reactor

An important step in the development of anaerobic wastewater reactors was the development of high-rate reactors. These are characterized by retention of the solids in the reactor, enabling high biomass concentrations and, consequently, high conversion rates to be realised. The biomass can be immobilized in several ways. It can be immobilized by solid surfaces, as used in in case of fluidized and fixed bed reactors. It can also be self-immobilized, as used in UASB (Upflow Anaerobic Sludge Bed) and EGSB (Expanded Granular Sludge Bed) reactors. In this study, we will focus on the successful UASB reactor (Lettinga et al., 1983).

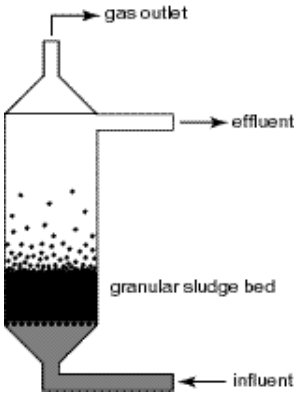

Fig. 2. Schematic picture of a UASB reactor.

The operation of a UASB reactor is schematically shown in fig. 2. Wastewater is pumped in from below. The biomass is present in the form of a dense bed of granules with a diameter ranging from 0.1 to 5 mm, thus preventing the biomass from being washed out of the reactor. Under optimal conditions, methane and carbon dioxide are the final products which are collected in the top of the reactor, and clean effluent leaves the reactor.

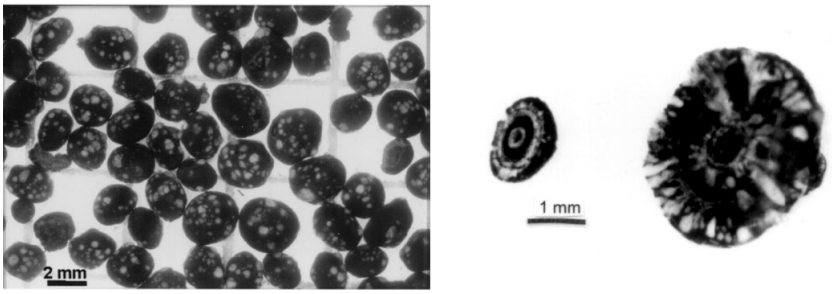

Fig. 3. Granules from an EGSB reactor, an anaerobic wastewater reactor similar to the UASB reactor. From: Gonzalez-Gil et al., 2001.

The granules (fig. 3) consist of a dense matrix of various microorganisms, inorganic precipitates and polymeric substances (Liu et al., 2002). The very specific microenvironment of the granule, with its chemical heterogeneity and biological diversity, is of great importance for the operation of the system. In the first place, a diversity of different microorganisms is brought together, enabling various synergetic features. For instance, products of some organisms can serve as substrates or growth stimulating factors for other organisms. This relationship can even be so strict, that different types of organisms can only grow in each other's vicinity (Stams, 1994).

Apart from the microorganisms involved in the conversions depicted in fig. 1, many other microorganisms are present. The sulfate-reducers form a group of microorganisms of particular importance. These convert sulfate into sulfide. The sulfide precipitates with metal ions, thus forming inorganic precipitates that contribute to the structure of the gran-

ule. Another important effect of the precipitation with sulfide is a strong decrease of the free ion concentration of the metals involved.

Apart from metal sulfide precipitates, many other precipitates can be formed, such as calcite. Furthermore, the matrix of the granule can be strongly influenced by the presence of so-called Extracellular Polymeric Substances (EPS), which are produced by microorganisms. All these components contribute to the dense and heterogeneous biofilm-type structure of the granule.

Metals and anaerobic wastewater treatment

The operation of UASB reactors and similar anaerobic wastewater treatment systems is highly dependent on the presence of metal ions, as was described by numerous authors (for reviews: Takashima & Speece, 1990; Oleszkiewicz & Sharma, 1990; Singh et al., 1999). First of all, the microorganisms require certain essential metals. More specifically, methanogenic archaebacteria are known to require Fe, Co, Ni, Mo and W (Jarrell & Kalmokoff, 1987). However, at higher concentrations, some of these metals can become toxic (e.g.: Jarrell et al., 1987; Bhattarachya et al., 1995; Mueller & Steiner, 1992). Apart from the effects on the level of the individual microorganism, the competition between microorganisms can be modified when one or some of them are affected by metals differently than others (e.g.: Florencio et al., 1994; Paulo et al., 2004; Speece et al., 1986; Takashima & Speece, 1990). Furthermore, since different metal ions are needed for different biochemical conversions, this also affects the conversions taking place (Florencio, 1994; Paulo, 2002; Takashima & Speece, 1990; Isa et al., 1986). Finally, the granular matrix is strongly affected by metal ions because of their role in the precipitates present. This can have important consequences for the granulation process (e.g. Oleszkiewicz, 1989; Yu et al., 2000; Sharma & Singh, 2001).

The distribution of the metals over different chemical forms or species, i.e. their *speciation*, strongly influences their effects in the bioreactor. Free metal ion concentrations can be very low due to precipitation and strong metal binding by various complexing compounds, potentially decreasing the bioavailability of these metals. Some examples of effects of metal binding compounds (ligands) include the decrease of inhibitory effects of toxic metals (e.g.: Carlson-Ekval & Morisson, 1995; Jarrell et al., 1987) and the decrease of the stimulating effect of Fe (Speece et al., 1986). However, the precise effects of metal speciation on bioreactor practice, especially in case of metal limitation, are not well known. The large number of chemical and biological interactions makes the system complex and difficult to describe.

In practice, it is important to understand metal effects for the optimal dosing strategies of methanogenic wastewater treatment systems. Metal limitation in these systems is known to occur. Industrial wastewaters can be devoid of one or more of the metal ions required, necessitating additional metal dosing. But even in cases where metal ions are present in the wastewater, limitations can be observed. An important reason for this could be the presence of metal precipitating ions such as sulfide and other metal binding substances, leading to very low bioavailable concentrations of metals.

In cases of metal limitation, the exact dosage of metals is critical. To stay on the safe side, metal ions are often dosed at high amounts. However, this has some important draw-

backs. In the first place, this strategy does not generally guarantee optimal bioreactor performance. In case of strong complexation or precipitation, bioavailability may remain low, so that even high metal dosing might not lead to optimal bioreactor performance. On the other hand, metal overdosing might lead to toxicity and suboptimal reactor performance. Furthermore, dosing high amounts of metal leads to high costs. And finally, in case of excessive metal dosing, the sludge formed will contain high amounts of metal.

Focus, aim and approach of this research

A well-known example of metal limitation in methanogenic wastewater treatment is the effect of Ni and Co on the conversion of methanol into methane. Methanol is an important feedstock for anaerobic wastewater treatment, especially as a waste product from breweries, and can furthermore be used to cometabolize other toxic compounds (Weijma & Stams, 2001; Paulo, 2002). The effects of Ni and Co on methanol degradation in bioreactor practice are well known (Lettinga et al., 1979; Florencio et al., 1993, 1994; Gonzalez-Gil et al., 1999; Paulo, 2002). In batch media, the effects of Ni and Co on methanogens have been studied in detail (Diekert et al., 1980a, 1980b, 1981; Scherer et al., 1981, 1983; Mazumder et al., 1986, 1987; Silveira et al., 1990, 1991a, 1991b; Nishio et al., 1992, 1993; Lin et al., 1989). From these data, the general role of Co and Ni in methanogenesis from methanol and orders of magnitude of cell contents and effects are known. However, a quantitative description linking speciation, uptake, and biological effects is still missing. Therefore, the aim of the research presented here is to determine the relationship between Co and Ni speciation, uptake and methanogenic activity. The approach is summarized in fig. 4. The main components of this approach will be discussed in more detail in the following subchapters. First, we have to understand the complex **speciation** in anaerobic bioreactor environments, as described in the next section. Secondly, the **microbiology** of the system is of great importance, with several factors playing a role such as uptake, eventual form and role of the metal ions within the microorganisms, and the mechanisms of metal limitation: these are described in the third section.

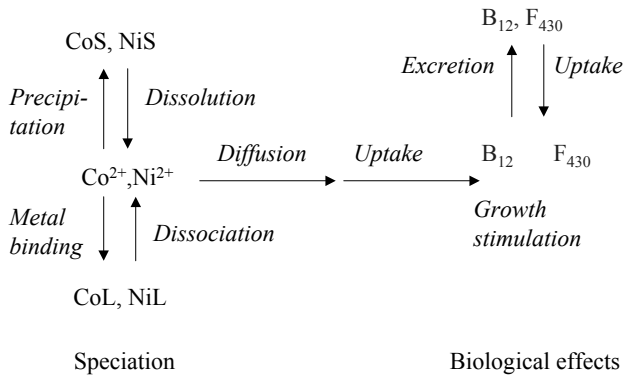

Fig. 4. Cascade of metal speciation, uptake, and biological effects of Co and Ni for methanogens.

The research described in this thesis was part of a broader multidisciplinary project 'Optimisation of trace element dosing for anaerobic wastewater treatment' undertaken at Wageningen University. While the research reported in this thesis focused on the relation between speciation and metal uptake and effects, in parallel to this, bioreactor implications were investigated by Marcel Zandvoort (Laboratory of Environmental Technology), while Bo Jiang (Laboratory of Microbiology) focused on microbiological aspects.

Broader scientific relevance

Apart from optimising metal dosing in anaerobic wastewater treatment, the research presented here is also interesting from other perspectives.

In the first place, understanding metal effects on methanogenesis is interesting within a broader context beyond anaerobic wastewater treatment itself. In many anoxic environments, such as sediments and peats, methanogenesis is an important process (Hoehler & Alperin, 1996). Currently there is also much interest in anaerobic methane oxidation (e.g.: Michaelis et al., 2002), a process which is still poorly understood and where Ni seems to play an important role (Krüger et al., 2003).

Furthermore, metal speciation and biological limitation are also interesting from a more fundamental point of view. Although the importance of metal speciation for biological availability and effects is well accepted, effects in anaerobic environments have only been scarcely studied so far. For example in deep-sea hydrothermal vents, metal-sulfide interactions seem to have great influence on biological processes (Luther et al., 2001; Atkins et al., 2002; Edgcomb et al., 2004). Metal speciation effects have been studied mainly within the context of toxicity, while studies on the limitation of metal ions are sparser. Finally, features of metal speciation dynamics, which are extremely important in the bioreactor environment, are often neglected.

METAL SPECIATION IN ANAEROBIC WASTEWATER MEDIA

Chemical medium composition

Some examples of typical media in anaerobic bioreactors or similar batch systems are described in Zandvoort et al., 2002a,b; Gonzalez-Gil et al., 1999 and in chapter 4 of this thesis. Table 1 gives an overview of the main components of these media.

Table 1. Components and their concentration levels of typical anaerobic bioreactor media for a UASB-reactor (Zandvoort et al., 2002a,b), a batch reactor (Gonzalez-Gil et al., 1999) and medium for growing methanogens in batch cultures (this thesis, chapter 4).

	UASB- reactor Zandvoort et al., 2002a,b	Batch-reactor Gonzalez-Gil et al., 1999	Batch medium This thesis, chapter 4
Macronutrients and salts (mM)			
Na^+	30	36	60
K^+	3	7	3
Ca^{2+}	0.09	5	0.75
Mg^{2+}	0.4	4	0.5
NH_4^+	5.2	15	5.6
PO_4^{2-}	1.5	7	5.8
Cl^-	5.3	25	13
HCO_3^-	30	36	48
S^{2-}	-	0.54	1
SO_4^{2-}	0.41	4	-
Micronutrients (μM)			
Fe^{2+}	2	50	7.5
BO_4^{3-}	-	4.0	1
Zn^{2+}	0.5	1.8	0.5
Cu^{2+}	4	1.1	0.1
Mn^{2+}	0.3	12.6	0.5
Ni^{2+}	0.04	variable	0.1
Co^{2+}	0.8	variable	0.1
SeO_3^{2-}	0.1	3.1	0.1
WO_4^{2-}	-	-	0.1
MoO_4^{2-}	-	-	0.1
$Mo_7O_{24}^{6-}$	0.0005	0.2	-
Additional agents			
Vitamins	-	-	+
Resazurin (mg/l)	-	-	0.5
Yeast extract (g l^{-1})	-	0.1	-
Gas phase			
N_2	-	0.7 atm	0.7 atm
CO_2	-	0.3 atm	0.3 atm
pH	6-8	7-7.2	6.9-7.1

The media contain large amounts of macronutrients, thus the ionic strength is generally high (typically in the order of 0.05 to 0.1 M). The high concentrations of carbonate and sulfide can cause precipitation of metals.

Trace elements are added in lower concentrations, but are still essential. Two groups can be distinguished: oxyanions, which are not prone to sulfide precipitation, and cations, which, for the greatest part, are.

Vitamins (such as: biotin, niacin, pyridocin, riboflavin, thiamin, p-aminobenzoic acid, panthothenic acid and vitamin B_{12}) are usually added in low concentrations (0.01-0.5 mg/l) in defined batch cultures. In most bioreactor systems, they are not added since in most of these cases, a consortium of microorganisms is present, and, if one species cannot synthesize a vitamin itself, others can provide it. In batch media, the redox indicator resazurin is often added. Complexing agents such as EDTA can be added to prevent precipitation of metals. Sometimes, additional growth stimulators such as yeast extract are added. They are not usually added in bioreactors because of the high cost; however, in microbial media these supplements are often added (Robb et al., 1995).

In table 1, organic substrates are not mentioned. In the research reported in this thesis, this is methanol, but in other anaerobic bioreactors the organic substrate can be any type of substrate summarized in fig. 1.

The pH is buffered by carbonate. If the gas phase changes, large amounts of acids are produced (such as VFAs, Volatile Fatty Acids), or CO_2 is consumed, and the pH might subsequently change. The pH typically ranges around 7.

The low redox potential is very important. In bioreactors, this mainly results from the action of sulfate reducers, which produce sulfide. In anaerobic batch media, first oxygen is removed by boiling and purging with nitrogen, after which a reducing agent such as sulfide, cysteine or titanium citrate is added. The low redox potential prevents metal ions such as Fe(II) and Co(II) from being oxidized. Due to protonation and gas/liquid distribution, S(II-) is distributed over S^{2-}(aq), HS^-(aq), H_2S(aq) and H_2S(g).

Apart from these components, high amounts of organic matter produced by the microorganisms can be present. These organic compounds can be present in solution (collectively known as SMP, Soluble Microbial Products), as gel matrix in the form of EPS (Extracellular Polymeric Substances), or in the form of biomass. The amounts and properties of these compounds are highly dependent on operating conditions (Bhatti et al., 1995).

Many features, such as anaerobicity and high carbonate and sulfide concentrations, are very similar to those of anoxic freshwater or deep-sea sediments. This is not surprising, since the organisms cultivated in anaerobic bioreactors are originally known from those natural environments. Thus, in considering chemical properties of the anaerobic bioreactor medium, we can also take into account much of the knowledge about the natural niches mentioned.

In the following two paragraphs we will discuss effects on metal behaviour of both the particulate and the dissolved ligands.

Particulate metal binding

Inorganic precipitates

At the concentration levels of the anaerobic media, metal ions can precipitate with sulfide, carbonate and phosphate. Metal sulfide precipitation is expected to be the most important process. The low solubility product of metal sulfides will result in extremely low free metal ion concentrations (Martell & Smith, 1989). However, in trying to predict free metal ion concentrations from these solubility products, one has to be careful. In the first place, to know the solubility product, the crystal structure of the metal sulfide precipitate should be known. Secondly, the errors in the literature values for the solubility products are very large, and different literature sources give different values. Thirdly, in many cases precipitation equilibrium is not actually reached due to kinetic reasons. And finally, size and ligand effects can affect precipitation. Apart from precipitation in the form of well-defined precipitates, in a mixture of metal ions, coprecipitation and adsorption are also expected to be important phenomena. These processes have been studied in sediments, typically containing excess of Fe over other metals (e.g. Cooper & Morse, 1999; Morse & Luther, 1999; Huerta-Diaz et al., 1998). Considerable adsorption and coprecipitation of Co and Ni on FeS (mackinawite) was studied in some detail (Morse & Arakaki, 1993). Kinetic factors can further influence coprecipitation (Morse & Luther, 1999).

Organic particles

Apart from inorganic ligands, organic ligands can be present in high amounts. A large proportion of the granules is made up of EPS, which can bind metal (e.g.: Shen et al., 1993). The nature and amounts of EPS vary with the operating conditions, thus also affecting metal binding (Shen et al., 1993; Schmidt & Ahring, 1994; Bhatti et al., 1995). Furthermore, the bacterial interface itself can also serve as an important metal binding surface (Aksu et al., 1991; Lester, 1987; Beveridge & Doyle, 1989).

Metal binding studies in anaerobic sludge

The overall chemical composition of granular methanogenic sludge was studied, amongst others, by Dolfing and coworkers (1985). FeS was shown to comprise 30% of the total ash content. The granules were very resistent to chemical disintegration, indicating the presence of different extracellular polymers and various groups of microorganisms. X-ray analysis of UASB granules has confirmed the existence of Cu, Fe, Zn and Ni sulfide precipitates (Fang & Liu, 1995; Liu & Fang, 1998, Gonzalez-Gil, 2001; Kaksonen, 2003). Metal sorption by anaerobic granules and similar aggregates has been studied by many authors (e.g.: Gould & Genetelli, 1978, 1984; MacNicol & Beckett, 1989; Artola et al., 2000; Gonzalez-Gil et al., 2001; Zandvoort et al., 2004; Osuna et al., 2003). Overall, these studies confirm strong sorption of metal ions in granules due to precipitation, coprecipitation, adsorption and binding by EPS and bacterial cells. However, the extent of binding can vary with chemical and biological conditions and is spatially heterogeneous.

Speciation in solution

Inorganic complexes

Sulfide is not only important because of the formation of metal precipitates, but also because of the formation of dissolved metal complexes. Dissolved metal sulfide species have only been studied over the last 15 years, and theoretical estimates date back only some 15 years (Dyrssen, 1988), while measurements are available only since 10 years (Zhang & Millero, 1994; Luther et al., 1996; Al-Farawati & Van den Berg, 1999). Although the data vary, they demonstrate that dissolved metal sulfide complexes are very strong and must be important.

Other important inorganic ligands are CO_3^{2-} and PO_4^{3-}. Although the complexes are less strong than the metal sulfide complexes, the importance of carbonate and phosphate complexes was demonstrated for anaerobic media (Callander & Barford, 1983a,b). Carbonate is very important because of its high concentration in reactor and microbial media, and its strong binding with metal ions, especially with Ni(II). Unfortunately, some disagreement about the binding constant exists in the literature (Fouillac & Criaud, 1984; Turner et al., 1981; Hummel & Curti, 2003).

The ligands OH^-, SO_4^{2-} and Cl^- are also present at large concentrations, but still are relatively unimportant because of their weak binding.

Organic complexes

In many anaerobic wastewater treatment systems, a high concentration of Soluble Microbial Products (SMPs) is present (Barker & Stuckey, 1999). They can consist of various types of compounds, such as humic and fulvic acids, polysaccharides, proteins, nucleic acids, organic acids, amino acids, antibiotics, steroids, exocellular enzymes, siderophores, structural components of cells and products of cellular energy metabolism. By definition volatile fatty acids (VFAs), possibly present in anaerobic wastewater treatment, are not counted in this category. The composition is highly dependent on the type of wastewater treatment system and the process parameters.

Metal binding by SMP was demonstrated for Ni (Kuo & Parkin, 1996; Kuo et al., 1996) and Cu (Bender et al., 1970); however, the data were collected for wastewater treatment systems different from those studied in the scope of this thesis, and thus cannot be directly applied here. Strong Zn-complexing ligands were detected by voltammetry in media after growth of sulfate-reducing bacteria (Bridge et al., 1999). The results suggest that excretion of metal binding SMP can serve as a mechanism to reduce metal toxicity. It is still an open question whether the microorganisms in anaerobic wastewater treatment systems actively excrete organic metal ligands in order to bind metals to overcome limitation, as is known for Fe (Neilands, 1995) and Co (Saito et al., 2002).

Apart from organic ligands produced by microorganisms, sometimes organic ligands are added. First, some organic substrates can have metal-binding properties, e.g. in the case of acetate. However, most of these complexes are weak compared to the strong complexes with sulfide (Martell & Smith, 1989). Furthermore, sometimes synthetic ligands such as EDTA, NTA or citrate are added. In some cases these are added deliberately, for

instance to keep metals in dissolved form (e.g.: Bretler & Marison, 1996; Hartung, 1992), in other cases these are reported to be present in the waste stream, possibly as waste products (e.g.: Nowack et al., 2002).

Finally, yeast extract or other supplements can be added. These generally consist of a complex mixture of compounds like amino acids, cofactors and vitamins, some of which can bind metal ions.

Organic ligands might influence precipitation. In general, in their presence, the concentration of dissolved metal is increased. However, conflicting data are also found. For instance, in a system containing Ag^+ and amorphous FeS in the presence of high concentrations of thiols, dissolved Ag^+ was smaller than predicted. A possible explanation for this could be the binding of the Ag-thiol complexes to FeS (Adams & Kramer, 1998). In another study, the solubility of CuS was increased by thiols, while that of PbS and CdS was not (Shea & MacCrehan, 1988a, b). Furthermore, organic ligands are reported to affect the size of the particles (e.g.: Peters & Ku, 1987) and the kinetics of precipitation and complex formation (see next paragraph).

Kinetics of precipitation and complex formation

Within the dynamic bioreactor environment, relative rates of the various processes can be of great importance, in particular the kinetics of particulate formation and dissolution.

Precipitation can be divided into 5 stages: (Nielsen, 1964)

- nucleation;
- growth of nuclei;
- aggregation;
- formation of irreversible aggregates;
- formation of larger crystals at the expense of smaller ones (Ostwald ripening).

In parallel with step 5, the precipitates tend to age, generally transforming from amorphous precipitates to more stable crystalline forms. Metal sulfide dissolution in near-neutral pH ranges has only been studied for mackinawite (FeS) (Pankow & Morgan, 1979, 1980) and for similar systems such as sediments (e.g.: Harper et al., 1998; Motelico-Heino et al., 2003).

Besides precipitation, the kinetics of adsorption onto and desorption from precipitates can be very important, as exemplified by the coprecipitation/adsorption kinetics of Cu, Zn, Pb and Cd to FeS (Davis et al., 1994).

The kinetics of precipitation and dissolution can be influenced by organic ligands. Organic ligands can cause a decrease in precipitation rate (Helz & Horzempa, 1983; Shea & Helz, 1987) or an increase in dissolution rate, e.g. in case of siderophores (e.g.: Liang et al, 2000; Kraemer & Hering, 1997; Cervini-Silva & Sposito, 2002).

Besides the kinetics of reactions involving the particulate matrix, the rates of processes within the dissolved fraction can be important. In aqueous solution, the rate of metal complex formation is largely dependent on the water-loss rate constants of the metal ions (Morel & Hering, 1993), and independent of the nature of the ligand. From this so-called Eigen mechanism, the order of the reaction rates of some of the relevant metal ions is estimated to be: $Fe^{2+}>Co^{2+}>Ni^{2+}$. Especially for Ni^{2+}, complexation reactions are known to be relatively slow (Margerum et al., 1978). Furthermore, in solutions containing high con-

centrations of competing ions such as Ca^{2+} and Mg^{2+}, exchange reactions can be inherently slow (Hering & Morel, 1989, 1990).

Analytical methods for speciation analysis

In the anaerobic bioreactor system, both particulate and dissolved metal speciation play an important role. The sizes and amounts of particulates can be determined using various separation techniques, such as filtration, centrifugation, field flow fractionation and Dynamic Light Scattering (DLS) (Buffle & Van Leeuwen, 1992, 1993). Methods to study the nature of the particulate matter are reviewed in Hullebusch et al. (2004). The biofilm can be fractionated, either by extraction of EPS-associated metals or by sequential extraction. Metal precipitates can be located within the biofilm matrix by use of microscopic techniques, such as Transmission Electron Microscopy (TEM), Scanning Electron Microscopy (SEM) or Confocal Laser Scanning Microscopy (CLSM). NMR can give information on diffusive properties in the granular matrix. Spectroscopic techniques such as X-ray or IR can give detailed information on elemental composition and structural properties such as crystal structure.

For studying the dissolved metal speciation, the low metal concentrations and properties of the mediumof anaerobic bioreactors set very specific demands on the experimental techniques chosen. In general, a number of methods is available, including:

- Techniques using semi-permeable gels, such as DET (Davison et al., 2000), DGT (Davison et al., 2000), SOFIE (Vink, 2002);
- Techniques using selective membranes, such as DMT (charge-selective) (Temminghoff et al., 2000), and PLM (ion-selective) (Buffle et al., 2000);
- Dynamic electrochemical techniques, such as voltammetries (Buffle & Tercier-Waeber, 2000) and (S)SCP (Van Leeuwen & Town, 2003) for metals that can amalgamate with mercury, or certain types of microelectrodes (De Beer, 2000);
- Combinations, such as GIME (porous gel in combination with voltammetry), CGIME (a GIME with a layer of metal binding resin) (Buffle & Tercier-Waeber, 2000) or certain types of microelectrodes (De Beer, 2000).

In this study, we assess the applicability of Competitive Ligand Exchange - Adsorptive Stripping Voltammetry (CLE-AdSV) to analysis of Co and Ni speciation in anaerobic bioreactor media. This technique has the advantages of selective detection of both Co and Ni at low concentrations and the possibility of analysing strong binding properties. It combines bulk equilibrium ligand exchange with a 2-step electrochemical technique. A ligand L_{ad} with well-defined complexation properties is added to form strong surface-active complexes with the metal ion(s) of interest, ML_{ad}. In the first step, the electrode potential is held at a constant potential for a fixed period of time, during which ML_{ad} adsorbs onto the mercury electrode at a given potential. After this accumulative preconcentration step, the adsorbed metal-ligand complexes are quantified by voltammetric reduction of the adsorbed complex. The size of the resulting signal gives information about the concentration and binding properties of other ligands in the solution. Furthermore, the in-built preconcentration step enables detection of metal concentrations down to very low values (for Co and Ni of the order of 1 nM). Although the technique has been applied to many freshwater and seawater matrices (e.g. Xue & Sigg, 2002), not much is known about its appli-

cation to the anaerobic wastewater matrix. The binding strength and concentration of the added ligand determine the range of binding strengths and concentrations of ligands in solution that can be detected: this range is called the detection window. Much is known about the equilibrium aspects of the detection window (Van den Berg et al., 1990; Town & Filella, 2002). However, kinetic factors will also be important and have been ignored to date.

BIOLOGICAL EFFECTS OF CO AND NI

Biochemical roles of Co and Ni in methanogenesis from methanol

Microorganisms from the genus *Methanosarcina* can convert methanol to methane via (Thauer, 1998):

$$4CH_3OH+2H_2O \rightarrow 3CH_4+CO_2+4H_2O \qquad (1)$$

Both Co and Ni are essential in this conversion. Apart from these, Fe is also needed, but here we will focus on Co and Ni. In the first step, Co in the form of vitamin B_{12} (fig. 5a) transfers a methyl group from methanol to the complex coenzyme M to form methyl-coenzyme M. In the next step, Ni in the form of factor F_{430} (fig. 5b) enables electron transfer by the enzyme methyl-coenzyme M reductase. This enzyme catalyzes the reduction of the methyl-coenzyme M complex, thus enabling the production of methane. The reaction occurs in all methanogenic archaebacteria, and this renders the presence of factor F_{430} indispensable for methanogens. Both vitamin B_{12} and factor F_{430} have a tetrapyrrole structure, in which the metal ion is strongly bound. In the biosynthesis, Co and Ni first have to be taken up by the cell, after which they are built into the cofactor structure during one of the subreactions of the cofactor synthesis (Raux et al., 2000; Telser, 1998). Apart from its role in factor F_{430}, Ni can be part of the enzymes hydrogenase and CO dehydrogenase (Watt & Ludden, 1999a), and furthermore Ni is thought to play a stabilizing role in the cell membrane (Jarrell & Kalmokoff, 1988).

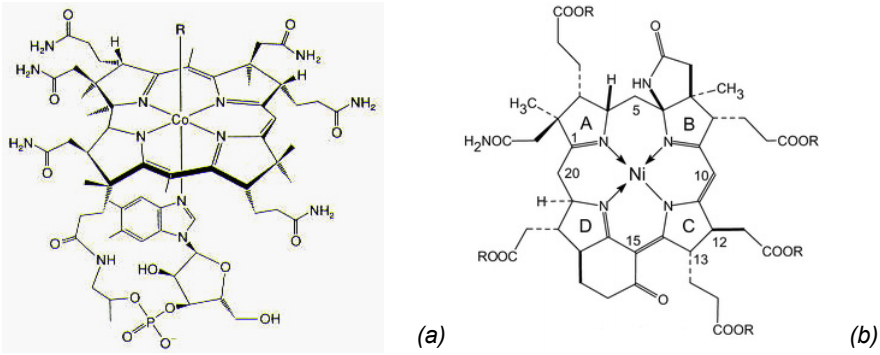

Fig. 5. Cofactors Vitamin B_{12} (a) and Cofactor F_{430} (b). Taken from Lippard & Berg, 1994 and Crabtree, 1988.

Effects of speciation of Co and Ni on methanogenic activity

Characteristics of uptake of Ni by methanogens were determined for two very different methanogens (Jarrell & Sprott, 1982; Baudet et al., 1988). Metal contents of 10 different methanogenic organisms were determined (Scherer et al., 1983) and for one methanogenic organism, the Ni cell content was shown to vary with varying added metal (Diekert et al., 1981). Optimal metal concentrations were determined under certain medium conditions (Silveira et al., 1991b), as well as the production and excretion of cofactors (Mazumder et al., 1987; Lin et al., 1989).

Notwithstanding these data, a mechanistic picture combining speciation, uptake and biological effects is still missing. Therefore we chose to link these components in model systems, to establish a more comprehensive description of metal speciation, uptake and effects on methanogenesis. In the next two subsections we will summarize relevant knowledge on mechanisms of metal uptake and biological effects.

Metal speciation and uptake

It is generally accepted that the speciation of metals has a huge impact on their uptake (and ensuing biological effects), as has been demonstrated in detail for algae and some microorganisms (Hudson, 1998; Sunda & Huntsman, 1998; Wilkinson & Buffle, 2004).

Two elementary approaches that are often used, are the Free Ion Activity Model (FIAM) (Morel & Hering, 1983) and the Biotic Ligand Model (BLM) (Pagenkopf, 1983; Playle, 1998; Paquin et al., 2002). The former relates biological effects to the free ion activity in solution, while the latter treats the organism as a ensemble of sorption sites, and relates biological effects to the equilibrium amount of metal adsorbed into and onto the organism. Both models rely on the assumption that the processes outside the organism are at equilibrium. These simple models have been criticized, since their applicability has been shown to be limited (Campbell, 1995; Campbell, 2002; Hassler et al., 2004; Wilkinson & Buffle, 2004). For instance, diffusion limitation, as expected to occur at low concentrations in combination with high-affinity uptake sites, will certainly cause deviations (Hudson, 1998). In these cases, labile species will also be bioavailable (Van Leeuwen, 1999). Secondly, not only free metal ions, but also lipophilic complexes, and even sometimes hydrophilic complexes, have been reported to be taken up as well (e.g.: Phinney & Bruland, 1994). Furthermore, biological responses can have significant impact on speciation and bioavailability in the exposure media due to excretion and changes of the uptake properties (Wilkinson & Buffle, 2004).

In general, the transport of metal ions is to a great extent determined by the properties of the transport systems (Köster, 2004). Transport of Co and Ni is reported to proceed either via specific Co and/or Ni transporters, or via magnesium transporters (e.g.: for Co: Komeda et al., 1997; Pogorelova et al., 1996; Degen et al., 1999; Saito et al., 2002; Kobayashi & Shimuzu, 1999; for Ni: Eitinger & Mandrand-Berthelot, 2000; Mulrooney & Hausinger, 2003; Watt & Ludden, 1999b). The uptake of metal ions by these specific transporters can be described by Michaelis-Menten kinetics, where the bioavailable metal ion is first bound by a transporter site and subsequently taken up. The binding properties determine the affinity of the transporter to the metal, while the amount of the transporter determines the maximum uptake rate. Both of these parameters can change with changing

chemistry or biology. Different metal ions can compete for the same uptake site, thus affecting the conditional affinity (e.g.: Sunda & Huntsman, 1998). Furthermore, the organism can actively decrease or increase the number of transporters in response to its environment, thus affecting the maximum uptake rates.

Metal limitation

In many cases of nutrient limitation of microorganism growth, a Monod relation between growth and external metal concentration is assumed (Kovarova-Kovar & Egli, 1998). However, since cell metal contents are known to vary, this relation is expected not to be widely applicable to metal limitation. Therefore, it would be more appropriate to link growth to the internal metal concentration. The Droop equation (Droop, 1983) relates growth rate to internal substrate concentration. It was applied to limitation of algal growth by several metal ions, nitrogen, phosphorus and vitamin B_{12} (e.g.: Droop, 1983; Flynn, 2003; Davidson et al., 1999), but has not yet been applied to limitation by Co and Ni in the anaerobic bioreactor environment. Also, the influence of limitation by dissolution or diffusion rate on the effects of metal speciation on growth limitation was seldomly studied.

SCOPE OF THIS THESIS

The aim of the research presented in this thesis was to study the impact of speciation on the uptake of Co and Ni by methanogens and its eventual effect on the methanogenesis from methanol in anaerobic bioreactor media. Because of the fundamental aspects involved, we have chosen one well-defined type of microorganism from the genus *Methanosarcina*. These microorganisms are active in methanol degrading wastewater reactors, and are sensitive to Co and Ni limitation. Part of the work was done using an enrichment culture from a bioreactor. Other experiments were performed with a pure culture of *Methanosarcina barkeri*, a well-defined model organism.

Chapters 2 and 3 address the dynamic metal speciation, both from an analytical point of view as well as from the bioreactor perspective. In **chapter 2**, the analytical technique CLE-AdSV is rigorously analysed with respect to its thermodynamic and kinetic detection windows, and the kinetic background of its basic speciation step. In **chapter 3**, this analytical technique is applied to anaerobic bioreactor media. Measurements in batch and reactor media are presented and consequences for bioreactor performance are discussed.

In **chapter 4**, the effects of metal sulfide precipitation/dissolution dynamics on metal uptake and methanogenesis are discussed. Experimental data are presented and analysed within the context of the speciation of metals including their sulfide precipitates and dissolved sulfide complexes. **Chapter 5** considers the effects of addition of yeast extract on Co and Ni bioavailability and relates them to the metal binding behaviour of the yeast extract as measured using AdSV. **Chapter 6** describes the effects of Co and Ni on a pure culture of *M. barkeri* under conditions of well-defined speciation in the absence of precipitates. A model is developed to quantitatively interpret the relationship between metal spe-

ciation, uptake and growth. In **chapter 7**, the model developed in chapter 6 is applied to different metal speciation conditions. Effects in the presence of cysteine and sulfide as sulfur sources are analysed. Furthermore, the influence of dissolution, dissociation and diffusion limitation are discussed.

The thesis is concluded with a summary in **chapter 8**.

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[2]

Dynamic aspects of metal speciation by Competitive Ligand Exchange - Adsorptive Stripping Voltammetry (CLE-AdSV)

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ABSTRACT

Competitive Ligand Exchange - Adsorptive Stripping Voltammetry (CLE-AdSV) measures metal speciation via an exchange reaction with an adsorptive ligand followed by electrochemical detection of an adsorbed metal complex. Although it is widely applied in practice to study metal speciation at low metal concentrations, some fundamental aspects of the technique have not been characterized to date. The ligand exchange step has important consequences with respect to the metal species contributing to the signal (also referred to as the detection window). Thermodynamic aspects of the detection window are well understood; kinetic aspects however have remained untouched so far. Furthermore, kinetic features of the adsorption step have not been analysed in proper detail yet.

The upper border of the detection window is formulated by the requirement of equilibration before adsorptive accumulation of the surface-active complex ML_{ad}. Kinetic analysis of the exchange step demonstrates that, due to the strong ligands typically added, any complexes with other sample ligands that remain in solution must be even more stable than those with the added ligand, thus they are nonlabile and will not contribute to the signal. Any residual free metal however does contribute to the accumulation process, but this contribution will in practice be negligible because of the low concentration after ligand exchange.

The adsorption of the surface-active complex ML_{ad} is demonstrated to be of a transient nature for only the first small fraction of the total adsorption time, after which the diffusive adsorption process is essentially steady state.

INTRODUCTION

Though usually classified as an electrochemical stripping technique, Competitive Ligand Exchange - Adsorptive Stripping Voltammetry (CLE-AdSV) is primarily a bulk ligand exchange method for metal speciation analysis. In the sample solution, an equilibrium is set up between the different species of the target metal M and a deliberately added ligand L_{ad} with (i) appropriate, well-defined complexation characteristics with respect to M, and (ii) sufficiently strong affinity for adsorption at an electrodic surface, usually Hg. The electrochemical adsorptive stripping experiment merely serves to measure the concentration of the complex between M and L_{ad} (fig. 1).

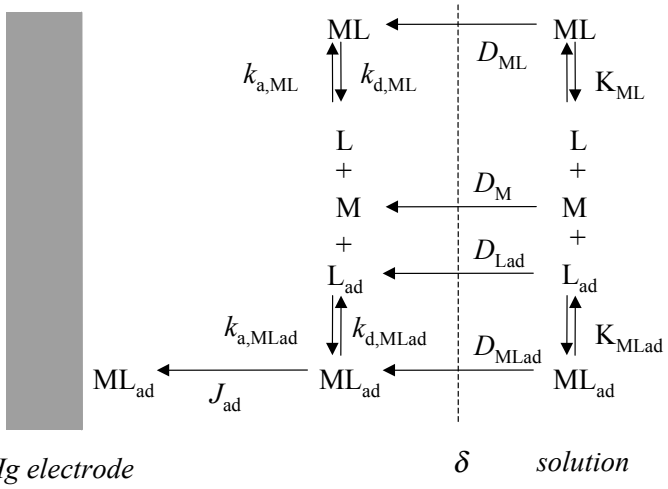

Fig. 1. Schematic outline of species and interactions near the Hg electrodic surface in a CLE-AdSV experiment for a solution containing a metal ion M, a ligand L with which M forms a complex ML, and an added ligand L_{ad} with which M forms a surface-active complex ML_{ad}. The parameters which determine the amount of ML accumulated during the adsorption step include the stabilities K of ML and ML_{ad}, the diffusion coefficients *D* for M, ML, L_{ad} and ML_{ad}, the rate constants of complex association (k_a) and dissociation (k_d) of ML and ML_{ad} , the adsorption flux (J_{ad}) and the diffusion layer thickness δ.

If the total metal concentration is low (typical of most CLE-AdSV applications), the concentration of ML_{ad} is so low that alternative methods of determination are not easily available, thus making the method very suitable for the study of metal speciation at low concentrations. The complexity of speciation by CLE-AdSV is generally not so much in the ligand exchange step, but in the following adsorptive accumulation and SVdetection steps. Under conditions of excess of L_{ad} over M, the bulk equilibrium distribution is prescribed by the conditional stabilities K' of the different species. Thus an effective thermodynamic cut-off window (also referred to as the detection window) is easily defined by comparing the conditional stability of the complexes in the original sample ($K'_{ML} = K_{ML}c_L^*$) to that of the complexes formed with the added ligand ($K'_{MLad} = K_{ML}c_{Lad}^*$) (Van den Berg et al., 1990; Town & Filella, 2002). However, the adsorptive accumulation at the electrode/solution interface is not allowed to attain equilibrium, but deliberately car-

ried out over a limited period of time. Consequently, the adsorbed amount reflects the time evolution of the flux of the adsorptive species, rather than simply the equilibrium concentration. Due to (mild) stirring, the larger part of the accumulation takes place under steady-state diffusion conditions, the characteristic time of the initial transient being relatively small compared to the total accumulation time t_{acc}. Apart from this, it has to be considered what is the possible contribution of the different species of M, other than the surface active ML_{ad}, to the accumulation flux. In other words, we have to face the question whether the various species of M are labile on the typical time scale of setting up steady state τ_{ss} and to what extent they consequently contribute to the accumulation via conversion into ML_{ad} inside the depletion layer. Here we address this issue by analysing the lability features of typical species distributions as met in CLE-AdSV and their ensuing impact on the diffusive accumulation process. In doing so, we shall define the corresponding kinetic detection window to complement the existing thermodynamic one.

THE TYPICAL SPECIES DISTRIBUTION AFTER THE CLE STEP

For CLE-AdSV to be successful in distinguishing between various metal species in a sample, the strength of the complex with the added ligand should be appropriately tuned to the thermodynamic window of interest. Improper choice of the nature and/or concentration of L_{ad} leads to limited information: with a too strong ligand L_{ad} one only obtains the total metal concentration, whereas with a too weak ligand one only measures the free metal ion (or even only part of it). By repeated measurement for various metal-to-(added) ligand ratios, that is in a titration sequence, one may vary to some extent the actual range of species stabilities covered, i.e. manipulate the width of the thermodynamic window. In line with the reasoning above, the meaningful situation of an AdSV metal speciation analyis is one where the concentration of ML_{ad} represents a significant fraction of the total metal, say, between 10 and 90%. From a kinetic point of view, the bulk equilibrium with ML_{ad} as the extra species will be established on a characteristic timescale $1/k_d$, with k_d being the dissociation rate constant of the complexes in the sample that undergo ligand exchange. The reason for this is that complexes with strengths up to that of ML_{ad} are subject to the ligand exchange $ML + L_{ad} \leftrightarrows ML_{ad} + L$, whereas stronger complexes are less affected. Therefore the conditions of ligand exchange equilibration can be formulated in terms of the k_a' for the formation of ML_{ad} (given by $k_a c_L$, the product of the rate constant of association k_a of ML_{ad} and the ligand concentration c_L) and the dissociation rate constant k_d of ML_{ad}. This defines the border of the thermodynamic window of the complexes ML that undergo the ligand exchange reaction. The basic condition would then read:

$$k_a' t_{eq}, k_d t_{eq} >> 1 \qquad (1a)$$

and since for all practical purposes K'(=k_a'/k_d) is well above unity, this comes to:

$$k_d t_{eq} >> 1 \qquad (1b)$$

The usual order of magnitude of t_{eq} is 10^3 s, meaning that for k_d up to O(10^{-2}) s^{-1}, the assumption of equilibrium would be correct. For a metal like Ni, with relatively low values for the complex formation rate constant (as can be calculated from the product of the rate of water loss, k_{-H2O} (≈ 3 * 10^4 s^{-1}) and the outer-sphere complex stability constant K_{OS} (≈ 3 mol^{-1} l) to be $k_a \approx 10^5$ l mol^{-1} s^{-1} (Margerum et al., 1978)) this means that complexes with stabilities up to K of O(10^7) mol^{-1} l obey the ligand exchange equilibration condition eq. (1). Likewise one finds that for Co(II), $k_a \approx 10^7$ l mol^{-1} s^{-1} (Margerum et al., 1978), resulting in an upper border of the thermodynamic window at O(10^9) M^{-1}. The real thermodynamic window, as defined by the conditional stability of ML_{ad} and the concentration of L_{ad}, should not exceed this upper border unless one extends the equilibration period.

THE ADSORPTIVE ACCUMULATION STEP

Lability aspects

After the ligand addition step, the sample solution contains at least three types of metal species, viz. ML, ML_{ad} and free M. Ideally, only one of these, the ML_{ad}, is surface active and hence it is the diffusive depletion of ML_{ad} and the coupled conversions of ML and M into ML_{ad} that need to be considered here (fig. 1).

In contrast with electrochemical conversion of M and questions on lability of electroinactive complexes ML, as applicable in the case of Anodic Stripping Voltammetry (ASV) (De Jong et al., 1987a,b), we now face the adsorptive accumulation of ML_{ad} where indirect contributions from the free metal (via association with L_{ad}) and complexes ML (via ligand exchange with L_{ad}) should be invoked.

For free M, lability with respect to ML_{ad} depends on the rate of association with L_{ad}, compared to the rate of diffusion of M itself. Following the conventional reaction layer concept, the flux J_{ass} ensuing from association of M with L_{ad} can be written as:

$$J_{ass} = k_a c_M c_{L_{ad}} \mu_{ML_{ad}} \qquad (2)$$

where k_a is the volume rate constant for $M+L_{ad} \rightarrow ML_{ad}$ and μ_{MLad} is the pertaining reaction layer thickness, defined by:

$$\mu_{ML_{ad}} = \sqrt{D_{ML_{ad}} / k_d} \qquad (3)$$

where k_d is the dissociation rate constant of ML_{ad}, which equals the reciprocal of the mean lifetime of ML_{ad} after its formation from M and L_{ad}. Combination of eqs. (2) and (3) with the maximum steady-state diffusion flux of M,

$$J_{dif}(\mathrm{M}) = D_{\mathrm{M}} c_{\mathrm{M}} / \delta_{\mathrm{M}} \tag{4}$$

yields the lability parameter L:

$$L = J_{ass} \Big/ J_{dif}(\mathrm{M}) = \frac{\left(k_{\mathrm{d}}^{1/2} \mathrm{K}' D^{1/2}\right)_{\mathrm{ML_{ad}}}}{\left(D/\delta\right)_{\mathrm{M}}} \tag{5}$$

For L>>1, M fully contributes to the diffusion of ML_{ad} (supposing L_{ad} to be in sufficient excess over M). For L<1, the contribution is of a predominantly kinetic nature.

For <u>all complexes ML</u> with stabilities beyond the detection window the rate constant for dissociation, k_d, is smaller than that for association of M to ML_{ad}. The reason is that, compared to ML_{ad}, ML is the stronger complex (otherwise it would not be present anymore after the ligand exchange step), hence $k_{d,ML} < k_{d,MLad}$. Then, since $K'_{MLad} > 1$ and thus $k'_{a,MLad} > k_{d,MLad}$, we certainly have:

$$k_{\mathrm{d,ML}} < k'_{\mathrm{a,ML\,ad}} \tag{6}$$

The significance of this is that in the sequence ML → M → ML_{ad} the step from ML to M is the rate-limiting one. Consequently, the lability of ML *with respect to conversion to* ML_{ad} derives from the dissociation of ML into M and L.

So for ML we can use the existing lability criteria, as valid for contibution of ML to electrodic conversion of M (Van Leeuwen, 2000, 2001):

$$L = J_{diss}(\mathrm{ML}) \Big/ J_{dif}(\mathrm{ML}) = \frac{k_{\mathrm{a}}^{'1/2} D_{\mathrm{M}}^{1/2} \delta_{\mathrm{ML}}}{\mathrm{K}' D_{\mathrm{ML}}} \tag{7}$$

In the previous section we found an approximate upper border for the thermodynamic window of O(10^7) M in case of Ni(II) and O(10^9) M for Co(II). If we compute the lability parameter L for Ni(II) and Co(II) complexes at ligand concentration levels of 10^{-5} M for a steady-state diffusion layer thickness δ_{ML} of O(10^{-4}) m (for details see next section) and $D_M \approx D_{ML} \approx 10^{-9}$ m^2 s^{-1}, we obtain values for L of about 3 * 10^{-2} and 3 * 10^{-3} for Ni(II) and Co(II) respectively. This would lead us to the conclusion that typically in an AdSV experiment the strong complexes, which are not subject to ligand exchange with L_{ad}, are not labile during the diffusive adsorption step. Only if the real thermodynamic window is by several orders of magnitude lower than the upper border defined in section 1, lability of the remaining complexes ML needs to be considered. For the upper borders of the thermodynamic windows for Ni(II) and Co(II), and a c_{Lad} of O(10^{-5}) M, we find from eq. (5) that for free M, L is 3*10^1 and 3 * 10^3 for Ni(II) and Co(II) respectively. This implies that any free metal must be expected to fully contribute to the supply of adsorbing ML_{ad}. Consequently, AdSV typically measures the <u>sum</u> of ML_{ad} and free M, a feature of practi-

cal importance e.g. if the c_{Lad} is not sufficient to warrant that $c_{MLad} >> c_{M}$.

If AdSV is applied in a metal titration of the ligand L, the overall lability picture of the method will not change due to changing metal-to-ligand ratios (Van Leeuwen, 1987). The reason for this is that over the whole titration range, the strong added ligand L_{ad} is kept in excess over M, thus the metal species detected as complexes are strong enough not to be labile.

Kinetics of the adsorptive accumulation

The interfacial rates of adsorption of surface active metal ion complexes onto mercury from aqueous solution are generally rapid (Delahay, 1965). Hence, the rate of adsorption of ML_{ad} is usually governed by mass transport parameters. Still, quite a few analyses of surface concentrations Γ (moles adsorbed per unit area) as a function of time (t) are a priori formulated in terms of interfacial adsorption rate constants. The difficulty of a proper analysis of the adsorption rate is compounded by the inherent complexity of diffusive accumulation into an adsorbed layer. Even for the simplest type of isotherm, the linear Henry case, the Γ,t function is involved due to the fact that the concentration of the adsorbing species just outside the adsorbed layer, $c^0(t)$, increases with time in proportion with $\Gamma(t)$. Under transient conditions, with a continuously growing diffusion layer, the combination of a linear isotherm

$$\Gamma(t) = K_H c^0(t) \tag{8}$$

with the space-dependent and time-dependent conservation equations (Fick's first and second law) yields, for $\Gamma(0) = 0$:

$$\Gamma(t) = \Gamma^{eq}\left\{1 - \exp\operatorname{erfc}\left[(t/\tau)^{1/2}\right]\right\} \tag{9}$$

with Γ^{eq} being the equilibrium value of Γ, i.e. $K_H c^*$; and the characteristic time constant τ being defined by:

$$\tau = K_H^2 / D_{ML_{ad}} \tag{10}$$

Since the adsorptive accumulation step is usually carried out under mild stirring, the evolution of Γ as a function of time follows eq. (9) only for the first couple of seconds. Even in the absence of stirring, natural convection in solution generally achieves steady-state diffusion towards the mercury drop electrode within a few tens of seconds. Within the steady-state convective diffusion regime, the flux of ML_{ad} only varies with time because of the steady increase of $c^0(t)$. Under the usual conditions of AdSV, the change of c^0(t) is generally slow compared to the timescale of adjustment of the concentration profile within the diffusion layer (typically of order δ^2/D, that is, on the order of 10 s). On the typical timescale of accumulation in AdSV (order 10^2 s) it is therefore reasonable to write

the flux of adsorbing species ($J_{ad,MLad}$) as:

$$J_{ad,ML_{ad}} = \frac{D_{ML_{ad}}\left[c^{0}_{ML_{ad}}(t) - c^{*}_{ML_{ad}}\right]}{\delta_{ML_{ad}}} \tag{11}$$

where $c^0_{MLad}(t)$ is the volume concentration of ML_{ad} just outside the adsorbed layer and δ_{MLad} is the convective diffusion layer thickness. For the latter parameter, explicit expressions are available for various hydrodynamic conditions (Levich, 1962). Practically, δ_{MLad} will be something like 100 μm for mild stirring and complex species ML_{ad} with diffusion coefficients not too much smaller than that of the free aquo ion. The change of Γ with t in a time interval short enough to have an essentially constant $c^0_{MLad}(t)$ is:

$$\Delta\Gamma(t) = -J_{ML_{ad}}(t) \times \Delta t \tag{12}$$

Combination of eqs. (8), (11) and (12) yields:

$$\frac{\Gamma(t)}{\Gamma^{eq}} = \frac{t/\tau}{1 + t/\tau} \tag{13}$$

In this case, the characteristic time constant τ is defined by:

$$\tau = K_H \delta_{ML_{ad}} / D_{ML_{ad}} \tag{14}$$

Fig. 2 shows the time dependencies for the transient and steady-state cases as defined by eqs. (9) and (13) respectively. Note the huge difference between the initial slopes and the similarity between (13) and an exponential decay for short times. Another point of interest is that the experfc is *never* linear with (t/τ): for very small t it approaches $2(t/\pi\tau)^{1/2}$ and for large t it approaches $1-(t/\pi\tau)^{-1/2}$. The transient and steady-state functionalities have in common that they very gradually approach the equilibrium value Γ^{eq}, which is physically understood from the continuous decrease in (c^*-$c^0(t)$) and the concomitant decrease in the flux ($d\Gamma/dt$). It should be underlined that these very gradual equilibration functions hold for the regime of linear adsorption. If Γ reaches values in the regime of decreasing $d\Gamma/dc$, the tailing of the Γ,t-functionality becomes even more serious (Reinmuth, 1961).

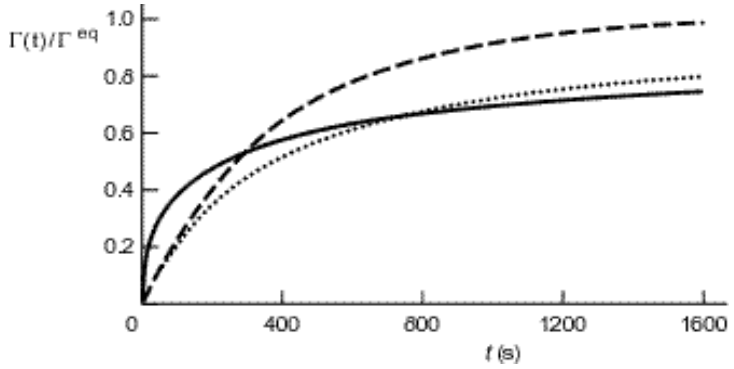

Fig. 2. Various model descriptions for the course of $\Gamma(t)/\Gamma^{eq}$ as a function of time for τ = 400 s: —— = transient: experfc functionality (eq. 9: 1-experfc$(t/\tau)^{1/2}$); — — = exponential decay functionality (1-exp(-t/τ)) (Ramirez et al., 1996); · · · = steady-state (eq. (13): $(t/\tau)/(1+t/\tau)$).

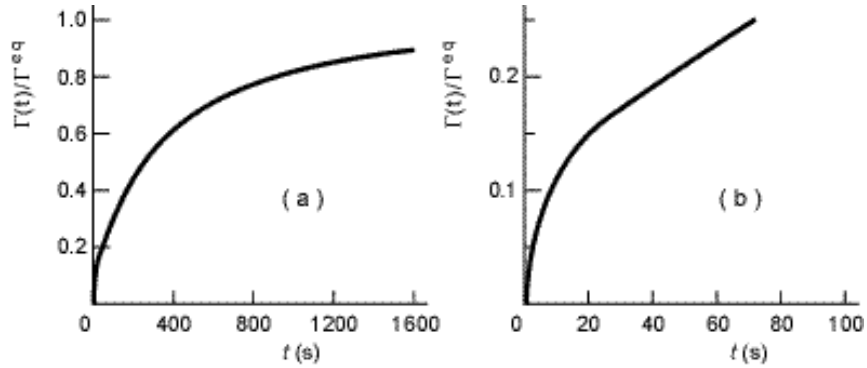

Fig. 3. Sketch of the dependence of $\Gamma(t)/\Gamma^{eq}$ on time: transient character in the initial stage; steady-state character in the later stages. a) Overall behaviour; b) Short term, showing the transient character of the initial part.

The common situation in the AdSV accumulation step is thus a mix of transient and steady-state diffusion. Depending on stirring conditions, the transient diffusive transport (eq. (9)) will be in effect over the first 1 to 20 s., and is then overruled by steady-state convective diffusion (eq. (13)). A sketch of this general behavior is given in fig. 3. The initial sharp rise of Γ is due to the transient contribution. With approaching steady state, the Γ,t-relation tends to linearity over a significant portion of time. Clearly, such a composite behavior does not lead to a reasonable fit of experimental data to either the transient expression (eq. (9)) or the steady-state equation (eq. (13)). For example, analysis in terms of the latter will generally result in a poor match for small t and a positive intercept (compare fig. 3). Fitting the full Γ,t-dependence to the transient expression (eq. (9)) is bound to be hopeless unless specific measures have been taken to avoid convection. Thus concluding that 'the adsorption process is not controlled by diffusion' (Ramirez et al., 1996), simply because the Γ,t data points do not fit to an experfc functionality is grossly incorrect. The experimental curves for the nickel/dimethylglyoxime system of Ramirez et al. (1996)

are actually quite well described by the convective diffusion equation (eq. (13)) with a diffusion layer thickness of $O(10^{-4})$ m and a Henry coefficient of $O(10^{-2})$ m, which confirms the rather strong adsorption of the complex (Pihlar et al., 1986).

CONCLUSIONS

The fundamental kinetic features of CLE-AdSV are analysed. The upper border of the thermodynamic window is defined by the requirement of equilibration before accumulation of the complex with the added ligand ML_{ad}. Lability criteria for the different types of metal species, relevant in a typical CLE-AdSV experiment are derived for the common case where only ML_{ad} is surface-active. The lability of sample complexes ML with respect to conversion to ML_{ad} always derives from the dissociation of ML into M and L. The strong complexes ML, which do not undergo ligand exchange with L_{ad}, are generally shown to be not labile on the effective timescale of the convective diffusion in the accumulation step. AdSV thus measures the sum of ML_{ad} and free M, with the latter being usually negligible. The evolution of the surface concentration of ML_{ad} with time is generally determined by the rate of mass transport which over the major part of the accumulation period is governed by steady-state convective diffusion.

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SYMBOLS AND ABBREVIATIONS

M	Metal
L_{ad}	Added ligand
L	Sample ligand
ML	Complex of metal and sample ligand
ML_{ad}	Complex of metal and added ligand
c_i	Concentration of species i (mol l^{-1})
D_i	Diffusion coefficient of species i (m^2 s^{-1})
J_{ass}	Association flux (mol m^{-2} s^{-1})
J_{ads}	Adsorption flux (mol m^{-2} s^{-1})
J_{dif}	Diffusive flux (mol m^{-2} s^{-1})
$k_{d,i}$	Rate constant of dissociation of species i (s^{-1})
$k_{a,i}$	Rate constant of association to species i (mol^{-1} l s^{-1})
$k'_{a,i}$	Conditional rate constant of association of species i ($=k_{a,i}c_L$) (s^{-1})
k_{-H2O}	Rate constant of water loss (s^{-1})
K_{OS}	Outer-sphere complex stability constant (mol^{-1} l)
K_H	Henry coefficient (m^{-1})
K_{ML}	Stability constant of complex ML (mol^{-1} l)
K'_{ML}	Conditional stability constant of complex ML ($= K_{ML}\ c_L$) (-)
L	Lability criterium (-)
t_{eq}	Equilibration time (s)
t_{acc}	Accumulation time (s)
δ_i	Diffusion layer thickness with respect to species i (m)
μ	Reaction layer thickness with respect to species i (m)
τ	Characteristic time constant, either for transient (eq. (10)) or steady state adsorption (eq. (14))
τ_{ss}	Time to reach steady state (s)
Γ	Adsorbed amount (mol m^{-2})
Γ^{eq}	Adsorbed amount at equilibrium (mol m^{-2})

Superscripts:

^{0}	Near the electrode surface
*	In the bulk

[3]

Speciation analysis of Co and Ni in anaerobic bioreactors using Competitive Ligand Exchange - Adsorptive Stripping Voltammetry (CLE-AdSV) and implications for bioavailability

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ABSTRACT

Competitive Ligand Exchange - Adsorptive Stripping Voltammetry (CLE-AdSV) is an electrochemical technique for the speciation analysis of metals such as Ni(II) and Co(II) down to nanomolar concentration levels. It has been applied to many environmental matrices, but there is a paucity of data on its application to anaerobic wastewater, for which the technique is particularly useful because of the low dissolved Ni and Co concentrations and the involved speciation.

This study presents AdSV measurements in anaerobic bioreactor and similar batch media. The stripping voltammetric peaks for Co and Ni are well-behaved. Sulfide interference, in effect above certain concentration levels, is avoided by acidification and degassing, after which is the pH is normalized again. Linear calibrations are obtained for both Co and Ni down to *ca*. 1 nM.

Comparison of metal concentrations measured using AdSV with total metal concentrations determined by ICP-MS gives information about the fraction of dissolved metal in strongly bound form. In the anaerobic matrices, up to 95% of the Co and Ni is present in strongly bound forms ($K_{MeL} \geq 2\text{-}3 * 10^{10}$ mol^{-1} l). In methanogenic media, no further binding was detected upon addition of Co and Ni. Presumably, the bound forms are the cobalt-containing vitamin B_{12} and the nickel-containing factor F_{430}, which are excreted by methanogenic archaebacteria. Effluent from sulfate reducing bioreactors however shows strong and extensive binding of added Co (c_L = 0.4 µM; $K_{CoL} \geq 5 * 10^9$ mol^{-1} l).

INTRODUCTION

For detection and speciation analysis of Ni(II) and Co(II) at low concentrations (nM-μM), Competitive Ligand Exchange – Adsorptive Stripping Voltammetry (CLE-AdSV, hereafter referred to as AdSV) is a very suitable technique (Xue & Sigg, 2002). The pre-concentration step confers adequate sensitivity for detection of very low concentrations, and the competitive equilibrium established with the added ligand enables detailed study of the binding characteristics of the sample ligands (Xue & Sigg, 2002). AdSV has been applied to many matrices, such as sea water (e.g.: Van den Berg & Nimmo, 1987; Zhang et al., 1990; Saito & Moffett, 2001), freshwater (e.g.: Qian et al., 1998; Xue et al., 2001; Lam et al., 1999), ground water (Xue et al., 2001) and wastewater (Sedlak et al., 1997). Its application to anaerobic matrices has not yet been investigated, although there are several reasons why this would be very interesting. In these environments, methanogenic archae-bacteria are present for which Co and Ni are essential elements due to their role in vitamin B_{12} and factor F_{430} (Thauer, 1998; Telser, 1998; Roth et al., 1996). Methanogenesis is a process of great interest: in anaerobic bioreactors, it can be used to efficiently degrade small organic molecules like methanol and acetate to methane (biogas) (Lettinga & Hulshoff-Pol, 1983), whilst in natural environments, it plays a role in the global carbon cycle (Hoehler & Alperin, 1996).

Actual limitation of methanogenesis by Co and Ni is known to occur (e.g.: Gonzalez-Gil et al., 1999; Florencio et al., 1993; Zandvoort et al., 2003), and is strongly affected by chemical speciation (Zandvoort et al., 2004a; Shen et al., 1993). Generally it is assumed that only the free metal ion is available for biouptake. In anaerobic environments, Co and Ni are largely precipitated by sulfide, leading to very low dissolved concentrations (Callander & Barford, 1983a; Hayes & Theis, 1978; Osuna et al., 2003) and they may interact with many other ligands in the matrix which will affect bioavailability and the ensuing bioreactor performance (Kuo & Parkin, 1996; Gould & Genetelli, 1978). To date, very little is known about ligands present in bioreactors. Many speculations on their existence and properties have been made (Callander & Barford, 1983b; Barber & Stuckey, 2000; Kuo & Parkin, 1996; Sedlak et al., 1997), but no detailed information is available. There can be a large pool of dissolved organic material in bioreactors, consisting mainly of soluble microbial products (SMPs) (Noguera et al., 1994). In particular, it would be interesting to establish whether the organisms are able to actively excrete metal binding substances to facilitate metal bioavailability, as is well known for organisms coping with Fe deficiency (Neilands, 1995) and recently found for algae suffering from Co limitation (Saito et al., 2002). Such knowledge would facilitate development of effective metal dosing in bioreactors.

In this work we have applied AdSV to determination of Co and Ni speciation in anaerobic media, and related these data to bioavailability and biological activity. A protocol is established that overcomes the challenges involved in AdSV measurements in such media, e.g. competitive adsorption of surface active compounds (Redinha et al., 1997) and the impact of degassing on the redox speciation.

THEORY

AdSV involves the addition of a ligand (in this case DMG, Dimethylglyoxime), which forms strong surface-active complexes with the metal ion. This complex is adsorbed onto a mercury drop electrode at a given potential for a fixed period of time. After this surface accumulation step, the potential is scanned cathodically, and the amount of adsorbed metal-DMG complex is measured by reduction of the complex.

The resulting peak current I_p is a function of the concentration of surface active complex $Ni(DMG)_2$ formed:

$$I_p = S \cdot [Ni(DMG)_2] \quad (1)$$

where S is the sensitivity (in A mol^{-1} l).

After addition of DMG to a sample containing several ligands L, the total amount of the metal will be distributed over different species resulting in the following mass balance:

$$[Ni]_{total} = [Ni^{2+}] + \sum_i [NiL_i] + [Ni(DMG)_2] \quad (2)$$

where $[Ni]_{total}$ = total nickel concentration, $[Ni^{2+}]$ = free nickel ion concentration and $[NiL_i]$ = concentration of nickel complexes with ligands L.

In case of strong binding by the ligands L and DMG, $[Ni^{2+}]$ is negligible, which simplifies eq. (2) to:

$$[Ni]_{total} = [NiL] + [Ni(DMG)_2] \quad (3)$$

Here, the nickel binding by the organic ligand (L) is formally written in terms of a 1:1 complex - the usual convention in macromolecular metal ion binding (Buffle, 1988). In reality, L likely includes various different ligands, leading to variation of K_{NiL} with the Ni/L ratio - a typical property of chemically heterogeneous systems.

Both [NiL] and $[Ni(DMG)_2]$ are determined by their stability constants K_{NiL} and $K_{Ni(DMG)2}$ respectively, and the concentrations of the competing ligands, [L] and $[DMG^-]$:

$$[Ni(DMG)_2] = K_{Ni(DMG)_2} \cdot [Ni^{2+}] \cdot [DMG^-]^2 = \alpha_{Ni,DMG} \cdot [Ni^{2+}] \quad (4)$$

$$[NiL] = K_{NiL} \cdot [Ni^{2+}] \cdot [L] = \alpha_{Ni,L} \cdot [Ni^{2+}] \quad (5)$$

The product of the stability constant and the ligand concentration equals the ratio of complex over free metal ion and is denoted by the complexation coefficient α.

The nickel will distribute over the DMG and the (possibly present) L. Then, rewriting eq. (1) in terms of $[Ni]_{total}$ using eq. (3), (4) and (5) gives:

$$I_p = S \cdot [Ni]_{total} \cdot \frac{[Ni(DMG)_2]}{[Ni(DMG)_2]+[NiL]} = S \cdot [Ni]_{total} \cdot \frac{\alpha_{Ni,DMG}}{\alpha_{Ni,DMG} + \alpha_{Ni,L}} \quad (6)$$

Metal titration

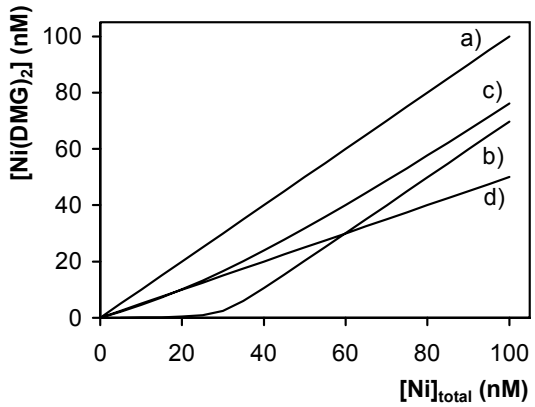

Fig. 1. Theoretical AdSV metal titrations in the absence and the presence of competing ligands with varying concentration and binding strength: a) no ligand present; b) low concentration of strongly binding ligands; c) low concentration of intermediately binding ligands; d): high concentration of intermediately binding ligands.

When a sample is titrated with metal, and I_p is plotted as a function of $[Ni]_{total}$, the outcome is dependent on the complexation coefficient of L and DMG (fig. 1). In the absence of L, addition of Ni will only result in the formation of $Ni(DMG)_2$, and the titration will be a straight line (fig. 1, line a). For the case $\alpha_{Ni,L} << \alpha_{Ni,DMG}$, a straight line is found with a slope equal to that of the reference line (fig. 1, line a). At the other extreme, i.e. $\alpha_{Ni,L} >> \alpha_{Ni,DMG}$, the titration will have a different form (fig. 1, line b). In the first part with an excess of L over Ni, all Ni will bind to L and the slope will be zero. However, as soon as L becomes saturated with Ni, the slope will increase and reach the reference slope. The ideal case for determination of both binding strength K and concentration of the ligand [L], is the case when $\alpha_{Ni,L}$ is only slightly bigger than $\alpha_{Ni,DMG}$, corresponding to fig. 1, line c. Under these conditions the Ni added will first distribute over both L and DMG. As soon as L starts to become saturated, the slope of the titration curve gradually increases, until the reference slope is reached. The curve can be linearized by plotting the calculated value for $[Ni^{2+}]/[NiL]$ against $[Ni^{2+}]$ (a linearized Langmuir isotherm). The slope is 1/[L] and the intercept equals $1/(K_{NiL} \cdot [L])$. This method is only applicable if only one class of ligands is present; in the presence of two or more ligands, the linearization is no longer valid. For more details, see Miller & Bruland, 1997. If saturation is not

reached (fig. 1, line d), $K_{NiL} \cdot [L]$ can be estimated by comparing the slope of the blank with the slope in presence of ligand.

Ligand titration

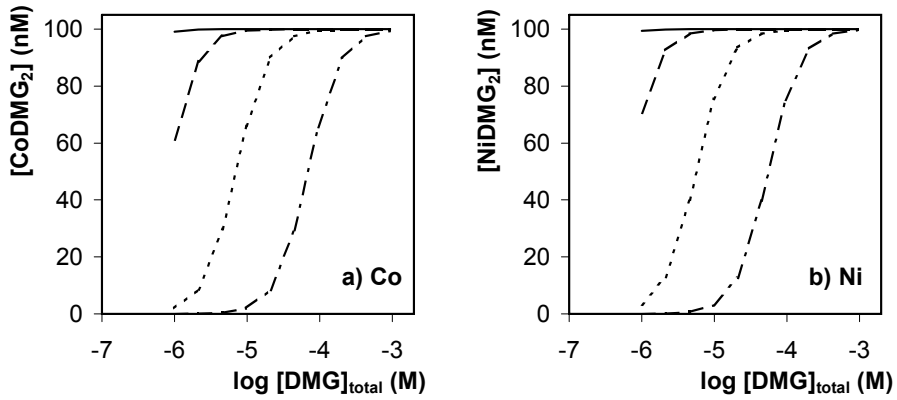

Fig. 2. Effect of pH on DMG titration of Co (a) and Ni (a) in absence of competing ligands. $[Co]_{total}$ and $[Ni]_{total}$ = 100nM. —・— : pH = 6; ・・・ : pH = 7; — — : pH = 8; —— : pH = 9.

Titration with DMG provides complementary information on the binding properties of the sample ligands, and facilitates selection of the optimal DMG concentration for a metal titration. For eq. (3) to be valid, Ni must be practically completely complexed by DMG. Fig. 2 shows the dependence of $Ni(DMG)_2$ and $Co(DMG)_2$ formation on $[DMG]_{total}$ as a function of pH.

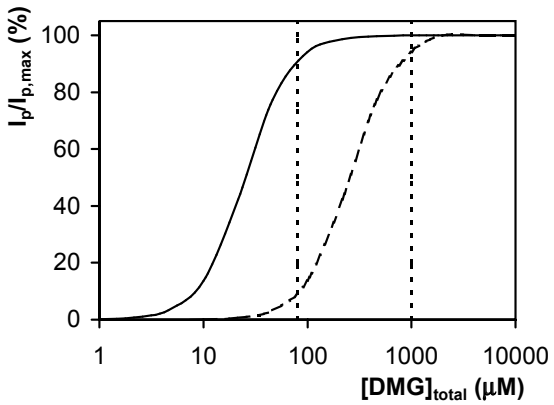

Fig. 3. DMG titrations in absence (—) and presence (- -) of competing ligand L. The range of DMG concentrations giving most information on binding constants of L is in between the vertical dotted lines. pH = 7; $[Co_{total}]$ = 100 nM; $\alpha_{Co,L}$= 100.

In the presence of a competing ligand, the amount of DMG required for complete complexation is increased (fig. 3). For obtaining information on the parameters of L, only the range where binding by both DMG and L are significant is useful, for which the lower and upper limits are indicated with dotted lines in fig. 3. This region defines the detection window, i.e. the range of values of complexation coefficients of L ($\alpha_{Ni,L}$) which can be detected at a certain complexation coefficient of DMG ($\alpha_{Ni,DMG}$). At the lower limit, $\alpha_{Ni,L} << \alpha_{Ni,DMG}$ and no information whatsoever can be obtained about L, since the AdSV signal is the same as in the absence of ligand. At the upper limit $\alpha_{Ni,L} >> \alpha_{Ni,DMG}$ and a metal titration provides some limited information about the ligand. If [L] is less than the maximum metal concentration added, the value for [L] can be found, while only a lower limit for K_{NiL} can be calculated. If [L] is larger than the maximum metal concentration added, then a lower limit for K_{NiL} . [L] is the only obtainable information.

Kinetic considerations

All these considerations are valid only if the system is at equilibrium. However, it is known that complexation reactions can be slow (e.g.: Margerum et al., 1978; Lam et al., 1999; Xue et al., 2001); therefore, complexation kinetics must be considered.

As discussed before, DMG is typically in excess over the free metal ion. In the absence of other ligands, the rate of complex formation is generally determined by the rate of water loss from $Ni(H_2O)_6^{2+}$ (Eigen & Wilkins, 1965). The complete rate equation is given by:

$$\frac{d[\mathrm{Ni(DMG)_2}]}{dt} = k_f\,'[\mathrm{Ni^{2+}}][\mathrm{DMG}]_{total} \tag{7}$$

with:

$$k_f\,' = k_f\,[\mathrm{DMG^-}]/[\mathrm{DMG}]_{total} \tag{8}$$

where: k_f = formation rate constant, k_f'= conditional formation rate constant.

After addition of a certain amount of Ni, the AdSV signal will increase with $Ni(DMG)_2$ according to eqs. (7) and (1). These equations can be combined and linearized as indicated in Xue et al., 2001 to give:

$$\ln\left(1-\frac{I_{\mathrm{p},t}}{I_{\mathrm{p,max}}}\right) = -k_f'[\mathrm{DMG}]_{total}\,t \tag{9}$$

where $I_{p,t}$ = the AdSV signal at time t, and $I_{p,max}$ = the final, maximum AdSV signal. In this way it is possible to obtain a value for k_f' from the I_p,time-dependence.

Sulfide complexation in anaerobic media

In anaerobic bioreactor media, sulfide typically reaches concentrations up to several mM. Although there is ongoing debate as to the exact values of the stability constants for Co(II) and Ni(II) sulfide complexes (e.g.: Luther et al., 1996; Davison, 1996; Al-Farawati & Van den Berg, 1999), the importance of complexation is unambiguous and a reasonable estimate of the competition for metal binding between DMG and sulfide is feasible (table 1), as shown in fig. 4. Calculations were made for 1 mM DMG_{total} and varying concentrations of sulfide for different pH values. It is evident that complexation by sulfide can have large effects on binding by DMG. Both the conditional binding strength of the DMG complexes, and that of the metal-sulfide complexes, and thus the competition between them, is strongly pH-dependent. pH will also affect the gas/liquid ratio for sulfide, a lower pH leading to a decrease of dissolved sulfide due to increased formation of $H_2S(g)$. In the anaerobic media at pH = 7 and the absence of added DMG, metal sulfide complexes constitute the majority of the total dissolved Co and Ni, $Co(SH)_2^0$ and $Ni(SH)_2^0$ being the main species. The ratio of total concentration of dissolved metal sulfide complexes over the concentration of free metal ion is between 100 and 1000.

Sulfide and its metal complexes may be adsorbed onto the mercury electrode surface, giving rise to adsorptive competition between the target metal-DMG complex and the sulfide (Redinha et al., 1997). Therefore, in matrices containing sulfide, the possible impact of this compound on the voltammetric response needs to be assessed (see experimental section).

Table 1. Stability constants used for calculation of speciation of Ni and Co as affected by DMG and sulfide (I = 0 M, T = 25 °C). When more than one value has been reported, that used for the calculations is indicated in italics.

Complex	Log K	Reference
HDMG	10.66	Martell & Smith, 1989
$Co(DMG)^+$	8.79	Sillen & Martell, 1964
$Co(DMG)_2^0$	17.64	Sillen & Martell, 1964
$Ni(DMG)^+$	9.44	Sillen & Martell, 1964
$Ni(DMG)_2^0$	17.84	Sillen & Martell, 1964
HS	13.9	Martell & Smith, 1989
H_2S	20.92	Martell & Smith, 1989
$Co(SH)^+$	19.53 / *20.06* / 21.33 / 19.23	Luther et al., 1996 / Zhang & Millero, 1994 / Al-Farawati & Van den Berg, 1999 / Dyrssen, 1988
$Co(SH)_2^0$	*39.15* / 40.95	Al-Farawati & Van den Berg, 1999 / Dyrssen, 1988
$Ni(SH)^+$	19.85 / *20.16* / 19.63 / 18.33	Luther et al., 1996 / Zhang & Millero, 1994 / Al-Farawati & Van den Berg, 1999 / Dyrssen, 1988
$Ni(SH)_2^0$	*39.55* / 40.15	Al-Farawati & Van den Berg, 1999 / Dyrssen, 1988

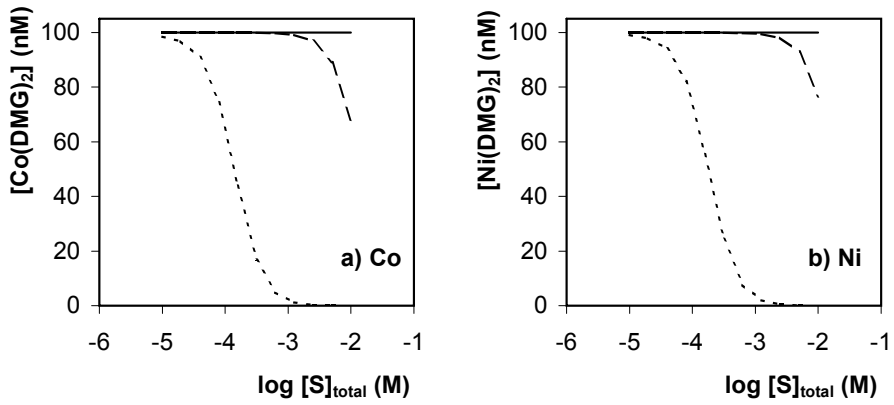

Fig. 4. Model calculations of competition between sulfide and DMG for Co (a) and Ni (b). ・・・: pH = 6; — —: pH = 7; ——: pH = 8.

MATERIAL AND METHODS

Chemicals

A stock solution of DMG (Dimethylglyoxime), 0.01 or 0.1 M, was prepared by dissolving the solid in 0.1 M NaOH. Co(II) and Ni(II) solutions were prepared by dissolving their chlorides into 0.1 M HNO_3 and diluting. Fresh buffers and DMG solutions were prepared on at least a monthly basis and refrigerated until use. All chemicals were of p.a. quality.

Total dissolved metal determination

Total dissolved metal concentration was determined by ICP-MS (Perkin Elmer ELAN 6000) after separation of cells or particles by filtration or centrifugation and acidification with HNO_3 to pH = 1.

Voltammetry

Voltammetric measurements were carried out using an Autolab potentiostat (Eco Chemie, The Netherlands), connected to a hanging mercury drop electrode (HMDE, Metrohm 663 VA stand, drop area=0.52 mm^2) and a graphite counter electrode. The cell was made of lead-free borosilicate glass and provided with a PTFE stirrer. Potentials are reported with respect to a Ag/AgCl reference electrode (3M KCl). The system was controlled by a PC using the program GPES32 (Eco Chemie, The Netherlands).

Prior to measurement, the solution in the voltammetric cell was stirred and purged with argon for about 5 min. The adsorptive accumulation was performed under stirring for 60 s, followed by a rest time of 5 s. The voltammetric scan was carried out in the DP mode, with a pulse height of 50 mV, a step potential of 5 mV and a scan rate of 10.2 mV/s. The deposition potential was –0.8 V and the scan range was from –0.4 to –1.2 V.

Media

Unless indicated otherwise, microbial batch cultures were grown in media as described elsewhere (Gonzalez-Gil et al., 2003), hereafter referred to as anaerobic batch media. Depending on the experiment, different amounts of Co or Ni were added. Sulfide was present in the medium as sulfur source at a concentration of 1 mM.

Biomass

In the batch experiments, two sources of biomass were used: (i) a methanogenic enrichment from an anaerobic wastewater reactor consisting mainly of *Methanosarcina* sp. (Gonzalez-Gil et al., 1999; Gonzalez-Gil et al., 2003; chapter 4 of this thesis), grown at 30°C, and (ii) a pure culture of *Methanosarcina barkeri* DSM 800, grown at 37°C.

Experimental setup

Effects of sulfide

Synthetic media containing 40 mM KNO_3, 60 mM Tris, 100 µM DMG and sulfide were prepared (pH = 7.0). Sulfide was freshly prepared by dissolving in argon preflushed water.

Kinetics of complexation of Co/Ni with DMG

Anaerobic batch medium containing sulfide as the sulfur source, and no added Co or Ni, were filtered using 0.2 µm filters and purged with argon under additions of HNO_3 to remove carbonate and sulfide. When the pH had stabilised, it was adjusted to 7.0 and buffered with 50 mM MOPS. Ni, Co and DMG were then added, and AdSVmeasurements were performed as a function of time.

DMG binding characteristics in anaerobic media

DMG titrations of synthetic medium were performed using mixtures of 50 mM MOPS, pH = 7.0, 50 mM KNO_3, 50 nM Ni and Co, and increments of DMG. The mixtures were left to equilibrate overnight before AdSV measurement.

DMG titrations of anaerobic batch medium containing sulfide were performed after the same pretreatment as in the studies of the kinetics of Co/Ni-DMG complexation. After the pretreatment, DMG was added stepwise and at each concentration the AdSV-signal was measured until a stable value was obtained.

In-situ speciation: batch cultures

In one experiment (Gonzalez-Gil et al., 2003) the enrichment was grown in the presence of 1 mM sulfide and a range of total Co and Ni concentrations (total Co, total Ni (µM, µM) = 0,0; 2,2; 40,40 and 400,200). After growth, the media were centrifuged anaerobically (10 min. at 42000 g) and the supernatant was acidified to pH = 1. For AdSV Co and Ni titrations, pH was adjusted to 8.3 using NaOH and borate buffer (50 mM) and DMG (15 µM) were added.

In a second experiment, batches of a pure culture of *Methanosarcina barkeri* DSM 800 were grown in presence of 0.2 µM Co and Ni and 1 mM sulfide. In the late exponential

phase, cultures were filtered using 0.2-µm filters. For AdSV analysis, the medium was pretreated in the same way as in the kinetics study. First a DMG titration was performed by adding Co and Ni (both to a concentration of 50 nM) and increasing DMG stepwise up till 280 µM. After this, the sample was titrated with Co and Ni.

In-situ speciation: reactor media

The first study of reactor media was carried out with wastewater effluent from a nickel-deficient methanogenic UASB reactor (Zandvoort et al., 2002). The influent contained 0.84 µM total Co, no added Ni and 0.4 mM sulfate, most of which was converted into sulfide. In the reactor an active methanogenic granular sludge bed was present. The effluent samples were centrifuged (10' at 5 * 10^4 g). The solution was buffered at pH 7.0 by addition of 50 mM Tris. Complexing ligand DMG was added to 100 µM and a Co titration was performed.

A second study of bioreactor media was performed with effluent from UASB-reactors with different Co loading (Zandvoort et al., 2004b). The sludge of one of these reactors (in the following called Co+) was loaded with Co before operation, while the sludge of the other reactor (Co-) was not. Effluent was filtered using 0.2-µm filters, diluted 4 x and added to a mixture containing, after addition of wastewater, 0.02 M PO_4 (pH = 7.0) and 100 µM DMG. The gas phase of the sample was changed with N_2 to remove CO_2 and sulfide. After this, AdSV-titrations with Co and Ni were performed. In parallel, samples were filtered using 0.2-µm filters, acidified and analysed for total Co and Ni using ICP-MS.

Apart from these two reactors in which methanogenesis was dominant, Ni and Co speciation was also studied in a reactor in which sulfidogenesis was dominant (Vallero & Lens, 2004). This UASB reactor was operated at 55 °C and pH = 7.5. Methanol was the sole electron donor and the reactors were operated under excess of sulfate. The sulfide concentration was around 20 mM. The reactor was fed with a mixture of metals, containing 48 nM Co and 0.2 nM Ni. EDTA was present in the influent at a concentration of 76 nM. Effluent and influent was centrifuged (10' at 5 * 10^4 g) and 5 ml of this supernatant was diluted to a mixture containing 35 mM Tris (pH=7.4) and 100 µM DMG. In this mixture a Co and Ni titration was carried out with AdSV.

Speciation modeling

Speciation was modeled using the speciation program ECOSAT. Stability constants used were taken from literature as indicated in table 1.

RESULTS

Effects of sulfide

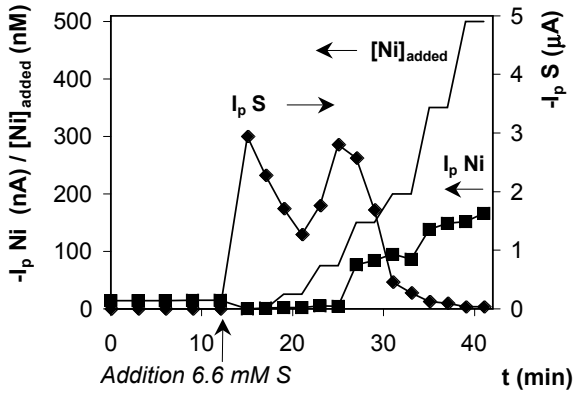

Fig. 5. Effects of sulfide on the Ni signal measured by AdSV.

Fig. 5 shows the effect of sulfide on the AdSV signal of Ni (results for Co were similar). At a high concentration (6.6 mM), freshly prepared sulfide strongly limits the Co and Ni signal. For instance, the clearly visible signal of Ni before addition of sulfide vanishes upon addition of sulfide. Upon further addition of Ni and Co, the AdSV signals only reappear again after a considerable amount of the sulfide has been removed. The effect of sulfide on the AdSV signal of Co and Ni is most likely due to competitive adsorption of the sulfide onto the mercury drop (Redinha et al., 1997). At this pH and DMG/sulfide ratio, binding of Co and Ni by DMG is stronger than binding by sulfide (fig. 4). Purging with N_2, in acid conditions, overcomes this interference: the sulfide in solution is quickly removed, and the Co and Ni signals are recovered rapidly.

Overall we can conclude that sulfide containing media can be best purged before measurement until most sulfide is removed, thus preventing competitive adsorption and binding.

Kinetics

From earlier studies (e.g.: Xue et al., 2001; Lam et al., 1999) it is known that the formation of metal-DMG complexes may be slow. AdSV measurements illustrate that the kinetics of complexation of Co and Ni with DMG in these typical anaerobic matrices can indeed be slow (fig. 6). As in earlier studies, the rate of complex formation is dependent on the DMG concentration (fig. 6c). Values for the conditional rate constant k_f' (table 2) were obtained by linearizing fig. 6a into fig. 6b and are in broad agreement with literature data. The intrinsic rate constant k_f was calculated using the protonation constant for DMG given in table 1. These results highlight the importance of taking kinetic features into account, for both Ni and Co. The rate of complexation does not appear to be affected by Ca^{2+} or by Ni complexants other than DMG. Co reacts more rapidly than Ni, but significantly slower than predicted on the basis of literature data.

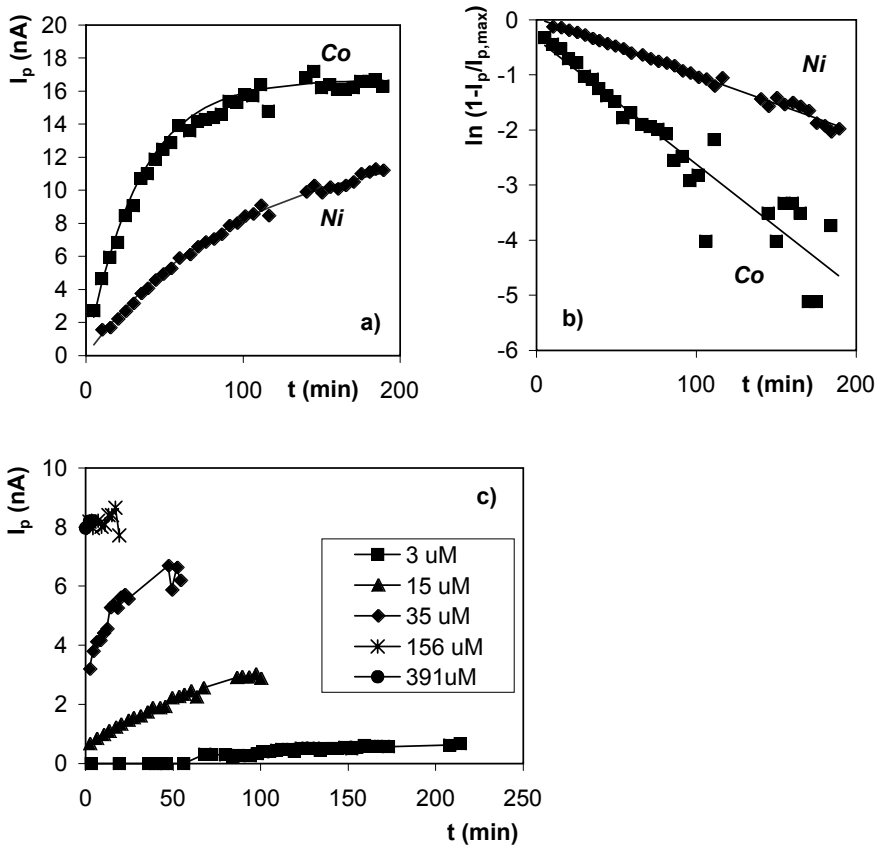

Fig. 6. Kinetics of $Co(DMG)_2$ and $Ni(DMG)_2$ formation in anaerobic medium. a) kinetics of $Co(DMG)_2$ and $Ni(DMG)_2$ formation in time ($[DMG_{total}]$ = 20 µM); b) linearization of fig. 6a for determination of rate constants; c) effect of DMG concentration on $Ni(DMG)_2$ formation rate.

Table 2. Rate constants k_f', k_f measured and taken from literature. Literature values for k_f were taken from experimental data by Xue et al., 2001, or calculated from K_{OS} and k_{-H2O} according to Morel & Hering, 1993 (K_{OS} taken to be 2 M^{-1} from Morel & Hering, 1993; k_{-H2O} taken to be 2 * 10^6 s^{-1} for Co and 3 * 10^4 s^{-1} for Ni from Margerum et al., 1978).

Metal	k_f' (min^{-1} μM^{-1})	k_f (s^{-1} M^{-1})	Source
Co	1.5 * 10^{-3}	1.1 * 10^5	This study
	n.d.	4 * 10^6	Margerum et al., 1978 and Morel & Hering, 1993
Ni	5 * 10^{-4}	3.8 * 10^4	This study
	n.d.	1.5 * 10^4	Xue et al., 2001
	n.d.	6 * 10^4	Margerum et al., 1978 and Morel & Hering, 1993

DMG binding characteristics in anaerobic medium

To estimate whether the binding behaviour of DMG in sulfide-containing microbial media is similar to that in background electrolyte, a DMG titration was performed in both types of media. This gives information on the possible binding in anaerobic background medium, and can serve as a reference for metal binding in actual media containing growing cells. Fig. 7 clearly shows that both for Ni and Co there is no extra binding in anaerobic medium as compared to indifferent background electrolyte. However, compared to the original anaerobic media, the metal speciation has changed, since sulfide and carbonate were stripped from the solution. In the undisturbed medium, binding by sulfide will be considerable, as demonstrated before. The carbonate that has been stripped also binds Co and Ni (Martell & Smith, 1989; Fouillac & Criaud, 1984), but this binding is negligible compared to the binding by sulfide or DMG. In spite of the change in speciation due to elimination of the ligands sulfide and carbonate, this method is preferable, because it allows for successful AdSV measurements. The impact of sulfide on the speciation can be estimated a posteriori using the stability constants from literature (see table 1).

These data confirm that, as calculated before and shown in fig. 2, for these media at pH = 7, the concentration of added DMG should be at least 100 µM.

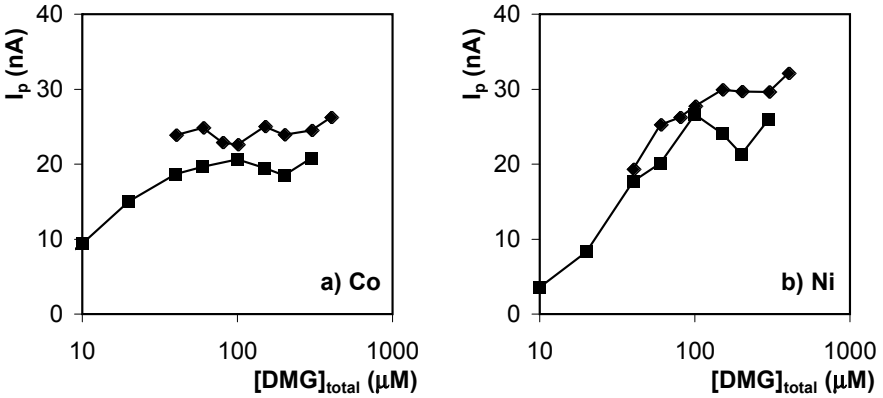

Fig. 7. DMG titration of solution containing only indifferent electrolyte (■) and purged, anaerobic medium, originally containing 1 mM sulfide (◆).

Assessment of Co and Ni speciation and binding capacity of batch cultures and bioreactor media

Anaerobic batch cultures

A *Methanosarcina* sp. enrichment culture grown in presence of 1 mM sulfide and a range of total Co and Ni concentrations (see Gonzalez-Gil et al., 2003) exhibited increasing methanogenic activity with increasing Co and Ni. The AdSV signal for Co increases with increasing total Co added; for Ni, however, the signal first increases but decreases again on going from 40 to 200 μM (fig. 8). No complexing capacity was detected in any of the media. Interestingly, for the 200/400 μM Ni/Co samples, total Co and Ni as measured using ICP-MS was higher than the value measured using AdSV (table 3). Apparently, a large fraction of the dissolved Co and Ni is present in strongly bound form.

Table 3. Ni and Co concentrations determined by AdSV and ICP-MS in batch media from a *Methanosarcina* sp. enrichment after growth in the presence of 400 μM Co and 200 μM Ni and 1 mM sulfide.

	$[Me]_{AdSV}$ (nM)	$[Me]_{ICP-MS}$ (μM)
Co	125	2
Ni	44	1

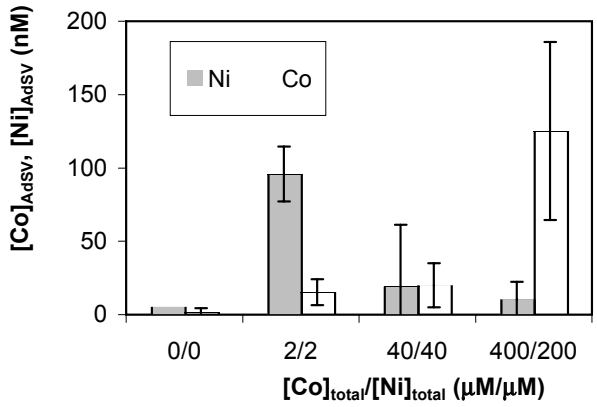

Fig. 8. AdSV signal of Co and Ni in supernatant of anaerobic enrichment cultures at different Co and Ni concentrations.

Second, a pure culture of *M. barkeri* was grown in the presence of 0.2 μM of both Co and Ni and 1 mM sulfide. Under these conditions, the growth is Co and Ni limited (Gonzalez-Gil et al., 1999, 2003; Silveira et al., 1991a) and there is good chance of finding metal binding by excreted ligands. First a DMG titration was performed. By comparing this titration (fig. 9a) to titrations in background electrolyte (fig. 7), we can conclude that there is no Co and Ni binding capacity in these cultures. Apparently, under these conditions, no metal binding ligands are excreted into the medium. After this, the same medium was titrated with Co and Ni (fig. 9b). Straight lines are found with a slope similar to that of the reference titration, indicating the absence of metal binding. Nevertheless, comparing the Co and Ni concentration determined by AdSV with the total dissolved Co and Ni value determined by ICP-MS (table 4), shows that most of these metals are present in strongly bound form.

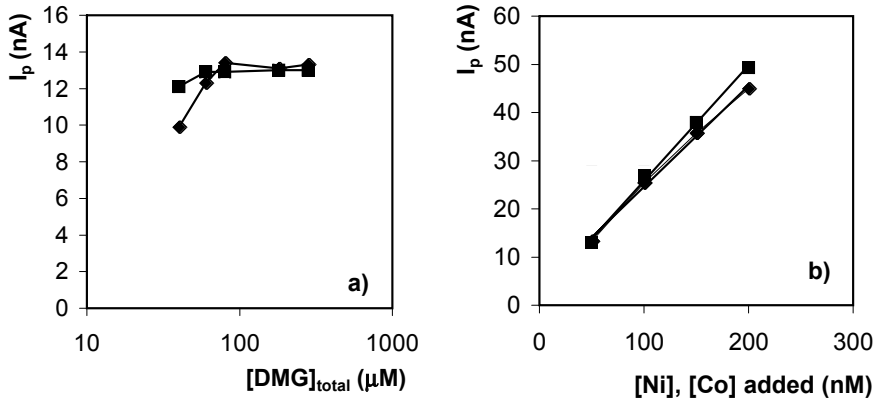

Fig. 9. Titration of sulfide containing batch media. a: DMG titration (Co_{added} = 50 nM; Ni_{added} = 50 nM); b: Co and Ni titration (DMG_{total} = 280 μM). ■ : Co; ◆ : Ni.

Table 4. Ni and Co concentrations determined by AdSV and ICP-MS determined in batch media from *Methanosarcina barkeri* after growth in the presence of 0.2 μM Co and Ni and 1 mM sulfide.

	$[Me]_{AdSV}$ (nM)	$[Me]_{ICP-MS}$ (nM)
Co	8	30
Ni	17	34

Anaerobic reactor media: 1. Methanogenic media

A first study was carried out with wastewater effluent from a nickel-deficient methanogenic UASB reactor (Zandvoort et al., 2002). Figure 10 shows a typical metal titration of this medium. These results indicate that Co is present in strongly complexed form: without metal addition no metal signal was measured, although the total amount of Co in the effluent was 54 nM. After metal addition no further binding by complexes was observed, indicating that the strong complexes present are inert. Further experiments showed that this is not due to kinetic effects (data not shown).

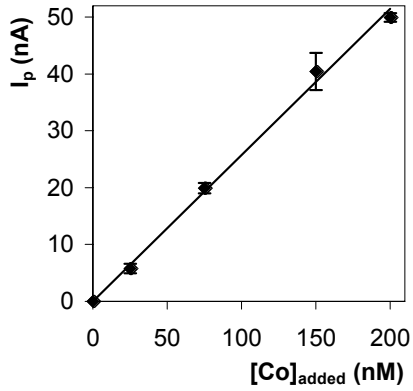

Fig. 10. Co titration of centrifuged nickel-deprived methanogenic bioreactor medium.

A second study was performed with two other reactors fed with methanol (Zandvoort et al., 2004b). The sludge of one of these reactors (in the following called Co+) was loaded with Co before operation. The other reactor (Co-) was not pre-loaded with Co.

Clear differences between the reactors in total dissolved Co and Ni as measured by ICP-MS ($[Co]_{ICP-MS}$ and $[Ni]_{ICP-MS}$) were found (fig. 11). In reactor Co- (fig. 11a), $[Co]_{ICP-MS}$ very rapidly decreases and stays very low (< 1 nM). The Co addition at t = *ca*. 60 d results in a clearly visible increase in $[Co]_{ICP-MS}$, which subsequently decreases. $[Ni]_{ICP-MS}$ remains constant throughout the run at a level of *ca*. 0.15 µM. In reactor Co+ (fig. 11b and c), $[Co]_{ICP-MS}$ starts extremely high but very quickly reaches a low value (~ 50 nM). $[Ni]_{ICP-MS}$ shows a remarkable pattern: after starting at approximately 0.15 µM, $[Ni]_{ICP-MS}$ increases in two steps (first to 0.4 µM, then to 0.55 µM). These steps seem to coincide with the increase in methanol loading of the reactor (Zandvoort et al., 2004b).

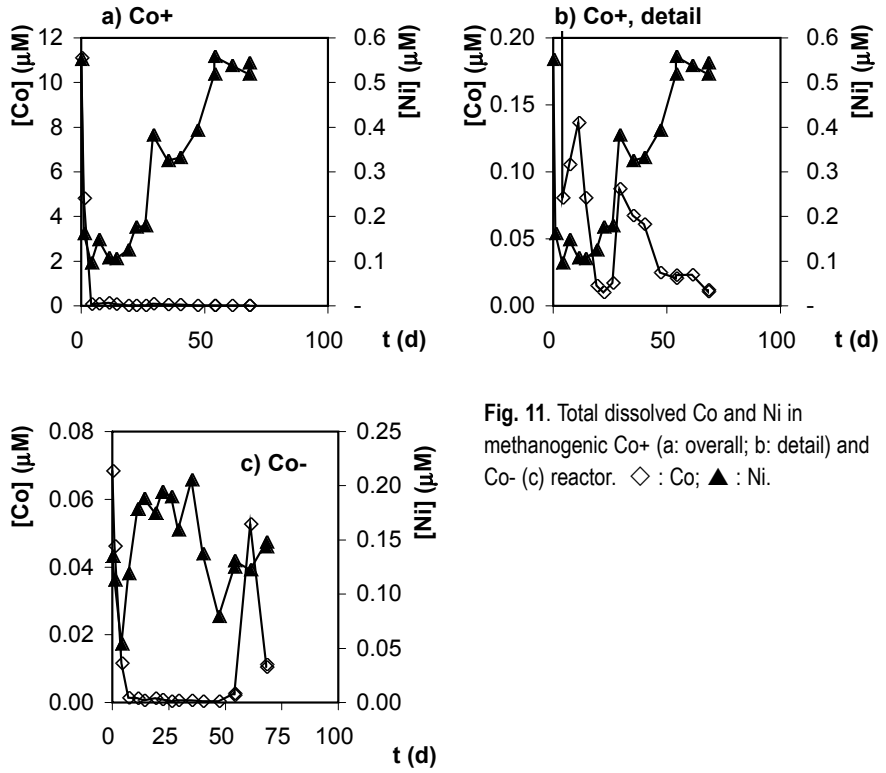

Fig. 11. Total dissolved Co and Ni in methanogenic Co+ (a: overall; b: detail) and Co- (c) reactor. ◇ : Co; ▲ : Ni.

AdSV titrations were performed for several points in time for both reactors (Co-: t = 0, 4, 14 and 54 d; Co+: t = 1, 4, 14, 61 and 68 d). In most of these cases, no Co or Ni binding ligands are detected and no indications for bound metal was found. In many of these cases, Co and Ni are below the detection limit of AdSV (*ca*. 1 nM). In the later stage of reactor Co+, dissolved Ni increases in two steps. In fig. 12, Ni and Co titration curves are shown for samples taken at t = 61 and 68 d, after the second increase of Ni in the effluent had taken place. Again, no metal binding capacity is present in the medium. When we compare the amount of Ni detected by AdSV($[Ni]_{AdSV}$) with $[Ni]_{ICP-MS}$ however (table 5), we see that $[Ni]_{AdSV} < [Ni]_{ICP-MS}$. As observed previously for Co, Ni is partly present in strongly bound form.

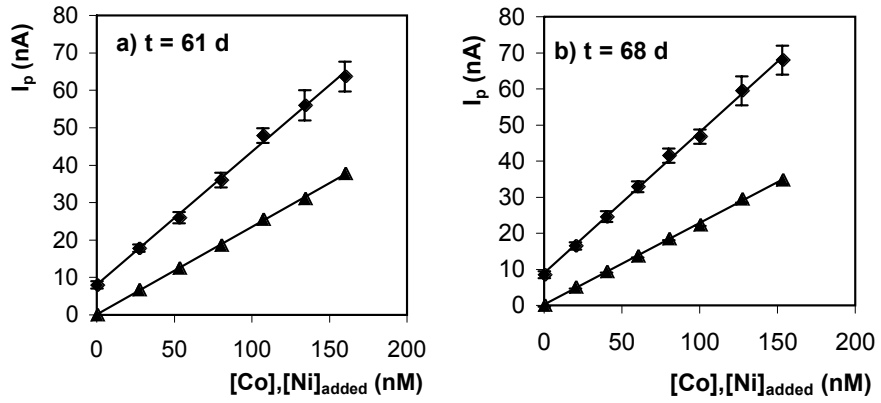

Fig. 12. Co and Ni titrations of filtered methanogenic reactor medium of Co+ reactor at t = 61 d (a) and t = 68 d (b). ▲ : Co; ◆ : Ni.

Table 5. Ni concentrations determined by AdSV and ICP-MS at different points in time in the UASB reactor Co+ preloaded with Co.

t (d)	$[Ni]_{AdSV}$ (nM)	$[Ni]_{ICP-MS}$ (nM)
61	20	145
68	18	120

Anaerobic reactor media: 2. Sulfidogenic media

Apart from two reactors in which methanogenesis was dominant, Ni and Co speciation was also studied in a UASB reactor in which sulfidogenesis was dominant.

AdSV titration of with Co and Ni are shown in fig 13a and b respectively. For Ni, no binding was observed, but for Co, very strong binding was observed up to an added concentration of 100 nM. This corresponds to a binding capacity in the original reactor medium of 0.4 µM. Furthermore, 50 nM Co was detected by ICP-MS, which was not detected by AdSV, indicating the presence of strongly bound Co. No strongly bound Ni was detected by ICP-MS.

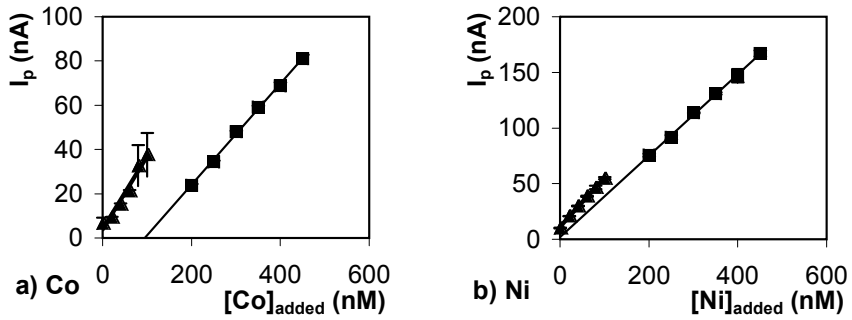

Fig. 13. Co (a) and Ni (b) titration of influent (▲) and effluent (■) of sulfidogenic reactor medium.

DISCUSSION

Experimental protocol for AdSV in anaerobic media

AdSV proves to be a suitable tool to study dissolved Co and Ni speciation in anaerobic bioreactors. For proper application of the technique, potentially perturbing influences of volatile and surface active components have to be eliminated. In many anaerobic media, volatile compounds are present in the form of carbonate and sulfide. It is difficult to keep their concentrations constant because of the sampling and measuring steps involved. Furthermore, competitive adsorption of sulfide onto the sensing electrode interferes with the analytical signal. Therefore, to avoid problems with these components, we chose to remove them from the solution by conversion to CO_2 and H_2S and degassing. This can be accelerated by addition of small amounts of acid. Afterwards, a non-volatile pH-buffer and other chemicals can be added and metal speciation in absence of sulfide and carbonate can be studied. Another way of taking samples which is especially well suited for bioreactors, is via a pre-flushed anaerobic syringe, followed by filtering over a 0.2-µm filter into a pre-flushed glass bottle, closed with a rubber stopper. After this, the gas phase of the solution in this bottle can be changed, thus removing carbonate and sulfide. Although this pretreatment inherently destroys any sulfide and carbonate complexes, the significance of these species is calculated from literature data (table 1).

Another point of concern is the high amount of particulate matter likely to be present. To remove this, two approaches were used in this study: centrifugation and filtration. Using filtration enables minimum handling and well-defined separation. However, often the bioreactor matrix is so dense that filtration is not easily achieved. In that case, anaerobic centrifugation is the optimal method.

Co and Ni distribution in anaerobic wastewater media

For the media tested, AdSV analysis gives interesting additional information on the speciation of Co and Ni.

First of all, in almost all cases, a proportion of the total Co and Ni was present in strongly complexed form. Likely candidates for these strong complexes are factor F_{430}

and vitamin B_{12}. These cofactors are the main molecules for which the methanogens require Co and Ni (Roth et al., 1996; Telser, 1998), and they can be excreted by the organisms (Mazumder et al., 1987; Silveira et al., 1991b). To test the hypothesis of the strongly bound Co and Ni being present in cofactors, a Co titration was performed in a solution containing 198 nM vitamin B_{12} and 1 mM DMG, pH = 7.0. The Co present in the vitamin B_{12} itself was not measured. Meanwhile, Co which was added to the solution was measured, providing a similar straight titration as fig. 10 (data not shown). Thus, Co present in the bioreactor effluent but not measured by AdSV, as was seen for the Ni-deprived reactor, could indeed be present in the form of vitamin B_{12}. Apparently, cofactors indeed play an important role in the metal speciation of anaerobic media containing methanogens.

Secondly, further Co and Ni binding is not very important in media containing mainly methanogens. Only in case of a sulfidogenic reactor were some indications found of strong Co binding. It is highly unlikely that the strong metal binding as observed in the sulfidogenic reactor is caused by sulfides. This can be partly ruled out by theoretical calculations as shown in fig. 4. Another reason why this is highly unlikely, is that the mixtures were thoroughly purged with N_2 before measurement, after which the voltammetric sulfide peak had almost completely vanished. Production of metal binding compounds by sulfate reducing bacteria is a plausible explanation for these observations, since this was reported in literature for Cu and Zn in media containing sulfate-reducing bacteria (Bridge et al., 1999).

From the data collected, not much information can be obtained about the binding strengths and concentrations of ligands present. For the strongly bound Co and Ni forms present in media containing methanogens, the lower limit of the binding strength K can be calculated on the basis of eq. (4) and (5) to be *ca*. 2-3 * 10^{10} mol^{-1} l for Co and Ni. As expected for cofactor-like compounds, the complexes are indeed very strong, and these complexes are expected to be chemically inert. For the metal binding in the sulfidogenic environment, further deductions are possible. For the strong ligands observed for Co, a ligand concentration of 0.4 µM and a lower limit for the binding strength K of around 5 * 10^9 mol^{-1} l can be calculated. This is again a very strong ligand and its concentration is rather high.

Overall, Co and Ni distribution in these matrices is mainly determined by sulfide precipitation, dissolved sulfide complexes, and to some extent by very strong dissolved complexes, probably cofactors. For sulfidogenic environments, there are indications that there are also other metal binding components.

Implications for bioavailability and bioreactor performance

As stated before, one of the major reasons of interest in the speciation of Co and Ni in anaerobic environments, is its impact on bioavailability.

First of all, no evidence was obtained to support the hypothesis that methanogenic archaea specifically excrete ligands to facilitate metal mobility and uptake (cf. siderophores for iron). It remains a question therefore, as to how these organisms are able to maintain considerable growth rates in the presence of the extremely low free metal concentrations of Co and Ni, even though these metal ions are indispensable for growth. In another chapter, evidence is presented for highly efficient uptake channels (chapter 6). In

sulfidogenic reactors, metal binding ligands do seem to be present. This is in accordance with previous findings for Zn binding by compounds excreted by sulfate reducing bacteria (Bridge et al., 1999). In general, the question on the importance of siderophores for metal bioavailability in anaerobic environments is still open, and AdSV is a suitable tool for its study.

The presence of Co and Ni in strongly bound soluble form, presumably cofactors, is a very important finding for metal bioavailability. The strong binding prevents the metals from being precipitated or sorbed otherwise. Within the anaerobic bioreactor environments, with its dense matrix of metal complexing solids, metals in this form will be more mobile than metals in free ionic form, which will be bound very easily. In case the strong complexes can be taken up, which is likely the case for cofactors (Roth et al., 1996), these complexes enhance Co and Ni bioavability in these environments. Furthermore, the specific role of the cofactors could be important within the ecosystem. Usually, the microorganisms live together in aggregates, composed of different types of organisms. Many of these organisms are not able to synthesize cofactors and are dependent on other organisms to acquire them. Within these aggregates, methanogens producing cofactors could thus have an important role. Meanwhile, the organisms that use the cofactors produced by the methanogens, can again be indispensable for the latter because of their conversions, e.g. sulfate reduction.

Analytical challenges and lookout for further research

This work has highlighted the utility of increased knowledge of Co and Ni speciation in anaerobic biosystems for improved mechanistic understanding in these systems. Yet, a lot of work is still to be done.

Firstly, it would be interesting to study systems with more complex mixtures of substrates. In systems treating only methanol we did not find any evidence of excretion of ligands; however, there are indications that in systems treating more complex mixtures of substrates (e.g. sulfate, glucose), there is a higher chance of ligand excretion, because of the high amount of EPS and SMP in these reactors (Noguera et al., 1994). Indeed, in a reactor treating glucose, Ni binding ligands were found (Kuo & Parkin, 1996). It would be extremely interesting to further investigate these processes, especially in the light of the relationships between bioavailability and reactor performance. AdSV can be a useful tool to scan bioreactor media for the presence of metal binding capacity.

A question of a more fundamental nature, is on the exact role of sulfide complexation. This has an important impact on metal speciation in a lot of different anaerobic environments, but the exact values of the binding strengths and the effects on bioavailability are not completely known. Addressing this issue directly requires overcoming the experimental challenges involved.

ACKNOWLEDGEMENTS

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The impact of Co and Ni speciation on methanogenesis in sulfidic media

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ABSTRACT

The speciation of Co(II) and Ni(II) in sulfide containing media impacts on the methanogenic activity of *Methanosarcina* sp. Dissolved metal concentrations and methane production rates were measured for a highly enriched culture at various total metal and total sulfide concentrations. The methanogenic activity increased with increasing total metal and decreased with increasing total sulfide. With increasing total metal, the dissolved Co concentration increased, whereas the dissolved Ni concentration remained constant. At various total concentrations of sulfide, the concentrations of total dissolved Co and Ni remained constant. Dissolution kinetics and the influence of soluble metal sulfide species explain the trends in the dissolved concentrations. The results suggest that the stimulation of the methanogenic activity is related to the free metal ion concentration. Calculations show the unlikeliness of limitation by slow dissolution of the solid metal sulfides. This was confirmed by direct measurement of Co and Ni uptake by *Methanosarcina barkeri* in the presence of metal sulfide precipitates. Growth limitation therefore seems to be controlled by biouptake characteristics

INTRODUCTION

Methanogenesis can be an efficient way to degrade small organic acids and alcohols (Lettinga & Hulshoff-Pol, 1983). In the methanogenic degradation of methanol, Co and Ni are essential elements because of their role in the cofactors vitamin B_{12} (Co) and factor F_{430} (Ni) (Thauer, 1998). In the practical application of methanogenesis in bioreactors converting methanol, the effects of Ni and Co are indeed well known (Lettinga et al., 1979; Florencio et al., 1993; Zandvoort et al., 2003). However, under the anaerobic conditions of the reactor medium, the availability of these metals is limited by precipitation with sulfide (Callander & Barford, 1983a,b). Although the importance of Ni and Co limitation is unambiguous, the details of Ni and Co bioavailability in these environments remain largely unclear. First, the distribution of the metals over their various chemical forms is not very well known. Although values for the solubility products of metal sulfide precipitates are available (Martell & Smith, 1989), it is not possible to make very precise estimates of the eventually dissolved concentrations. There are several uncertain factors such as the nature and degree of crystallinity of the precipitate, the size of the particles, the extent of coprecipitation and adsorption, and kinetic effects (for a review on these difficulties see Thoenen, 1999). Also, not many experimental data exist on methanogenic activity in relation to metal speciation. Most studies merely report the effect of total added metal (e.g.: Speece et al., 1983; Diekert et al., 1981; Silveira et al., 1991) and only some go into more detail. Gonzalez-Gil et al. (1999a) showed that the dosing strategy affects Co and Ni bioavailability in the bioreactor: continuous dosing was more efficient than pulsed dosing. This suggests that kinetic effects play an important role and that precipitation might hamper bioavailability. In another study, the addition of yeast extract was shown to improve the bioavailability of Ni and Co (Gonzalez-Gil et al., 2003). The same effect was obtained if sulfide is replaced by cysteine, which forms soluble complexes with Co and Ni. This seems to be related to higher concentrations of both total dissolved metal and the free metal ion. A couple of studies aimed at the direct measurement of the actual metal uptake by methanogens (instead of only considering effects of metal via methanogenic activity). These studies only dealt with short-term uptake (Jarrell & Sprott, 1982; Baudet et al., 1988), or a limited number of total metal concentrations or other chemical conditions influencing metal speciation (Diekert et al., 1980, 1981; Scherer et al., 1983). Generally, the data on dissolved metal concentrations in relation to biological uptake or activity are limited or incomplete and do not allow conclusions on the underlying speciation and bioavailability.

The objective of this study is to find relations between metal speciation and methanogenic activity in a model metal-sulfide system, in order to find out which metal species determines biouptake rates. For this purpose, the speciation of dissolved metal and methanogenic activity are studied in batch cultures as a function of Ni, Co and sulfide concentrations. To obtain information about the underlying basis of the metal limitation, uptake fluxes are compared to rates of metal sulfide dissolution.

MATERIALS AND METHODS

Biomass

Most experiments were performed using a highly enriched *Methanosarcina* sp. culture. It was obtained from anaerobic sludge by continuous transfer of inoculum (10%) into fresh medium over a period of 6 months at 37^{o} C. The sludge treated the wastewater from a chemical factory that produces formaldehyde out of methanol (Caldic Europoort, The Netherlands). The culture was enriched in a medium containing 104 mM methanol and 1 μM of both Ni and Co. Routine maintenance of the culture was performed under these conditions. Prior to an experimental run, the culture was repeatedly transferred (3 to 4 times) to nickel- and cobalt-deficient media. Eventual experimental runs were performed at 30^{o} C. This enrichment culture was especially used to demonstrate effects of the metals on microorganisms actually present in anaerobic bioreactors.

For more direct determination of cell Co and Ni uptake in the presence of metal sulfide precipitates, a pure culture of *Methanosarcina barkeri*, as described in chapter 6, was used. Prior to the experiments, this culture was grown 3 times in media containing a limiting amount of Co and Ni (both 0.1 μM). To prevent temperature limitation of growth, these experiments were performed at 37^{o} C.

Medium composition

A bicarbonate buffered mineral medium was used containing: 3 mM KH_2PO_4, 3 mM Na_2HPO_4, 5 mM NaCl, 0.5 mM $MgCl_2$, 5.6 mM NH_4Cl, 0.89 mM $CaCl_2$, 50 mM $NaHCO_3$, 1 mM Na_2S, as well as acid and alkaline solutions of trace elements (1 ml of each per liter) and vitamins (0.2 ml/liter). The alkaline trace element solution contained: 0.1 mM Na_2SeO_3, 0.1 mM Na_2WO_4, 0.1 mM Na_2MoO_4, and 10 mM NaOH. The acid trace element solution contained: 7.5 mM $FeCl_2$, 1 mM H_3BO_4, 0.5 mM $ZnCl_2$, 0.1 mM $CuCl_2$, 0.5 mM $MnCl_2$, and 50 mM HCl. The vitamin solution contained: 0.02 mg/l biotin, 0.2 mg/l niacin, 0.5 mg/l pyridoxin, 0.1 mg/l riboflavin, 0.2 mg/l thiamine (B_1), 0.1 mg/l *p*-aminobenzoic acid, 0.1 mg/l pantothenic acid 0.1 and 0.1 mg/l cyanocobalamin (B_{12}). Vitamins and sulfide were filtered-sterilized, other components were sterilized by heat. Nickel(II) and cobalt(II) were added as required in the form of chloride salts. Incubations were carried out in glass serum bottles sealed with butyl rubber septa and aluminum crimp caps. The bottles contained a gas phase (1 atm) of N_2-CO_2 (70:30, vol/vol). Under these conditions the pH was 7.0 ± 0.2.

The medium for the experiments with the pure culture was similar to the media used for the experiments with the enrichment culture. Cells were pre-grown in the presence of 200 μM EDTA, which was carried over during inoculation, leading to a concentration of 20 μM EDTA in the final experiment. Furthermore, the concentrations of all trace elements were five times higher than in case of the experiments with the enrichment culture, except for Fe, Co and Ni, which were dosed to concentrations of 50 μM (Fe) and 5 μM (Co and Ni). At these concentrations, the metals are known not to be limiting growth, even in case of metal sulfide precipitation (chapter 6). Vitamin B_{12} was left out of the medium. Further details on the medium and experimental setup can be found in chapter 6.

Biogas production

The biogas produced was measured over time as the increase in pressure in the serum bottles. These measurements were conducted with a membrane pressure sensor WAL BMP 040A (Wal Mess-und Regelsysteme Gmbh, 26123, Oldenburg). It was shown previously that this is a good measure of the amount of methane produced (Angelidaki et al., 1998). Furthermore, measurements showed that the production of methane was directly related to the amount of biomass formed and the amount of methanol consumed.

Metal concentration

Total dissolved Ni, Co and Fe were determined by inductively coupled plasma-mass spectrometry (ICP-MS) (Perkin Elmer ELAN 6000) in samples acidified to 0.1 M HNO_3.

Experimental setup

Co, Ni and Fe precipitation kinetics were studied in bioreactor-type media containing (in total concentrations) 0.1 µM Ni, 0.5 µM Co, 7.5 µM Fe and 1mM S, without adding cells. Precipitates were separated from the dissolved phase by filtration through a 0.2 µm filter.

The effects of total Co and Ni on growth of the biomass were studied by inoculating cultures containing total concentrations of Co/Ni of 0/0, 2/2, 40/40 and 400/200 µM. In this experiment, 208 mM methanol (=10 g COD/l) was used. The dissolved concentrations of Co and Ni under these circumstances (except for 400/200 µM) were measured after centrifugation for 10 min. at 10000 g, both for media with and without cells. The sampling and centrifugation were performed in an anaerobic chamber (Coy Type, Coy Laboratory Products Inc., Michigan) with N_2-H_2 headspace (96/4).

The effects of total sulfide were tested by inoculating cells at total sulfide concentrations of 0.25, 0.5 and 1 mM, 2 µM of both Co and Ni, and 166 mM methanol (= 8 g COD/l). Dissolved Co and Ni were measured after centrifugation for 10 min. at 10000 g by ICP-MS analysis of the supernatant, both for media with and without biomass.

A more direct test of Co and Ni uptake and possible limitation by dissolution was done using a pure culture of *Methanosarcina barkeri*. Aliquots of the medium of the inoculum and the cultures of growing biomass were sampled and filtered over 0.2 µm filters for determination of EDTA, methanol and dissolved Co, Ni and Fe. When 65 to 75% of the expected maximum pressure was reached, the medium was divided into three aliquots of approximately 80 ml. Each aliquot was centrifuged for 20 min. at 32000 g. A portion of the supernatant was collected for determination of dissolved Co, Ni, Fe and methanol. The pellet was washed with 10 ml 0.02 M EDTA (pH 7), and centrifuged again for 20 min. at 32000 g. The supernatant was stored for determination of cell-adsorbed Co, Ni and Fe. The respective pellets from the three aliquots were used for determination of dry weight, cofactor content and total metal content. Further details on the experimental setup and analysis of methanol and cofactor content can be found in chapter 6.

RESULTS

Metal precipitation: time scales and concentration levels

Fig. 1 shows the evolution of dissolved Fe, Ni and Co at total concentrations of 0.1 µM Ni, 0.5 µM Co, 7.5 µM Fe and 1mM S in the absence of microorganisms. Ni does not precipitate, whereas Fe and Co do. Fe precipitates more slowly and stays at a final dissolved concentration higher than that of Co (Fe: ~ 1.5 µM; Co:~ 20 nM).

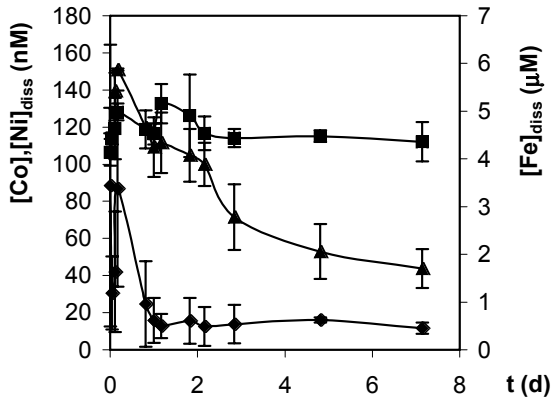

Fig. 1. Decrease in dissolved Fe (▲, right axis), Co (◆, left axis) and Ni (■, left axis) over time at total concentrations of 1 mM S, 100 nM Ni, 500 nM Co and 7.5 µM Fe.

Variation of total Co and Ni

Methanogenic activity increases with increasing total Co and Ni concentration in the range of 0 to 400 µM (fig. 2). At all concentrations (except for 400 µM Co/ 200 µM Ni), sulfide is in excess over metal which, under conditions of chemical equilibrium, should result in an fixed concentration of dissolved metal. So long as such conditions prevail, the differences in Ni and Co bioavailabilities cannot be explained by the concentrations of total dissolved or free metal. To verify the assumption of equilibrium, dissolved Ni and Co were measured.

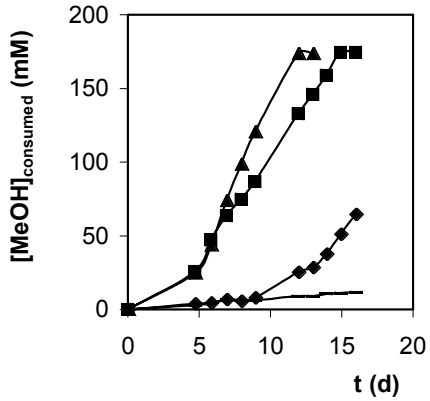

Fig. 2. Methanogenic activity at different total Co and Ni concentrations (µM Co / µM Ni): – : 0 / 0; ◆ : 2 / 2; ■ : 40 / 40; ▲ : 400 / 200.

Dissolved Co is slightly higher at 40 µM than at 2 µM total Co and Ni, while dissolved Ni does not significantly differ between 2 and 40 µM total Co and Ni (fig. 3). The increase in methanogenic activity thus seems to be related to the increased concentration of dissolved Co.

In spite of the known metal need, in the presence of growing biomass, dissolved metal concentrations do not decrease strongly over time (or, in case of Co, even seem to increase). Furthermore, the dissolved metal concentration in these media is not significantly lower than in the media without biomass. These observations suggests that the pool of dissolved metal is quickly replenished by metal sulfide dissolution.

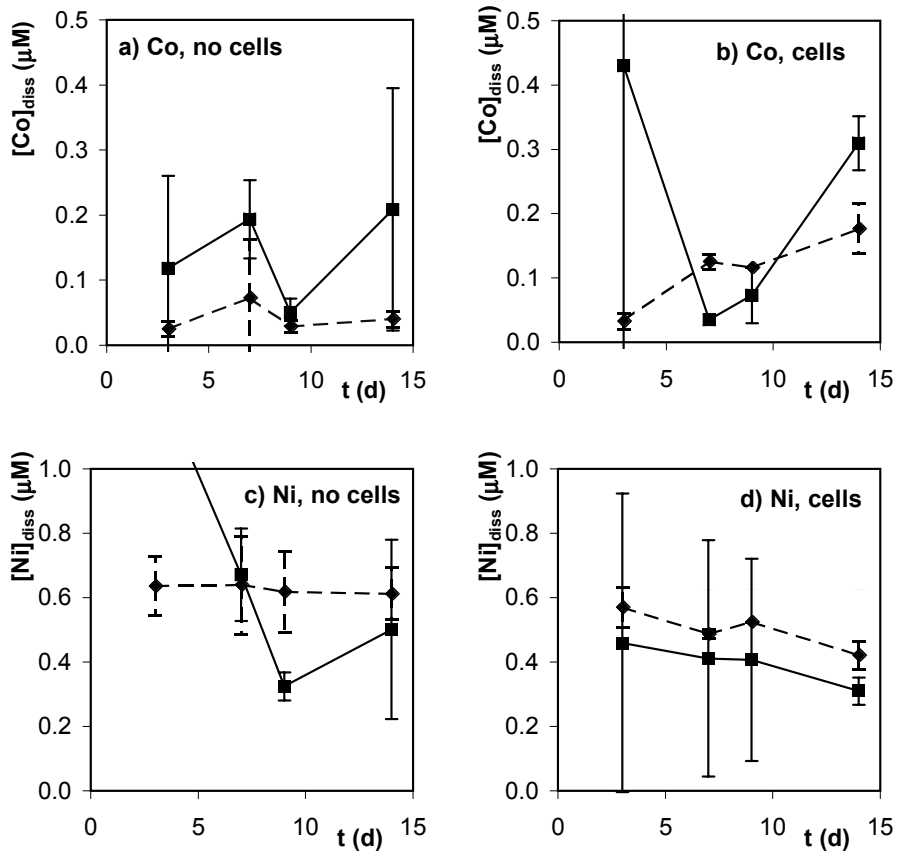

Fig. 3. Total dissolved Co and Ni concentrations at 2 and 40 µM total Co and Ni in the presence and absence of growing methanogens (- -◆- - : 2 µM total Co and Ni; —■— : 40 µM total Co and Ni; a: Co, no cells; b: Co, cells; c: Ni, no cells; d: Ni, cells).

Variation of S(-II) total

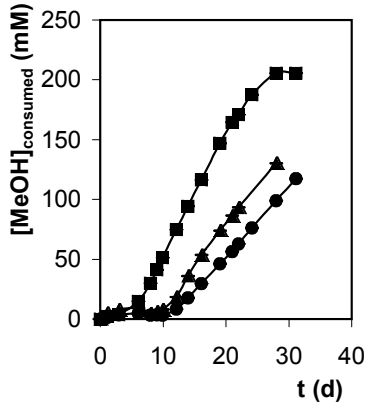

Fig. 4. Methanogenic activity, expressed in terms of methanol (MeOH) consumption, calculated from the gas pressure due to methane formation, at different total sulfide concentrations (■ : 0.25; ▲ : 0.5; ● : 1 mM).

As total S(-II) increases from 0.25 mM to 1 mM the methanogenic activity decreases (fig. 4). This is unlikely to be due to sulfide toxicity effects, since 1 mM was previously found to be below toxic concentrations (Mountfort & Asher, 1979). For 0.25 mM S the total sulfide consumption by the microorganisms at late growth is expected to be of the same order of magnitude as the total amount available (Scherer et al., 1983). Thus the corresponding dissolved metal data can be analysed only if growth rates are sufficiently low to exclude sulfide depletion. Dissolved Co and Ni are shown in fig. 5. Both metal ions show a fast initial decrease in dissolved concentration: within 6 hours Co goes from 2 to 0.4 µM and Ni from 2 to 0.7 µM. After that, the concentrations of both metals decrease more slowly, reaching the 0.1 µM level after more than 20 days. Contrary to expectation, there is no significant difference in dissolved metal concentration decay between media containing 0.5 or 1 mM sulfide. Like in the experiments with various total Co and Ni concentrations, there is no significant difference in dissolved metal between media with and without biomass, again suggesting fast replenishment of the dissolved metal pool.

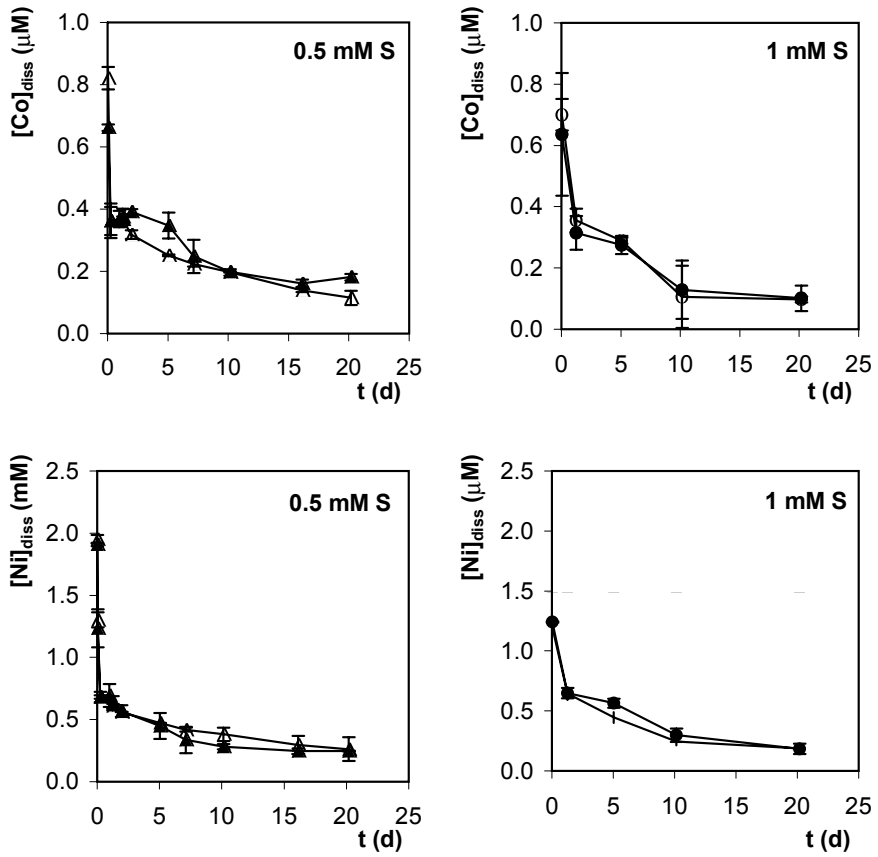

Fig. 5. Decrease in dissolved Co and Ni at different total sulfide concentrations (0.5 (▲) and 1 mM (●)) in presence (closed symbols) and absence (open symbols) of growing biomass. Initial total concentrations: 2 μM.

Co and Ni uptake by *M. barkeri*

Growth rates of *M. barkeri* and total dissolved Co, Ni and Fe in the presence of metal sulfide precipitates were measured simultaneously (fig. 6). In the first 2 days, dissolved metal concentrations change, most probably due to the carry-over of some EDTA with the inoculum. During this phase hardly any methanogenic activity takes place yet and no biouptake is expected to occur, as was also demonstrated in parallel experiments with the same biomass (chapter 6). All three metal ions are mainly present in the form of precipitates. During growth, the dissolved metal concentrations remain remarkably constant for all of the three metals. To exclude the possibility that this effect is completely due to the fact that cell metal requirements are negligibly small, we need to know the internalized amount of metal. Determination after separation of the cells by filtration of centrifugation is difficult because of the presence of metal sulfide precipitates. Therefore, we determined cofactor contents, which are a direct measure for the amount of Co and Ni taken up. The relation between the cofactor content and corresponding total metal content was deter-

mined over a broad range of metal concentrations (chapter 6). The values of cofactor contents and the estimated Co and Ni content are given in table 1. For both metals, the requirements of the cells are larger than the total dissolved concentrations, while the latter were observed not to change significantly during growth. This provides direct proof for the fast replenishment of the dissolved metal through metal sulfide dissolution.

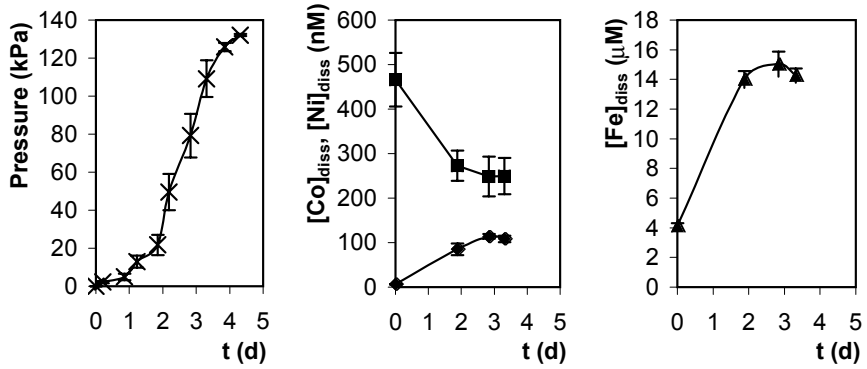

Fig. 6. Simultaneously measured pressure due to methane formation (X) and dissolved concentrations of Co (■), Ni (◆) and Fe (▲) for a culture of *Methanosarcina barkeri* growing in the presence of metal sulfide precipitates.

Table 1. Cell cofactor content and estimated Co and Ni content for *Methanosarcina barkeri* grown in presence of metal sulfide precipitates. Biomass increase measured: 0.6 g/l. Further details: see text.

$[Me]_{dissolved,total}$ (nM)	100	250	Measured
Cell cofactor content (μmol/g)	0.28	0.043	Measured
Cofactor content/metal content (%)	90 ± 10	10 ± 5	Chapter 6
Cell metal content (μmol/g)	0.28 – 0.35	0.29 – 0.86	Calculated
Metal consumption (nM)	170 – 210	174 – 520	Calculated

DISCUSSION

Dissolved metal levels in sulfide containing media

All results show that the metal sulfide precipitation processes range from fast (e.g. for Co) to slow (e.g. for Fe) on the time scale of interest. After 1 week, the order of the dissolved concentrations (Fe>Ni>Co) is in accordance with literature data for the solubility products of the corresponding sulfides (table 2). However, the absolute values are much higher than expected on the basis of these solubility products alone.

Table 2. Literature values for solubility products of metal sulfides and calculated values for free metal ion concentrations at 1 mM S total and all other conditions as used in this study.

Me	Formula	Name	Log K_{SP} $((Me^{2+})*(S^{2-}))$	Log (Me (aq)) (M)	Ref.
Fe	FeS FeS Fe_3S_4 FeS FeS	Amorphous (α) Mackinawite Greigite Pyrrhotite Troilite	-16.85 -17.5 -18.3 -19 -19.15	- 6.65 - 7.3 - 8.1 - 8.8 - 8.95	Davison, 1991
Ni	NiS NiS NiS	Amorphous (α) β γ	-19.4 -24.9 -26.6	- 9.2 -14.7 -16.4	Martell & Smith, 1989
Co	CoS CoS	Amorphous (α) β	-21.3 -25.6	-11.1 -15.4	Martell & Smith, 1989

Carbonate and phosphate complexes are not strong enough to explain the differences (table 3a and b). Probably, dissolved metal sulfide complexes of the type $MeSH^+$ and $Me(SH)_2^0$ account for the difference: the scarce literature available on these species indicates that the stability constants are large enough to account for substantial concentrations of soluble SH^- complexes around pH 7 (Al-Farawati & Van den Berg, 1999; Dyrssen, 1988; Luther et al., 1996; Zhang & Millero, 1994: table 3a and b). In previous studies on metal speciation in bioreactor media the discrepancy between the actual and expected dissolved metal concentrations has sometimes been ascribed to dissolved metal complexes of biological origin (Barber & Stuckey, 2000; Callander & Barford, 1983b). However, the present results indicate that even in the absence of biological activity the dissolved metal concentrations are high and that these can be explained by dissolved metal-hydrogen-sulfide complexes. Similar data on the importance of dissolved metal sulfide complexes in these environments were presented for Zn-sulfide precipitation in an anaerobic bioreactor (Veeken et al., 2003).

Table 3a. Literature values for stability constants of dissolved metal-sulfide, -phosphate and –carbonate complexes (I = 0 M). All K values defined as: $K = (Me(H_xL)_y^{(2-y(n-x))})/((Me^{2+})(L^{n-})(H^+)^{xy})$, with $L^{n-} = CO_3^{2-}$, PO_4^{3-}, or S^{2-} and log $K(HS^-) = 13.9$.

Complex	Log K			Reference
	Ni(II)	Co(II)	Fe(II)	
$MeCO_3^0$	4.57	4.22	4.73	Ni, Co:Martell & Smith, 1989; Fe: Fouillac & Criaud, 1984
$MeHCO_3^+$	12.42	12.22	11.43	Martell & Smith, 1989
$MeHPO_4^0$	15.36	15.41	15.95	Martell & Smith, 1989
$MeH_2PO_4^+$	-	-	22.25	Martell & Smith, 1989
$MeSH^+$	19.63 20.16 18.33 19.85	21.33 20.06 19.23 19.53	20.63 19.99 15.93 19.81	Al-Farawati & Van den Berg, 1999 Zhang & Millero, 1994 Dyrssen et al., 1988 Luther et al., 1996
$Me(SH)_2^0$	39.55 40.15	39.15 40.95	- 37.65	Al-Farawati & Van den Berg, 1999 Dyrssen, 1988

Table 3b. Calculated values for the ratio of activity of complexes to the activity of free metal ion as calculated on the basis of the constants given in table 3a and the total concentrations used in this study. Due to the scatter in the literature data the same estimates for the metal sulfide stability constants were used for all three metal ions (i.e. log K (MeSH) = 19.9 and log K ($Me(SH)_2$) = 38.8).

Complex(es)	Ratio complex(es)/free metal ion		
	Ni(II)	Co(II)	Fe(II)
$MeCO_3^0 + MeHCO_3^+$	2	2	0.5
$MeHPO_4^0 + MeH_2PO_4^+$	1	1	2
$MeSH^+$	10^2	10^2	10^2
$Me(SH)_2^0$	10^3	10^3	10^3

What pool determines metal bioavailability in sulfidic media?

Table 4 gives a summary of the results of the experiments in which total metal and sulfide were varied. It provides us with information about the species that control Co and Ni bioavailability in the presence of their sulfide precipitates. With variation of total Ni and Co concentrations, total dissolved and free Ni concentrations stay approximately constant, whereas total dissolved and free Co concentrations slightly increase. Apparently, Ni reaches a state of (pseudo)equilibrium, whereas Co does not.

Table 4. Summary of effects of changes in total metal and sulfide on methanogenic activity and metal speciation.

Condition	Response in: Methanogenic activity	Total metal	Total dissolved metal	Free metal ion
Total metal increase			Ni; constant;Co: increase	Ni: constant; Co: increase
Sulfide increase	Decrease	Constant	Constant	Decrease

The increase in methanogenic activity seems to be correlated with increasing free or total dissolved Co concentration. For different total sulfide, the change in methanogenic activity is not in accordance with a corresponding change in total dissolved metal. However, due to increasing sulfide complexation, there will be a decrease in the free metal ion concentrations of both Co and Ni (fig. 7). These results make it most likely that in metal sulfide containing media, the bioavailability of Co and Ni is determined by their free forms. Dissolved metal sulfide species have to be taken into account when relating total dissolved metal to eventual biological effects. This is in accordance with studies on metal toxicity to microorganisms in presence of sulfide (Atkins et al., 2002; Edgcomb et al., 2004).

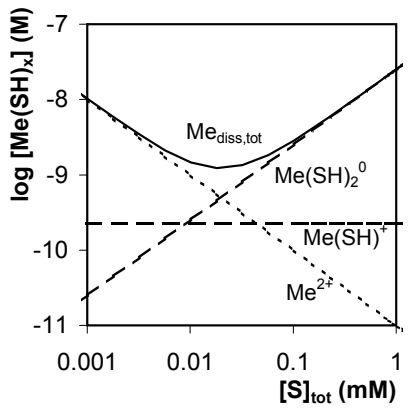

Fig. 7. Speciation diagram for the dissolved metal-sulfide species as a function of total dissolved sulfide concentration. Stability constants used: log K (MeSH) = 19.9; log K ($Me(SH)_2$) = 38.8). Total metal concentration: 2 μM.

Metal biouptake and the influence of dissolution

When combining the results for different total metal and total sulfide concentrations, it is remarkable that there is considerable growth (and concomitant Ni and Co uptake) while dissolved metal concentrations are not significantly lower than those in absence of cells. The data for *Methanosarcina barkeri* confirm that the amount of metal taken up is larger than any decrease in the dissolved concentration. Apparently, dissolution of the metal sul-

fides is fast enough to maintain a constant dissolved metal concentration on the time scale of growth of the biomass. In order to test this hypothesis, we can compare the rates of metal biouptake to those of metal sulfide dissolution. In the case of *M. barkeri*, the metal uptake flux J_{upt} (in mol m^{-2} s^{-1}) for a certain cell density x (in g/l, dry weight) can be estimated from the biomass growth rate dx/dt, the cell metal content (Me_{cell}, in mol g^{-1}) and the specific surface area (A_{sp}, in m^2 g^{-1}):

$$J_{upt} = \frac{dx/dt\, Me_{cell}}{A_{sp} x} \qquad (1)$$

The biomass at the end of growth was measured to be 0.6 g l^{-1}. Assuming linear growth over two days (fig. 4a), the growth rate dx/dt can be calculated to be O(10^{-6}) g l^{-1} s^{-1}. As estimated from the amount of cofactor determined (table 1), the metal contents for Co and Ni are O(10^{-7}-10^{-6}) mol g^{-1}. Combining these figures, the biouptake rate is found to be O(10^{-13}-10^{-12}) mol l^{-1} s^{-1}. The specific area A_{sp} can be calculated by assuming a spherical cell with a radius of the order of 10^{-6} m (as demonstrated by SEM measurements (Gonzalez-Gil et al., 1999b) and a density of the order of 10^5 g dry weight m^{-3}. This gives a value for the specific area of O(10) m^2 g^{-1}. Under the conditions above the measured biomass concentration develops from 0.04 g l^{-1} (start of experiment) to 0.6 g l^{-1} (end of growth). These values give an estimate of the metal uptake flux O(10^{-12}-10^{-11}) mol m^{-2} s^{-1}.

Metal sulfide dissolution can be estimated by using literature values for the dissolution rate of mackinawite (Pankow & Morgan, 1979, 1980) at pH = 7 to be O(10^{-11}) mol m^{-2} s^{-1}. The particle radius can be estimated from literature values for iron sulfide particles (Herbert et al., 1998; Neal et al., 2001) to be O(10^{-6}) m. For total amounts of 5 µmol metal sulfide per liter dispersion and a density of 4 * 10^6 g m^{-3} (Pankow & Morgan, 1979, 1980) the estimated dissolution rate is 4 * 10^{-11} mol l^{-1} s^{-1}. Comparison of these values with the rates estimated above for the biouptake flux (10^{-12}-10^{-11} mol l^{-1} s^{-1}), confirms the observation that dissolution limitation is absent here.

Implications for bioreactor practice

This study clearly shows, that metal dissolution rates of freshly formed sulfide precipitates in many cases do not limit methanogenic activity. Limitation is more likely to occur in case of stabilization of the precipitates due to coprecipitation or ageing, lowering of the dissolution rate due to a larger particle sizes and thus, lower specific area, or increased biomass concentration. In the dynamic anaerobic bioreactor environment however, we expect mainly less stable amorphous metal-sulfide phases to be formed, and concomitantly higher dissolution rates. Therefore, in case of high metal dosing and subsequent precipitation in the form of metal sulfides, this precipitate fraction could very well serve as a source of metal over a long period of time, as is supported by data on Co-loaded reactors (Zandvoort et al., 2004).

Still, metal limitation can occur. For the present bioreactor conditions, the metal limitation is consistent with control by biouptake characteristics.

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Effect of Yeast Extract on speciation and bioavailability of nickel and cobalt in anaerobic bioreactors

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ABSTRACT

The speciation of metals plays an important role in their bioavailability. In the case of anaerobic reactors for the treatment of wastewaters, the ubiquitous presence of sulfide leads to extensive precipitation of metals like nickel and cobalt which are essential for the metabolism of the anaerobic microorganisms carrying out the mineralization of the pollutants present in the wastewater. In practice, nickel, cobalt and iron are added in excessive amounts to full scale installations. This study is concerned with the complexation of nickel and cobalt with yeast extract and its effect on the biogas production by methanogenic biomass. Adsorptive stripping voltammetry was used to get information about the stability and complexing capacity of the metal-yeast extract complexes formed. Nickel and cobalt form relatively strong organic complexes with yeast extract. The bioavailability of these essential metals in anaerobic batch reactors was dramatically increased by the addition of yeast extract. This is due to the formation of dissolved bioavailable complexes which favours the dissolution of metals from their sulfides. Trace doses of yeast extract may be effective in keeping additions of essential metals to anaerobic reactors at a minimum.

INTRODUCTION

Usually anaerobic reactor media are primarily designed to sustain optimal bacterial growth. An important feature in this is the mode of supply of essential trace metals such as Co(II) and Ni(II), which, in case of (industrial) wastewater, to a large extent precipitate with sulfide. To overcome such removal of metals from the medium, complexing agents are often added in order to increase the dissolved fraction of the metals. Under such conditions, the speciation and bioavailability of the metals may become rather involved functions of type of complexing agent, mode of addition, time, etc.

Frequently, yeast extract is added to mineral reactor media in order to 'enhance' microbial growth. The stimulatory effect of yeast extract on the growth of methylotrophic (Hutten et al. 1980, Mah et al. 1978) and acetogenic (Bainotti et al. Bomar et al. 1991, Savage and Drake 1986) microorganisms has been discussed. However, the mechanism of the stimulatory effect is still largely unclear and possibly different for different microorganisms. It was reported that yeast extract is more effective than a cocktail of vitamins in enhancing the growth rate of acetogenic bacteria growing on methanol (Bainotti et al. 1996). Yeast extract has also been mentioned to form complexes with metals (Farrell et al., 1993; Ramamoorthy and Kushner 1975) but this has never been experimentally measured in sufficient detail yet.

The speciation of metals may play a decisive role in their bioavailability. Particularly in the case of anaerobic reactors for the treatment of wastewaters, the ubiquitous presence of sulfide resulting from sulfate reduction and organic matter mineralization will lead to extensive metal precipitation in the form of sulfides, from which the metals are not (directly) available for uptake by microorganisms. A key factor to consider in the anaerobic treatment of wastewaters from chemical industries is that (contrary to those from food industry and agriculture), often they are deficient in macronutrients as well as in trace metals. This is of importance since most of the microorganisms involved in the anaerobic treatment process are highly dependent on the presence and availability of trace elements like iron, cobalt, nickel and molybdenum (Friedman et al. 1990, Kumar et al. 1995, Vorholt et al. 1996). Hence mineral addition may be required to sustain microbial growth during the process. In practice to fulfil trace nutrient requirements (i.e. Fe, Ni, Co), metals are added in excessive amounts to ascertain the functioning of the treatment reactors. Metal dosing strategies that consider the rates of bioconversion and the chemical forms (speciation) of the metals should be explored in order to achieve a more rational addition of key trace metals in treatment systems.

In this study we investigate the metal bioavailability for methanogenic microorganisms in the presence of different metals at various concentrations with or without yeast extract. We explore the relation between the effect of yeast extract on the growth of biomass and its complexation with the essential trace metals Co(II) and Ni(II). As a part of this, the metal binding properties of the yeast extract are studied by adsorptive stripping voltammetry (Lam et al. 1999) which yields information on both the stability and the complexing capacity of the complex species.

MATERIALS AND METHODS

Biomass

A highly enriched *Methanosarcina* sp. culture was obtained from anaerobic sludge by continuous transfer of inoculum (10%) into fresh medium over a period of 6 months at 37^{O} C. The sludge treated the wastewater from a chemical factory that produces formaldehyde out of methanol (Caldic Europoort, The Netherlands). The medium contained methanol (104 mM) and 1 mM of both nickel and cobalt. Routine maintenance of this culture was performed under these conditions. This enrichment was used in the experiments described here. Prior to an experimental run, several (3 to 4) successive subcultures in nickel- and cobalt-deficient media were generated.

Medium composition

The basal medium contained the following components (mM): KH_2PO_4 3, $NaHPO_4$ 3, NaCl 5, MgCl 0.5, NH_4Cl 5.6, $CaCl_2$ 0.89, $NaHCO_3$ 46.7, Na_2S 1, as well as acid and alkaline solutions of trace elements (1 ml of each per liter) and vitamins (0.2 ml/liter) was used. The alkaline trace element solution contained the following (mM): Na_2SeO_3 0.1, Na_2WO_4 0.1, $Na_2MoO_4.2H_2O$ 0.1, NaOH 10. The acid trace element solution contained the following (mM): $FeCl_2$ 7.5, H_3BO_4 1, $ZnCl_2$ 0.5, $CuCl_2$ 0.1, $MnCl_2$ 0.5, HCl 50. The following vitamins were added (mg/l): Biotin 0.02, Niacin 0.2, Pyridoxin 0.5, Riboflavin 0.1, Thiamine (B1) 0.2, *p*-aminobenzoic acid 0.1, Pantothenic acid 0.1, Cyanocobalamin (B_{12}) 0.1. Vitamins and sulfide were filtered-sterilized, other components were sterilized by heat. Nickel(II) and cobalt(II) were added as required in the form of chloride salts. Incubations were carried out in 120-ml glass serum bottles sealed with butyl rubber septa and aluminum crimp caps. The bottles contained 25 ml medium and a gas phase (1 atm) of N_2-CO_2 (70:30, vol/vol). All experiments were performed in triplicate at 30^{O}C and an initial pH of 7 to 7.1.

Ashed yeast extract

A stock solution was prepared by ashing 5 g of yeast extract (Becton Dickinson, Cockeysville, Maryland) in a furnace at 550^{O}C for 12 hr. The ash was dissolved and neutralized with HCl. This concentrated solution was added to the medium to give equivalent concentrations as when added as the original intact yeast extract.

Hydrolyzed yeast extract

A stock solution was prepared by adding to 3 ml of 6N HCl, 0.8 g of yeast extract and flushing with N_2 for 1 min. The solution was digested 24 h in an oven at 100^{O}C. After digestion, about 20 ml of demineralized water was added and the pH was adjusted to 7. The solution was filled up to a final volume of 50 ml. This concentrated solution was added to the medium to give equivalent concentrations as when added as intact yeast extract. Table 1 shows the amino acids present in the yeast and hydrolyzed yeast extract. The amino acids were analyzed by HPLC as described by Kengen and Stams (1994).

Table 1. Amino acid composition of the yeast extract used in the experiments.

Amino acid	Yeast extract µmol/g	Hydrolized yeast extract µmol/g
Asparagine	144	298
Gutamic acid	483	778
Serine	180	124
Threonine	88	145
Glycine	212	439
Alanine	445	583
Proline	227	399
Arginine	188	424
Valine	192	314
Methionine	49	24
Isoleucine	183	300
Leucine	320	395
Phenylalanine	80	166
Cystine	130	219
Lysine	160	422
Histidine	45	83
Thyrosine	66	24

Biogas production

The biogas produced was measured over time as the increase in pressure (atm) in the serum bottles. The measurements were conducted with a membrane pressure sensor WAL BMP 040A (Wal Mess-und Regelsysteme Gmbh, 26123, Oldenburg).

Methodology for assessing the metal-complexing capacity of yeast extract

The nickel- and cobalt-complexing capacities of intact as well as hydrolyzed yeast extracts were studied by nickel and cobalt titrations with detection by adsorptive stripping voltammetry (AdSV). Details on the principles of this particular analytical technique are presented elsewhere (Lam et al. 1999, Nimmo and Chester 1993). The method essentially measures the free metal plus the weakly complexed metal in the system. The stability window of the complex species detected derives from the nature and concentration of the surface-active added ligand. For details we refer to the specialized literature (Miller and Bruland 1997, Lam et al. 1999, Zhang et al. 1990, Van den Berg and Nimmo 1987).

In case of an AdSV measurement with dimethylglyoxime (DMG) as the added ligand, the peak current I_p measured is a function of the concentration of surface active complex $Ni(DMG)_2$ formed:

$$I_p = S \cdot [Ni(DMG)_2] \quad (1)$$

where S is the sensitivity (in A mol^{-1} l), which is determined by calibration.

In a sample containing several binding agents the total amount of the metal, in this case nickel, will be distributed over different species resulting in the following mass balance:

$$[\mathrm{Ni}]_{total} = [\mathrm{Ni}^{2+}] + \sum_i [\mathrm{NiL}_i] + [\mathrm{Ni(DMG)_2}] \quad (2)$$

where $[\mathrm{Ni}]_{total}$ = total nickel concentration, $[\mathrm{Ni}^{2+}]$ = free nickel ion concentration, $[\mathrm{NiL_i}]$ = concentration of complexes, $[\mathrm{Ni(DMG)_2}]$ = concentration of nickel complexed with added dimethylglyoxime (DMG).

Here we consider the case where yeast extract is the sole ligand (L_i =YE). In case of very strongly binding organic ligand YE and DMG, $[\mathrm{Ni}^{2+}]$ is generally negligible (Van den Berg and Nimmo, 1987) compared to the complexes [NiYE] + $[\mathrm{Ni(DMG)_2}]$, which simplifies eq. (2) to:

$$[\mathrm{Ni}]_{total} = [\mathrm{NiYE}] + [\mathrm{Ni(DMG)_2}] \quad (3)$$

Both [NiYE] and $[\mathrm{Ni(DMG)_2}]$ are defined by their conditional stability constants K_{NiYE} and $K_{Ni(DMG)2}$ respectively, and the ligand concentration [L]:

$$[\mathrm{Ni(DMG)_2}] = K_{\mathrm{Ni(DMG)_2}} \cdot [\mathrm{Ni}^{2+}] \cdot [\mathrm{DMG}^-]^2 \quad (4)$$

$$[\mathrm{NiYE}] = K_{\mathrm{NiYE}} \cdot [\mathrm{Ni}^{2+}] \cdot [\mathrm{YE}] \quad (5)$$

Here, the nickel binding by yeast extract (YE) is formally written in terms of a 1:1 complex, as usual in macromolecular metal ion binding (Buffle, 1988). Actually, the binding behaviour of YE involves various different ligands, which leads to variation of K_{NiYE} with the metal-to-ligand ratio. In the absence of YE, addition of Ni will only result in the formation of $Ni(DMG)_2$. According to eq. (1), then the titration will be a straight line (fig. 1, curve A).

In the presence of YE, the nickel will be distributed over the DMG and the YE. Then, rewriting eq. (1) in terms of $[\mathrm{Ni}]_{total}$ using eq. (3), (4) and (5) will give:

$$I_p = S \cdot \frac{[\mathrm{Ni}]_{total} \cdot [\mathrm{Ni(DMG)_2}]}{[\mathrm{Ni(DMG)_2}] + [\mathrm{NiYE}]} = S \cdot \frac{[\mathrm{Ni}]_{total} \cdot K_{\mathrm{Ni(DMG)_2}} \cdot [\mathrm{DMG}^-]^2}{K_{\mathrm{Ni(DMG)_2}} \cdot [\mathrm{DMG}^-]^2 + K_{\mathrm{NiYE}} \cdot [\mathrm{YE}]} \quad (6)$$

When performing a metal titration, and plotting I_p as a function of $[\mathrm{Ni}]_{total}$, the outcome is dependent of the effective binding strength and capacity of the YE.

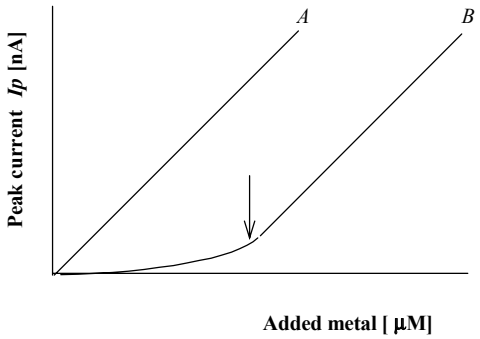


Fig. 1. Idealized calibration curve in the absence of ligands, except dimethylglyoxime (curve A), and titration of a sample containing ligands (curve B). The arrow indicates the saturation of the ligands present in the sample with the added metal. At this point, the slope of the curve reaches the slope of the calibration curve.

In case $K_{NiYE} \cdot [YE] << K_{Ni(DMG)2} \cdot [DMG^-]^2$, a straight line is found with a slope equal to that of the reference curve. In case $K_{NiYE} \cdot [YE] >> K_{Ni(DMG)2} \cdot [DMG^-]^2$, the curve will change. In the first part with an excess of YE over Ni, the slope will be lower compared to that of the reference (fig. 1, curve B). However, when YE becomes saturated with Ni, the slope will increase and finally reach the reference slope (fig. 1, curve B). From these titrations, binding strength and capacity of YE or the ligand (L) can be calculated. In cases where saturation of the ligands in the titration curve is reached, the curve can be linearized by plotting the calculated value for $[Ni^{2+}]/[NiL]$ against $[Ni^{2+}]$ (in accordance with a linearized Langmuir isotherm). The slope is 1/[L] and the intercept equals $1/(K_{NiL} \cdot [L])$. For more details, see Zhang et al., 1990. In case saturation is not reached, $K_{NiL} \cdot [L]$ can be estimated by comparing the slope of the blank with the slope in presence of ligand.

Voltammetry

Mineral medium without Ni (II) or Co (II) was prepared in the same manner as for the bioreactor experiments (except that sulfide was omitted). The medium contained either no yeast, intact yeast or hydrolyzed yeast extract. Voltammetric measurements were carried out using an Autolab voltammeter (Eco Chemie, The Netherlands), connected to a hanging mercury drop electrode (HMDE, Metrohm 663 VA stand, drop area=0.52 mm^2) and a graphite counter electrode. The voltammetric cell was made of lead-free borosilicate glass type 3.3 (DIN-ISO 3585) and the cell solution was stirred using a PTFE rotor. Potentials are given with respect to a Ag/AgCl reference electrode (3M KCl). The system was controlled by a personal computer using the program GPES32 (Eco Chemie, The Netherlands). The deposition potential was –600 mV (deposition time 60 s), followed by a relaxation time of 5 s. Differential pulse was used at a pulse height of 50 mV, a step potential of 5 mV and a scan rate of 10.2 mV/s. The scan range was –0.8 to –1.2 V.

Mineral medium samples (15 ml) were placed in the voltammetric cell, dimethylglyoxime was applied as the surface-active added ligand at 0.085 mM, TRIS buffer at 0.025 M and Ni or Co were added stepwise from stock solutions (1 mM). The solution in the

voltammetric cell was stirred and purged with argon for about 10 min prior to measurement. The pH was kept constant at 7.65. Scans to measure Co were carried out after 10 min, while Ni measurements were performed after 30 min in order to reach pseudo-equilibrium conditions (Lam et al. 1999). Scans were recorded 3 to 6 times.

Total dissolved metal concentration

Total dissolved Ni and Co were determined by inductively coupled plasma-mass spectrometry i.e. ICP-MS (Perkin Elmer ELAN6000) in samples acidified with 0.1 M HNO_3. These samples were taken from bottles without biomass, which were left to equilibrate for 7 days. Sampling was performed in an anaerobic chamber (Coy Type B, Coy laboratory products Inc.) with a N_2-H_2 atmosphere(96:4) to prevent oxidation of sulfide. Precipitates were separated from the dissolved phase by centrifugation at 10000 g for 10 minutes.

RESULTS

Nickel and cobalt limitations in the presence of sulfide

We first studied the uptake of nickel and cobalt as reflected by the biogas production by an enriched culture of *Methanosarcina* sp. Batch tests were performed in which mineral medium and 10% (v/v) of biomass were added. At time zero, methanol was added as sole carbon source at a concentration of 166 mM (8 g methanol-COD/l). The gas production (CH_4 + CO_2) due to the conversion of the substrate was measured over time. Tests were performed for nickel and cobalt additions in the range of 0 to 400 µM, and sulfide was added at 1mM.

In the presence of sulfide, metals seem to be limiting the rate of methanogenesis from methanol, even at total concentrations as high as 40 µM. The biogas production rate increases with increasing metal concentration (fig. 2a).

Effect of yeast extract

When 0.1 g/l of yeast extract was added in the batch tests with sulfide, nickel and cobalt limitations seem to be overcome (fig. 2b). Interestingly in the presence of yeast extract, the gas production was independent of the added metal concentration, and the lag phase was shortened.

Effect of ashed yeast extract

As indicated in the previous section, the addition of yeast extract strongly reduces Ni and Co limitations. In order to investigate whether this effect is due to organic or to inorganic components of the yeast, experiments were conducted in which the same amount of yeast extract was added but in ashed form. The results of the tests with ashed yeast extract and sulfide are shown in fig. 2c. The addition of ashed yeast did not cause any stimulatory effect and the trends of biogas production were similar to those obtained in the absence of yeast extract (fig. 2a).

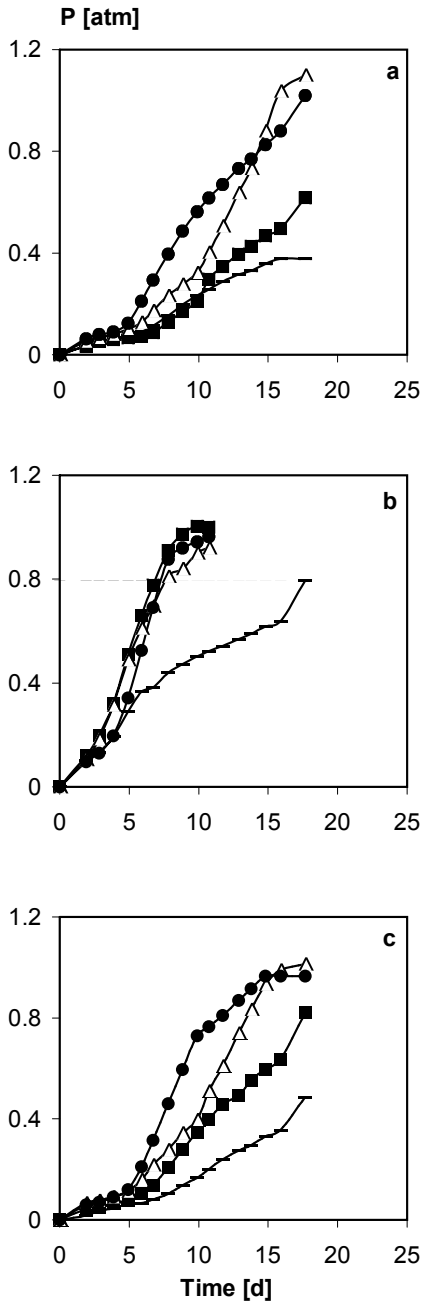


Fig. 2. Gas production due to the anaerobic conversion of methanol by a *Methanosarcina* sp enrichment culture at different Ni and Co additions. Sodium sulfide was added as sulfur source at 1 mM. Ni and Co were added each at: 0 μM (-), 2 μM (■), 40 μM (△), and 400 μM* (●). (a) No yeast extract was added. (b) Yeast extract was added at 0.1 g/l. (c) Ashed yeast extract was added at 0.1 g/l.
*nickel was added at 200 μM

Effect of hydrolyzed yeast extract

Results of the experiments with ashed yeast extract suggest that the alleviation of the Ni and Co limitations for the microorganisms by intact yeast extract is due to organic components of yeast. The positive influence of organic molecules might be due to (i) metal binding properties and/or (ii) stimulatory properties of the yeast components themselves (e.g. cofactors). Here we hypothesize that the first mode of action is possibly due to amino acid type of molecules, while the second is mainly due to more complex (and larger) organic molecules. In principle, a discrimination between these two mechanisms can be made by comparing the effect of addition of hydrolyzed yeast extract. After hydrolysis, complex molecules will be largely destroyed, while most amino acids except tryptophan will remain intact (Wu and Tanoue 2001).

Run tests in mineral medium with sulfide were performed at nickel and cobalt additions in the range of 0 to 400 μM. The tests were conducted (i) in the absence of yeast extract, (ii) with yeast extract at 0.1 g/l, and (iii) with hydrolyzed yeast extract at 0.1 g/l. The results of these experiments showed that hydrolyzed yeast extract also stimulates the gas production (fig. 3a versus fig. 3c). However its stimulatory effect was weaker than that observed when intact yeast extract was added (fig. 3b versus fig. 3c).

The results also suggest that both amino acids and other organic compounds are involved in the observed stimulatory effect of yeast extract. The role of the latter group seems to be important in shortening the lag phase (fig. 3b) and might be due to the presence of some coenzymes (i.e. cofactors). Addition of mere coenzyme M, (a methyl transferring enzyme involved in methanogenesis from methanol), did not give the same stimulatory effect as yeast extract or hydrolyzed yeast extract (data not shown). On the other hand, amino acids may play an important role via complexation of the metals. The formation of metal-amino acid complexes from yeast components may be expected to give rise to a certain extent of dissolution of metal sulfides, and a concomitant increase of the bioavailability of the metals involved. In support of this, results showed that the total dissolved concentrations of both nickel and cobalt in the presence of yeast extract are indeed higher than in its absence (table 2).

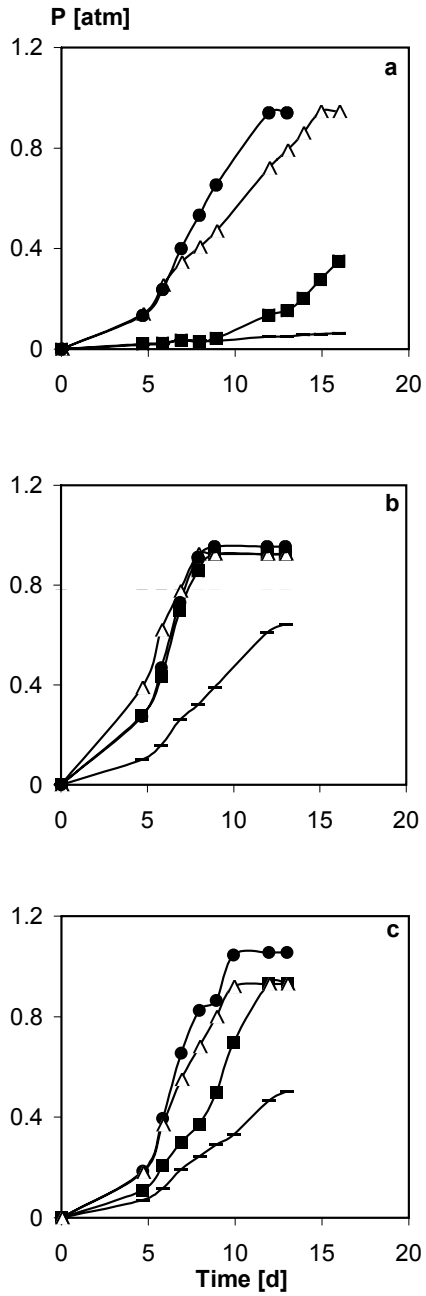


Fig. 3. Gas production due to the anaerobic conversion of methanol by a *Methanosarcina* sp enrichment culture at different Ni and Co additions. Sodium sulfide was added as sulfur source at 1 mM. Ni and Co were added each at: 0 μM (-), 2 μM (■), 40 μM (△), and 400 μM* (●). (a) No yeast extract was added. (b) Yeast extract was added at 0.1 g/l. (c) Hydrolyzed yeast extract was added at 0.1 g/l.
*nickel was added at 200 μM

Table 2. Measured total dissolved concentrations of Co and Ni in the presence and absense of yeast extract. The measurements were conducted in bottles with mineral medium and without biomass after 7 days of the additon of the metals.

Co and Ni added [μM]	Co dissolved [μM]		Ni dissolved [μM]	
	Yeast extract	No yeast extract	Yeast extract	No yeast extract
2	0.15 ± 0.06	0.07 ± 0.09	1.16 ± 0.04	0.64 ± 0.15
40	0.19 ± 0.06	0.03 ± 0.01	0.67 ± 0.14	0.28 ± 0.07

Nickel and cobalt bioavailability in the presence of cysteine

The use of cysteine instead of sulfide as sulfur source would, in principle, replace the formation of metal-containing precipitates by the formation of dissolved metal complexes. In experiments in which instead of sulfide the organic ligand cysteine was added to the batch reactor, metal concentrations as low as 2 μM were already beyond the range of metal limitations, and a clear exponential increase of the gas production was observed (fig. 4a). In the presence of cysteine, metal concentrations as high as 40 μM even appear to generate some inhibitory effect. The addition of yeast extract to the cysteine batch systems shortened the lag time, and decreased the metal inhibition observed at 40 μM when yeast extract was not added (fig. 4b). The addition of ashed yeast extract on the other hand, did not show any effect (fig. 4c).

Nickel- and cobalt-complexing capacity of yeast extract

Among the physical and chemical factors affecting the bioavailability of trace metals, complexation by organic ligands can be an important factor. It is hypothesized that the capacity of yeast extract to form metal complexes plays an important role in the observed stimulatory effect described in the previous sections.

The extent of organic complexation of nickel and cobalt by the yeast extract was determined by comparing the difference between AdSV data of the metals in mineral medium with and without addition of yeast extract. A blank titration (i.e. reference) curve was obtained by stepwise addition of either Ni(II) or Co(II) to the mineral medium without yeast.

In the presence of yeast extract the slope of the cobalt titration curve decreased compared to the blank. The decrease of the slope was directly related to the concentration of the yeast extract (fig. 5a). In the case of nickel titrations, the titration curves eventually reached the slope of the blank (fig. 5b). In these curves, two regions could be distinguished. A first region showing a strongly reduced slope as compared to the blank, and a second region in which added nickel is at excess so that the slope becomes similar to the slope of the blank, indicating that the metal-binding sites from the yeast extract have become saturated (fig. 6).

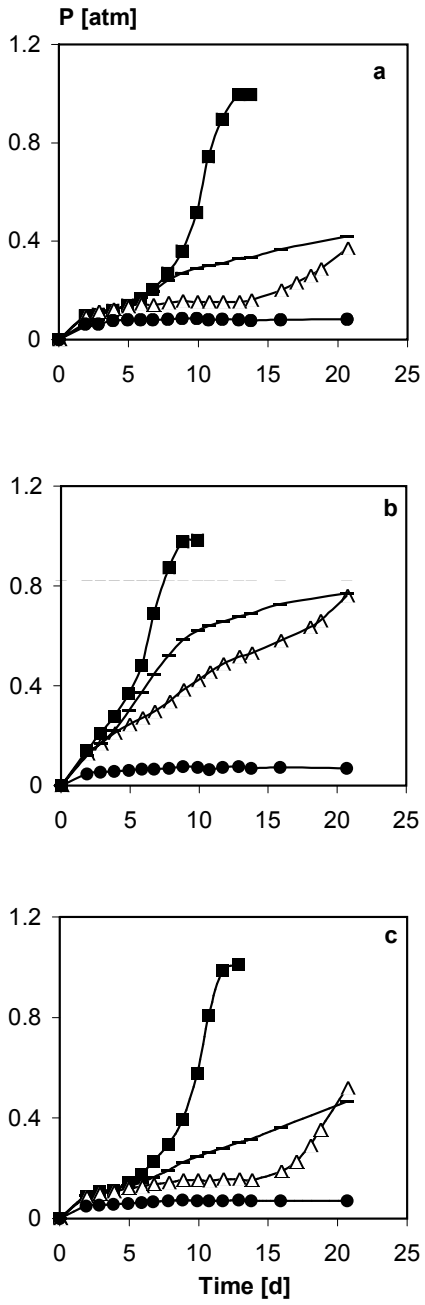
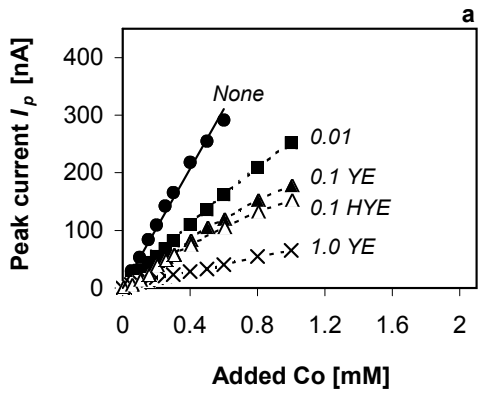


Fig. 4. Gas production due to the anaerobic conversion of methanol by a *Methanosarcina* sp enrichment culture at different Ni and Co additions. Cysteine was added as sulfur source at 1 mM. Ni and Co were added each at: 0 μM (-), 2 μM (■), 40 μM (△), and 400 μM* (●). (a) No yeast extract was added. (b) Yeast extract was added at 0.1 g/l. (c) Ashed yeast extract was added at 0.1 g/l.
*nickel was added at 200 μM



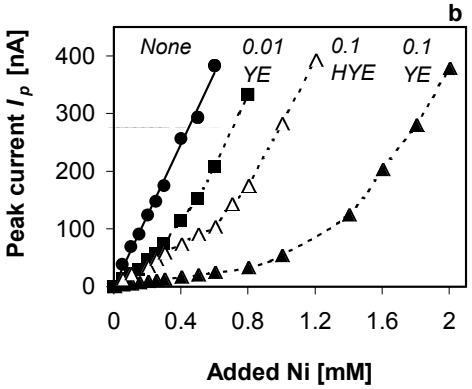


Fig. 5. Cobalt (*a*) and Nickel (*b*) titration curves in the presence of different concentrations of yeast extract (in mg/l) as indicated in the plot area. YE and HYE refer to yeast extract and intact yeast extract, respectively.

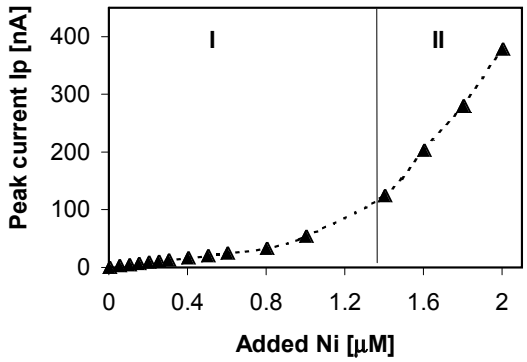


Fig. 6. Nickel titration curve in the presence of 0.1 g yeast extract/l. Different slopes could be observed. Region I shows a much lower slope as compared to the blank (fig 5b). Region II indicates the saturation of the metal-bindig sites from the yeast extract. This is reached when at sufficient excess of added nickel, the slope becomes similar to the slope of the blank.

The cobalt-complexing properties of the yeast extract were similar independent of the form in which yeast extract (0.1 g/l) was present, i.e. either intact yeast extract (YE) or hydrolyzed yeast extract (HYE) (fig. 5a). However, the nickel-complexing properties of the yeast extract were substantially reduced when the yeast extract (0.1 g/l) was hydrolyzed (fig. 5b). It should be noted that during hydrolysis, peptides are denatured so that the binding characteristics of the original amino acids if present as peptides could have been modified. Any modification of the binding properties of yeast extract due to acid hydrolysis seemed to affect its nickel binding properties but apparently not its cobalt binding properties.

DISCUSSION

Metal limitations

The various results clearly show that the metabolic activity of the anaerobic biomass is metal-limited. The chemical form of the metals (speciation) will determine their availability for the microorganisms. Metal sulfide precipitates are not directly available for uptake by biomass and therefore in practice, in order to increase metal availability, the mode of metal addition should be optimized such that rates of metal precipitation are minimized. In the presence of organic ligands like cysteine, the total concentrations of dissolved metals are higher, resulting in higher bioavailability. Under conditions where hardly any precipitate may be formed, only 2 μM of nickel and cobalt were required for the conversion of the added substrate. This value corresponds to a metal to methanol ratio of 0.25 μmol/ g methanol-COD and agree with previous studies in which precipitation was avoided by adding nickel and cobalt continuously (Gonzalez-Gil et al. 1999).

Enhancement of metal bioavailability due to yeast extract

Nickel and cobalt bioavailability in anaerobic treatment reactors is dramatically increased by the addition of yeast extract as reflected by the enhancement of the bioconversion rates. It was observed that yeast extract forms complexes with both metals. The relevant organic components of the yeast extract probably are of an amino acid and peptide nature. In the case of nickel, peptides can be important since the binding capacity is substantially decreased after yeast extract hydrolysis. Considering the central metabolic role of amino acids and the possible presence of specific membrane-bound transport systems for these metabolites, it is possible that metals pass through the biological membrane in the form of lipophilic metal-amino acid complexes.

Nickel and cobalt binding properties of yeast extract

We should emphasize that the ligands of the yeast extract as detected in the voltammetric analysis probably represent a range of compounds with various metal complexing functional groups. The definition of an overall stability constant for nickel therefore merely describes the collective behavior of the yeast extract. As stressed before, such overall conditional stability constants provide us with a broad estimate of the metal binding strength of the yeast extract components, rather than with individual affinities of specific groups (Buffle, 1988).

The voltammetric titration curves show that both nickel and cobalt form strong complexes with yeast extract components as compared to the DMG. For cobalt, all titration curves yield straight lines (fig 5a), indicating that the cobalt binding sites of the yeast extract were not saturated in the concentration range studied. For nickel, the slope of all titration curves comes back to the value of the blank at high added amounts of nickel (fig 5b), so for the pertaining binding sites there is saturation in this concentration range. In the case of cobalt the hydrolysis of the yeast extract does not give a difference in the titration curve, indicating little difference in binding behaviour, while for nickel the titration curve lies significantly higher after hydrolysis, indicating a decrease in binding strength (fig. 5).

To have an idea of the complexing strength of the yeast extract (YE), van den Berg-Ruzic plots (Van den Berg and Nimmo, 1987; Donat et al., 1994) were used to estimate a conditional stability constant for nickel (K_{NiYE}). Using a log ($K_{Ni(DMG)2}$) of 17.18 (Martell and Smith 1979). The estimated K_{NiYE} is conditional and based on 1:1 stoichiometry (table 3). The resulting value for log K_{NiYE} of 10 illustrates that yeast extract components bind nickel relatively strongly (table 4).

Table 3. Estimated conditional stability constant K_{NiYE} for yeast extract. The estimation was based assuming a 1:1 stoichiometry of the metal with respect to ligands present in the yeast.

Added YE (g/l)	Ligand concentration (μM)	Log K'_{NiYE}
0.01	1.7	10
0.1	2.5	10
1.0	20	10

Table 4. Stability constants for Ni obtained from different water samples.

Sample	Ni complexed %	Log K	Reference
Bay water	30-50	>17	Donat et al. 1994
Wastewater treatment plant	>75	>12.6	Sedlak et al. 1997
Fresh water systems (river affected by sewage effluents and agriculture	>99	12.1-14.9	Xu et al. 2001
Humic substances		3.1-4.2	Schnitzer et al. 1970
Biopolymers produced by activated sludge		4.6-5.4	Rudd et al. 1984
Mineral medium with 0.1 g yeast extract/l		10	This study
EDTA		12.5	Martell and Smith 1989

The K_{CoYE} for the Co-complexes could not be estimated by the same rationale since the saturation point was not reached. However, the value of $K_{CoYE} \cdot [YE]$ was estimated instead. This is a useful parameter, since it reflects the ratio of the concentration of cobalt complexed with yeast extract [CoYE] over the concentration of free cobalt $[Co^{2+}]$ at this concentration of yeast extract [YE], and therefore the factor by which the dissolved Co concentration is expected to be increased. These values were $1.9 \cdot 10^3$, $3.5 \cdot 10^3$ and $1.3 \cdot 10^4$ at YE additions of 0.01, 0.1 and 1 g/l respectively. It is remarkable that the Co titration curve does not reach the reference slope at the concentration levels comparable to those for Ni. This suggests that the complexing capacity for Co is larger than that for Ni. The ensuing overall picture is thus that YE forms a limited amount of strong complexes with Ni and a larger amount of weaker complexes with Co.

The metal complexing properties of yeast extract as found using AdSV should be reflected in the measured dissolved concentration in the presence and absence of yeast extract (table 2). Although the errors are substantial, indeed the dissolved concentrations for both Co and Ni are higher in the presence of yeast extract than in its absence. We have found however, that the concentrations are not in exact agreement with the aforementioned data on stability constants and complexation capacities found for yeast extract. There may be several reasons for this. First of all even after 7 days the metal speciation in the presence of sulfide will not have reached complete equilibrium, which makes equilibrium constants as found by AdSV be only partially applicable. Secondly it is possible that the ongoing precipitation process is not only governed by speciation aspects, but that the YE ligands present also influence the process indirectly by influencing the particle size. Such effects have actually been demonstrated for Zn, Cu and Cd precipitation by sulfide as influenced by citrate (Peters & Ku, 1987).

Bioreactor applications

Addition of yeast extract leads to the formation of dissolved complexes and consequently facilitates the dissolution of metals from their sulfides. Thus it may provide an attractive approach to keep additions of essential metals at a minimum while assisting on their bioavailability. This approach will mainly be attractive (provided that it is economically feasible), when treating chemical industrial wastewaters in which no bioavailable organic ligands, and normally no essential metals are present.

Not only can yeast extract modulate the growth/activity of microorganisms by its metal-binding properties as shown in this study, but it has also been reported to modulate the growth and metabolism of several acetogenic species (Bomar et al., 1991; Savage & Drake, 1986; Leclerc et al., 1998). The mechanism of that modulation however has not been indicated. Growth of several acetogenic bacteria on H_2-CO_2 was directly proportional to the concentration of yeast extract in the medium. In some of the strains, replacement by a vitamin solution restored growth and H_2-CO_2 metabolism, but in other strains vitamins could not replace yeast extract. In some strains, in the absence of yeast extract, glucose fermentation was shifted to metabolites more reduced than acetate, such as lactate and ethanol (Leclerc et al., 1998).

Interestingly, the results of this study also suggest that yeast extract can have a dual effect (fig. 4b *vs* 4a). Yeast extract may act like a metal-buffer, hence releasing metal when

needed while maintaining the free metal concentration low in case of total dissolved concentrations at potentially toxic levels. In practice, addition of trace amounts of yeast extract could be used in waste streams in order to alleviate toxic effects.

In general, the following aspects are of fundamental importance: (i) It is clear that the added essential metal should be in an available form and not all aminoacid-metal complexes may be directly bioavailable for all microorganisms. Still the increased dissolved concentration might increase the microbial activity in an indirect way. It is likely that the dissociation-rate controlled production of bioavailable metal from the dissolved yeast complexes is much higher than that from dissolution of metal sulfide precipitates, thus enabling a larger microbial activity. (ii) Direct addition of compounds like cysteine and/or other amino acids to full-scale anaerobic reactors treating (petro-) chemical wastewaters may not be economically feasible. However, as shown in this study, it is tempting to speculate whether optimized additions of yeast and/or metals could be an attractive option to enhance and eventually manipulate metal bioavailability in full-scale treatment reactors.

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[6]

Co and Ni bioavailability and growth effects for *Methanosarcina barkeri*: experiments and model description

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ABSTRACT

The uptake of Co and Ni and its influence on vitamin B_{12} and factor F_{430} content and methanogenesis from methanol by Methanosarcina barkeri was studied under conditions of controlled metal speciation. The cells were grown in the presence of 1 mM sulfide and 200 µM EDTA, thus preventing metal sulfide precipitation and enabling direct determination of metal content of the cells. Growth limitations by Co and Ni are demonstrated and evidence is found for competition between Ni and Co uptake. Vitamin B_{12} and factor F_{430} broadly follow the corresponding metal content, but at low metal contents the percentage of metal in the cofactor is distinctly lower. For Co, the fraction present in vitamin B_{12} is high (40-100%), whereas for Ni, the fraction in factor F_{430} is considerably lower (5-15%).

A model consisting of Michaelis-Menten uptake kinetics and Droop growth limitation successfully describes the data. Minimum Co and Ni contents $Q_{Co,min}$, $Q_{Ni,min}$ are found to be 85 and 75 nmol (g dry wt)$^{-1}$ respectively. The uptake is best described by competitive, high affinity/low capacity uptake of Co and Ni. Cell efflux of metals (presumably in the form of cofactors) is shown to be significant in a complete description of the data.

INTRODUCTION

In anaerobic reactors removing methanol from wastewater, Co and Ni are essential nutrients (Lettinga et al., 1979; Florencio et al., 1993; Zandvoort et al., 2003). These metals play an important role in methanogenic archaebacteria (Thauer, 1998) especially because of their presence in the cofactors vitamin B_{12} (Co) (Roth et al., 1996) and factor F_{430} (Ni) (Telser, 1998). Metal availability in wastewater environments, however, can be limited by precipitation with sulfide or by binding to other components present in anaerobic sludge (Callander & Barford, 1983a,b; Hayes & Theis, 1978; Osuna et al., 2003). For optimal metal dosing, it is therefore important to know the relationship between chemical speciation, biological uptake, incorporation into cofactors and eventual growth rate stimulation. Information is available on several of these aspects, but the overall picture is far from comprehensive.

Only two studies of characteristics of Ni uptake by methanogens are available (Jarrell & Sprott, 1982; Baudet et al., 1988). However, the methanogens studied are so different, that it is hard to draw general conclusions about metal uptake by methanogens. For one of these organisms (*Methanosaeta concilii*, former *Methanothrix concilii*), uptake is mainly related to chemical adsorption onto the sheath structure of the methanogens, while uptake for the other organism (*Methanobacterium bryantii*) is mainly caused by actual uptake via transporters in the biological membrane. Furthermore, both studies do not take into account speciation. Apart from the limited amount of information on the characteristics of Ni uptake by methanogens, some information is available on the uptake of Co and Ni by other microorganisms (e.g.: for Co: Komeda et al., 1997; Pogorelova et al., 1996; Degen et al., 1999; Saito et al., 2002; Kobayashi & Shimuzu, 1999; for Ni: Eitinger & Mandrand-Berthelot, 2000; Mulrooney & Hausinger, 2003; Watt & Ludden, 1999a). However, it cannot be stated a priori that these data can be applied to methanogens.

Metal contents of the cells (or cell quota, expressed by Q) of methanogens have been studied, albeit not in a very systematic way. Scherer et al. (1983) report cell quota for 10 different types of methanogens which were harvested in the early stationary phase after growth on different substrates. Ni uptake and incorporation into F_{430} by *Methanothermobacter thermoautotrophicus* (former *Methanobacterium thermoautotrophicum*) and other methanogens was studied by Diekert et al. (1980a,b, 1981). From these studies, no complete picture can be obtained for the relationship between Co and Ni speciation in solution and the resulting Q. The effects of Co and Ni on *Methanosarcina barkeri* in the presence of cysteine have been studied (Silveira et al., 1990, 1991a, 1991b; Nishio et al, 1992; Mazumder et al., 1986, 1987; Lin et al., 1989). These studies provide interesting data on the stimulation of methanogenesis (Silveira et al., 1991a, 1991b; Nishio et al, 1992) and the production and excretion of cofactors (Mazumder et al., 1986, 1987; Silveira et al., 1990, 1991a; Lin et al., 1989), but lack data on Co and Ni uptake and speciation.

The various components outlined above have not been combined into a single model description. To describe metal bioavailability and limitation, models are known from microbiological and algal literature (e.g., Flynn, 2003, Sunda & Huntsman, 1998; Droop, 1983). Short-term metal uptake can be described with Michaelis-Menten uptake kinetics,

which can be extended to include competition (Sunda & Huntsman, 1998). Nutrient-limited microbiological growth can be described with a Monod equation (Monod, 1949). This is valid if the growth yield is constant; however, if cell quota vary, different models have to be applied, such as Droop (Droop, 1983).

The aim of the present study is to quantify Co and Ni uptake, cofactor content and methanogenic activity of *Methanosarcina barkeri* under well-defined conditions of metal speciation in the medium, and to interpret these data within the context of a unified conceptual framework.

MATERIAL AND METHODS

Biomass

Methanosarcina barkeri (DSM 800) was grown at 37^{o}C. Prior to the experiments, three successive subcultures were generated in a medium containing 0.1 µM of both nickel and cobalt and 200 µM EDTA. The inoculum size was always 10%.

Medium composition

A bicarbonate buffered mineral medium was used containing: 3 mM KH_2PO_4, 3 mM Na_2HPO_4, 5 mM NaCl, 0.5 mM $MgCl_2$, 5.6 mM NH_4Cl, 0.89 mM $CaCl_2$, and 50 mM $NaHCO_3$, as well as acid and alkaline solutions of trace elements (5 ml/liter) and vitamins (1 ml/liter). The alkaline trace element solution contained: 0.1 mM Na_2SeO_3, 0.1 mM Na_2WO_4, 0.1 mM $Na_2MoO_4.2H_2O$, and 10 mM NaOH. The acid trace element solution contained: 1 mM H_3BO_4, 0.5 mM $ZnCl_2$, 0.1 mM $CuCl_2$, 0.5 mM $MnCl_2$ and 50 mM HCl. The vitamin solution contained: 20 mg/l biotin, 200 mg/l nicotinamid, 500 mg/l pyridoxamine, 100 mg/l riboflavin, 200 mg/l thiamine (B1), 100 mg/l *p*-aminobenzoic acid, 100 mg/l pantothenic acid, 50 mg/l lipoic acid and 20 mg/l folic acid. As a sulfur source, sulfide was added (1 mM). EDTA was added to a total concentration of 200 µM. This concentration was chosen to be in excess over the sum of the micronutrients but lower than the concentrations of the macronutrients, thus preventing precipitation of the former without causing limitations due to decrease of the latter. Vitamins and sulfide were filter-sterilized; other components were sterilized by heat. Ni(II) and Co(II) were added as chloride salts. Fe(II) was added in the form of $Fe(NH_4)_2(SO_4)_2.6H_2O$ to a final concentration of 50 µM. After preparation and sterilization the medium was left to equilibrate for at least 24 hours before inoculation.

All experiments were carried out in glass serum bottles with butyl rubber stoppers. One-liter bottles containing 300 ml medium and a gas phase (1.75 atm) of N_2-CO_2 (80:20, vol/vol) were used. Under these conditions the pH was 7.0 ± 0.2.

Experimental setup

Three parameters were varied independently (table 1): total Co concentration (0.03 to 5 µM), total Ni concentration (5 and 0.1 µM) and total EDTA concentration (200 and 20 µM).

Table 1. Experimental conditions
a) Free metal concentrations for Co and Ni in absence of EDTA calculated from dissolution products given by Martell & Smith, 1989 for amorphous CoS and NiS.
b) Complexation capacities for Co and Ni in absence of EDTA calculated from the ratio of the measured dissolved metal concentration over the calculated free metal concentration.

Condition	$[Co]_{total}$ (μM)	$[Ni]_{total}$ (μM)	$[EDTA]_{total}$ (μM)	Co		Ni	
				(1+K') (-)	$[Me^{2+}]$ (nM)	(1+K') (-)	$[Me^{2+}]$ (nM)
Co 0.03	0.03	5	200	$2 * 10^5$	$1.5 * 10^{-4}$	$4 * 10^7$	$1.3 * 10^{-4}$
Co 0.05	0.05	5	200	$2 * 10^5$	$2.5 * 10^{-4}$	$4 * 10^7$	$1.3 * 10^{-4}$
Co 0.1	0.1	5	200	$2 * 10^5$	$5.0 * 10^{-4}$	$4 * 10^7$	$1.3 * 10^{-4}$
Co 0.5	0.5	5	200	$2 * 10^5$	$2.5 * 10^{-3}$	$4 * 10^7$	$1.3 * 10^{-4}$
Co 5	5	5	200	$2 * 10^5$	$2.5 * 10^{-2}$	$4 * 10^7$	$1.3 * 10^{-4}$
Ni 0.1	5	0.1	200	$2 * 10^5$	$2.5 * 10^{-2}$	$4 * 10^7$	$2.0 * 10^{-6}$
NoL	5	5	20	$1 * 10^{4}$ b)	$1 * 10^{-2}$ a)	$5 * 10^{2}$ b)	1 a)

Before inoculation, an aliquot of the medium was sampled and filtered over a 0.2 μm filter for analyses. After inoculation, a remaining portion of the inoculum was analyzed as described below. Growth was followed by measuring pressure due to methane formation. During growth, aliquots of the medium were sampled and filtered over 0.2 μm filters for determination of EDTA, methanol and dissolved Co, Ni and Fe. When 65 to 75% of the expected maximum pressure was reached, the medium with cells was divided into three aliquots of approximately 80 ml. Each aliquot was centrifuged for 20 min. at 32000 g. A portion of the supernatant was collected for determination of dissolved Co, Ni, Fe and methanol. The pellet was washed with 10 ml 0.02 M EDTA (pH 7), and centrifuged again for 20 min. at 32000 g. The supernatant was stored for determination of cell-adsorbed Co, Ni and Fe. The respective pellets from the three aliquots were used for determination of dry weight, cofactor content and total metal content.

For Co = 0.03, 0.1, 0.5 and 5 μM and Ni = 0.1 μM non-inoculated bottles were prepared and treated in the same way as inoculated bottles to serve as blanks.

Analytical methods

The biogas produced was measured over time as the increase in pressure (kPa) in the serum bottles using a Chrompack FP-meter (Chrompack B.V., the Netherlands).

Methanol concentration was measured by gas chromatography using a Chrompack gas chromatograph (Model CP9000) equipped with Sil5 CB column (25 m x 0.32 mm) and flame ionization detector at 300^{o}C. The column temperature was 50^{o}C and the injection port temperature was 250^{o}C. The carrier gas was N_2 saturated with formic acid.

For dry weight determination, cells were resuspended after centrifugation in 2 ml H_2O and transferred to a dry aluminum cup of known weight. After drying in a stove at 103^{o}C for 24 hr, the filled cups were left to cool down in an exsiccator and weighted.

EDTA was measured by HPLC according to Bergers & De Groot (1994).

Metal concentrations (dissolved, cell-adsorbed, internalized) were determined by ICP-MS (Perkin Elmer ELAN6000). The samples for determination of dissolved and cell-

adsorbed metal were acidified to 0.14 M HNO_3. The samples for cell-adsorbed metal were diluted 10x before acidification to prevent EDTA precipitation.

Metal content of the cells was determined after microwave digestion (Milestone Ethos E, Monroe, CT, USA) in a mixture of 1.5 ml HNO_3 (65%) and 4.5 ml HCl (37%). After digestion, the samples were diluted 10x before ICP-MS determination.

Vitamin B_{12} and factor F_{430} were analyzed by HPLC according to previous methods (Stupperich et al., 1986; Kengen et al., 1988 and Holliger et al., 1992), modified as below. An amount of 0.5 g (wet weight) of cell pellet was washed with phosphate buffer (50 mM, pH = 7.5). The cell pellet was resuspended in 2.5 ml acetate buffer (0.5 M, pH = 5.0) with 1% KCN. The cofactors were extracted by autoclaving the cell suspension at 121^{O}C for 20 min. Cell debris and denatured protein were separated by centrifugation and extracted by the same buffer again. The resulting supernatant was pooled and loaded onto C18 reverse phase sep-Pak cartridge (Waters Chromatography, Millipore). The loaded column was washed with distilled water and bound cofactors were eluted by 80% methanol. This fraction was dried by flash evaporation and the residue was dissolved in 1 ml distilled water. HPLC analysis was performed with a Chromosampler C18 column (300 x 3 mm), eluted with a methanol gradient in 0.1 % acetate from 20 to 49 % for 20 min., 60 % for the next 5 min. and 20 % for the last 7 min. Vitamin B_{12} and factor F_{430} were recorded at 540 nm and 430 nm respectively.

Speciation

The speciation of Co and Ni was fixed by complexation with EDTA. Speciation was calculated using the speciation program ECOSAT. The estimated values for the ratio between total metal and free metal (1+K') are $2 * 10^5$ and $4 * 10^7$ respectively for Co and Ni, as calculated using stability constants from Martell & Smith (1989). Corresponding free ion concentrations can be found in table 1.

THEORY

In the experiments described, different processes are studied in batch cultures. To obtain information on characteristics of uptake and growth limitation, we need to combine descriptions for growth, limited by metal uptake, plus the mass balances for the relevant components.

Microbial growth rate is defined as:

$$\frac{\partial x}{\partial t} = \mu x \tag{1}$$

where: μ = growth rate constant (hr^{-1}); t = time (hr) and x = biomass concentration (g l^{-1}, dry weight).

Under the conditions of the present study, growth rates can be limited by methanol, Co and Ni according to:

$$\mu = \mu_{max} \frac{c_{MeOH}}{c_{MeOH} + K_{MeOH}} \frac{(Q_{Co} - Q_{Co,min})}{Q_{Co}} \frac{(Q_{Ni} - Q_{Ni,min})}{Q_{Ni}} \tag{2}$$

where: μ_{max} = maximum growth rate constant (hr^{-1}); c_{MeOH} = methanol concentration (mol l^{-1}); K_{MeOH} = methanol Monod constant (mol l^{-1}); Q_{Co} = Co content (mol g^{-1}); $Q_{Co,min}$ = minimum Co content (mol g^{-1}); Q_{Ni} = Ni content (mol g^{-1}) and $Q_{Ni,min}$ = minimum Ni content (mol g^{-1}).

We chose to describe methanol limitation with a Monod-type equation and to describe Co and Ni limitations by Droop-type factors. In many cases of nutrient limitation, the Monod equation is used to describe the relationship between the nutrient concentration in solution and the growth rate. This approach is valid for most macronutrients, for which the amount per cell is approximately constant. For many micronutrients however, the amount per cell can vary and the Monod equation thus may no longer be valid; the Droop equation offers a more valid approach in these cases (Droop, 1983).

Note that in eq. (2) limitations by methanol, Co and Ni are taken into account simultaneously. It is not known whether these limitations indeed are cooperative, or that the single most limiting factor is rate determining. In literature, support for both types of limitation can be found (Droop, 1983). In our results, limitation by two or more substrates at the same time hardly ever occurs because of the experimental conditions chosen. Therefore, on the basis of these results we cannot distinguish between simultaneous or separate limitation. Eq. (2) offers the most useful description of growth rate, since it combines all possible limitations in one equation.

Finally, we note that under the experimental conditions of this study, methanol hardly ever becomes limiting, since the initial concentration is much larger than the K_{MeOH} value. Only when this substrate is almost completely consumed does limitation occur.

The uptake flux of Co and Ni can be assumed to take place through saturable transporters and can be described using a Michaelis-Menten equation. Because these metal ions

might compete for the same transporter, competition has to be taken into account. Thus, the uptake flux, J_{in} (mol g^{-1} hr^{-1}), can be described by (e.g. for Co):

$$J_{Co,in} = J_{max} \frac{c_{Co^{2+}} / K_{JM,Co^{2+}}}{c_{Ni^{2+}} / K_{JM,Ni^{2+}} + c_{Co^{2+}} / K_{JM,Co^{2+}} + 1} \quad (3)$$

where: J_{max} = maximum uptake flux (mol g^{-1} hr^{-1}); $K_{JM,Co^{2+}}$ = Co Michaelis-Menten constant (mol l^{-1}); $K_{JM,Ni^{2+}}$ = Ni Michaelis-Menten constant (mol l^{-1}) ; $c_{Co^{2+}}$ = free Co^{2+} concentration (mol l^{-1}); $c_{Ni^{2+}}$ = free Ni^{2+} concentration (mol l^{-1}).

In case of a constant concentration of the competing ion, eq. (3) can be written as:

$$J_{Co,in} = J_{max} \frac{c_{Co^{2+}}}{c_{Co^{2+}} + K'_{JM,Co^{2+}}} \quad (4)$$

where $K'_{JM,Co^{2+}}$ is a conditional Co Michaelis-Menten constant (mol l^{-1}), defined as:

$$K'_{JM,Co^{2+}} = \frac{K_{JM,Co^{2+}}}{K_{JM,Ni^{2+}}} c_{Ni^{2+}} + K_{JM,Co^{2+}} \quad (5)$$

(For this example, Co uptake with competition by Ni (and vice versa) is described. Eq. (3) can also be generalized with competition by other ions. Thus, the observed Michaelis-Menten constant will be conditional in most cases, since it is dependent on environmental conditions.)

Methanogens are able to excrete Co and Ni (e.g. Mazumder et al., 1986, 1987; Silveira et al., 1991a; Lin et al., 1989). We approach the efflux J_{eff} (mol g^{-1} hr^{-1}) by first order kinetics (e.g. for Co):

$$J_{Co,eff} = k_{eff,Co}(Q_{Co} - Q_{Co,eff,min}) \quad (6)$$

where $k_{eff,Co}$ = the efflux rate constant (hr^{-1}). Since efflux is more likely to happen at higher cell quota, a threshold value ($Q_{Co,eff\,min}$) below which no efflux takes place is included.

Having defined equations for growth rate and metal fluxes, we need to establish mass balances for the metal content of the cells, the dissolved metal concentration and the dissolved methanol concentration.

The metal content of the cells changes over time, to an extent which is dependent on the uptake flux J_{in}, the efflux J_{eff}, the growth rate μ and the metal content Q (e.g. for Co):

$$\frac{\partial Q_{Co}}{\partial t} = J_{Co,in}(t) - J_{Co,eff}(t) - \mu(t) Q_{Co}(t) \quad (7)$$

In a batch system, the uptake may give rise to depletion of the total amount of dissolved metal in the medium (e.g. for Co):

$$\frac{\partial c_{\mathrm{Co,total}}}{\partial t} = -\left(J_{\mathrm{Co,in}}(t) - J_{\mathrm{Co,eff}}(t)\right)x(t) \tag{8}$$

The methanol consumption rate $\partial_{\mathrm{MeOH}}/\partial t$ is related to the biomass concentration *x(t)* and the growth rate $\partial x/\partial t$ by:

$$\frac{\partial c_{\mathrm{MeOH}}}{\partial t} = \frac{1}{Y}\frac{\partial x}{\partial t} + mx(t) \tag{9}$$

where: Y = growth yield on methanol (g biomass (mol methanol)$^{-1}$) and m = maintenance factor (mol methanol (g biomass)$^{-1}$ hr^{-1}).

Fitting procedure

The value of the maximum growth rate μ_{max} was obtained from the non-limited growth curves. Orders of magnitude of minimum metal contents $Q_{\mathrm{Co,min}}$ and $Q_{\mathrm{Ni,min}}$ were estimated from directly measured metal contents. Values of J_{max}, $K_{\mathrm{JM,Co}^{2+}}$ and $K_{\mathrm{JM,Ni}^{2+}}$ were estimated by fitting of the data for growth and metal uptake from the medium. Finally, k_{eff} was estimated from the discrepancy between modelled and measured metal uptake at high metal contents. The growth yield Y was determined by comparing the change in biomass with the amount of methanol consumed, taking into account a correction for maintenance via eq. (9). The maintenance factor m was determined from the pressure change (and thus methanol consumption) during the lag phase, when $\partial x/\partial t = 0$.

RESULTS

Experimental results

Growth rate (fig. 1) increases as total Co increases from 0.03 to 0.5 μM and as total Ni increases from 0.1 to 5 μM. For total Co, there is no further change in growth rate from 0.5 to 5 μM Co. Unlike growth rate, cell yield is not affected by the amount of Co and Ni added. Growth rate under non-metal-limiting conditions (5 μM total Ni and Co) is the same in the presence or absence of EDTA (data not shown), meaning that the EDTA itself is not toxic at this concentration.

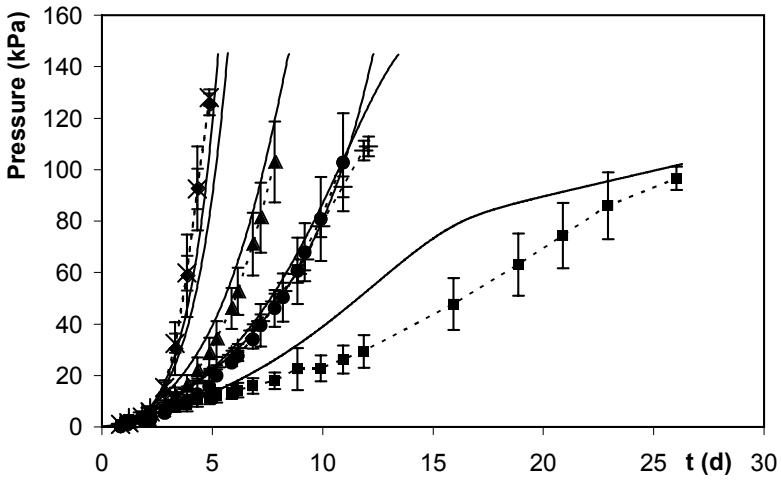


Fig. 1. Pressure increase in time due to methane formation during growth at different total. Co and Ni concentrations. ■ = 0.03 μM Co; + = 0.05 μM Co; ▲ = 0.1 μM Co; X = 0.5 μM Co; ◆ = 5 μM Co; ● = 0.1 μM Ni. Further details: table 1. Dashed lines connect the datapoints; solid lines represent the model description.

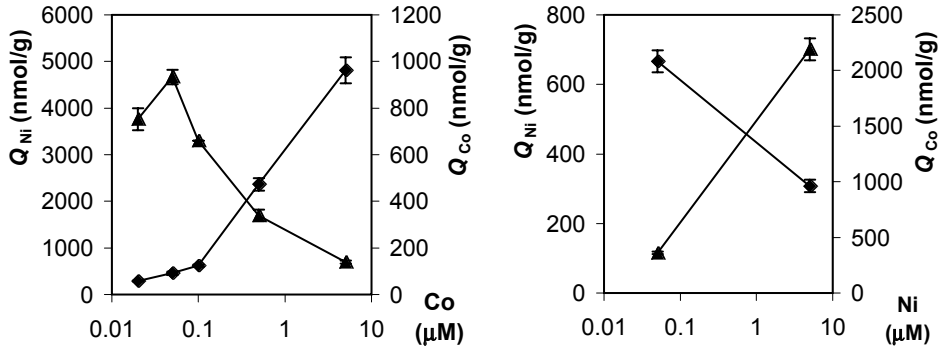
With increasing Co or Ni concentration in the medium, the corresponding contents in the cells increase, as expected (fig. 2a). However, while Co content in the cells increases with increasing total Co in the medium, Ni content in the cells decreases, and vice versa. This inverse relationship between Ni and Co content is partly due to time effects: under conditions of limitation by one of the metals, cells were left to grow for a longer time and were able to accumulate a larger amount of the other metal. However, the difference in Ni content between the cells grown in the presence of 0.5 and 5 μM Co cannot be explained in this way, since they have grown at the same growth rate for the same time. This provides strong evidence for competition between Ni and Co at the stage of uptake. Compared to the overall trends observed, the Ni content at 0.03 μM Co is atypically low, which might be due to different metabolic processes in these seriously limited cells. Iron content does not vary much for the different conditions; for all conditions, the Fe content is 20 ± 5 μmol g^{-1}. Apparently, there is no significant influence of Co and Ni on the iron uptake.

The overall trends for vitamin B_{12} (fig. 2b) follow the same pattern as the Co content. The factor F_{430} content is less closely related to the Ni content. With decreasing Ni in the medium, the Ni content and the factor F_{430} content both decrease, but with increasing Co in the medium, the decrease of the factor F_{430} content sets in later than the decrease of the total Ni content. Like the Ni content, also the factor F_{430} content at 0.03 μM Co is lower. The ratio between metal content and the corresponding cofactor content (fig. 2c) is much higher for B_{12}/Co (40-100%) than for F_{430}/Ni (5-15%). Interestingly, for both metals this ratio decreases with increasing limitation. Apparently, cells do not respond to limitation by using the metal ions in the cell more efficiently with regard to vitamin B_{12} and factor F_{430}. For Co, at higher metal contents, B_{12}/Co remains constant in the order of 95 %. For Ni however, with increasing Ni content at lower Co in the medium, F_{430}/Ni decreases again.

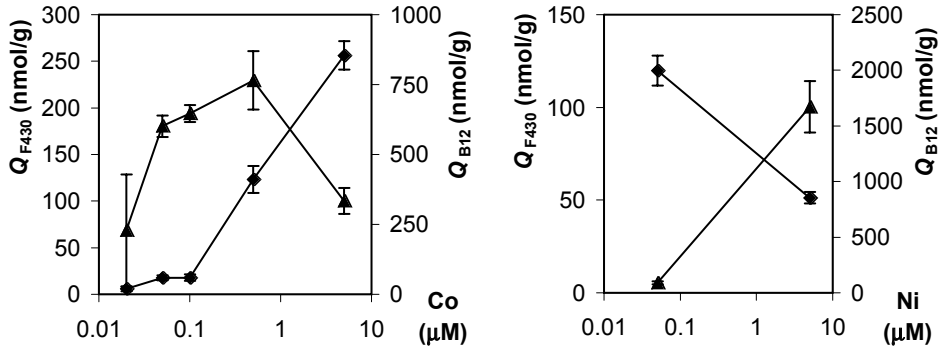
Under most conditions there is significant bulk depletion of dissolved Co and Ni (fig. 3), which is caused by uptake by cells, as can be concluded by comparing with blanks without cells. These data can be used to characterize the uptake characteristics by modelling.

Binding of Co, Ni and Fe to the cell walls, as measured via a washing procedure with 0.02 M EDTA, is insignificant compared to total uptake (data not shown).

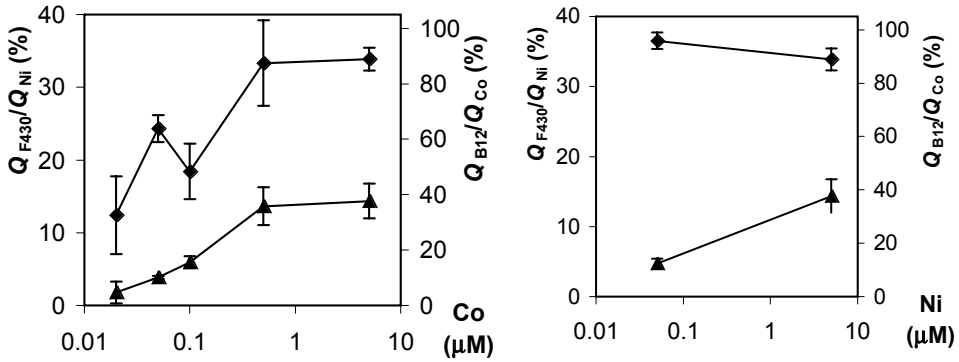
Dissolved methanol decreases in accordance with the growth rate as estimated from the pressure increase (data not shown).



a) Co (◆) and Ni (▲) content



b) Vitamin B_{12} (◆) and factor F_{430} (▲) content



c) B_{12}/Co (◆) and F_{430}/Ni (▲) content ratio

Fig. 2. Co and Ni content (a), factor F_{430} and vitamin B_{12} content (b), and ratio of cofactor to corresponding metal content (c) of the cells at the end of growth with variation of total Co and Ni concentration. Left: total Co concentration varied, total Ni concentration constant at 5 μM; right: total Ni concentration varied, total Co concentration constant at 5 μM.

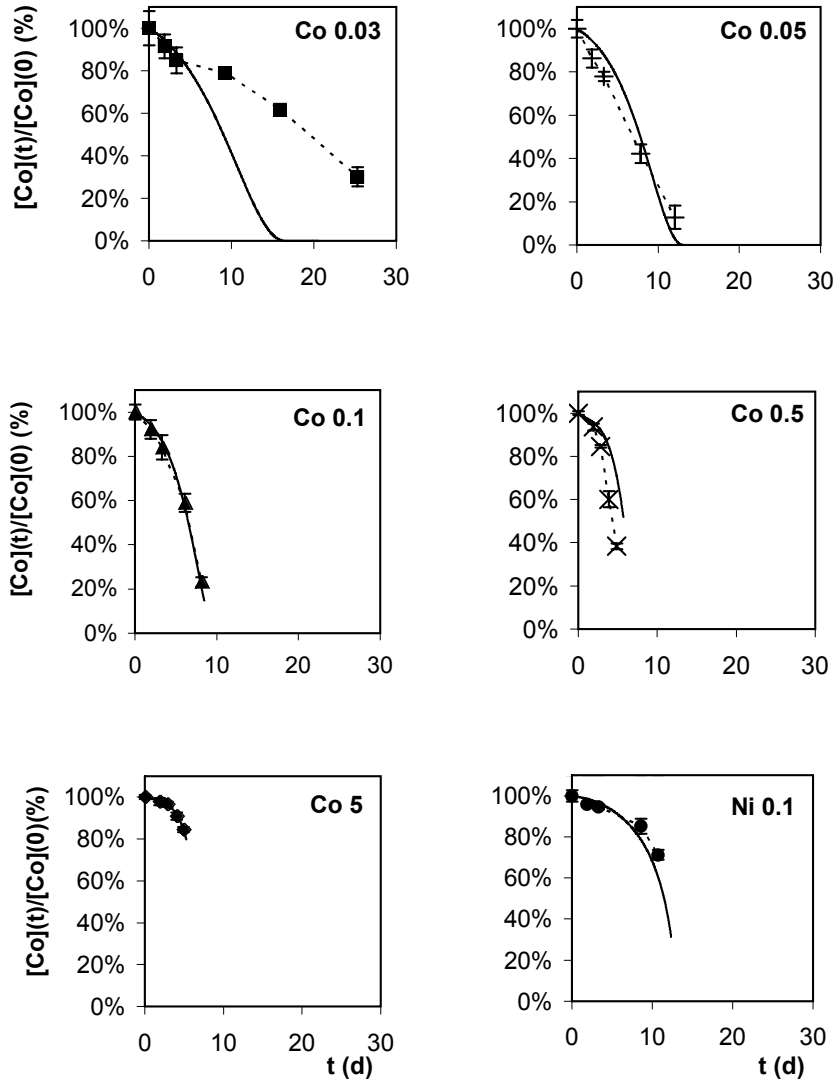


Fig. 3a. Total dissolved Co concentration [Co](t) in time, expressed as the percentage of the total initial concentration, [Co](0). Experimental conditions referred to as in table 1. Dashed lines connect the data points; solid lines represent the model description.

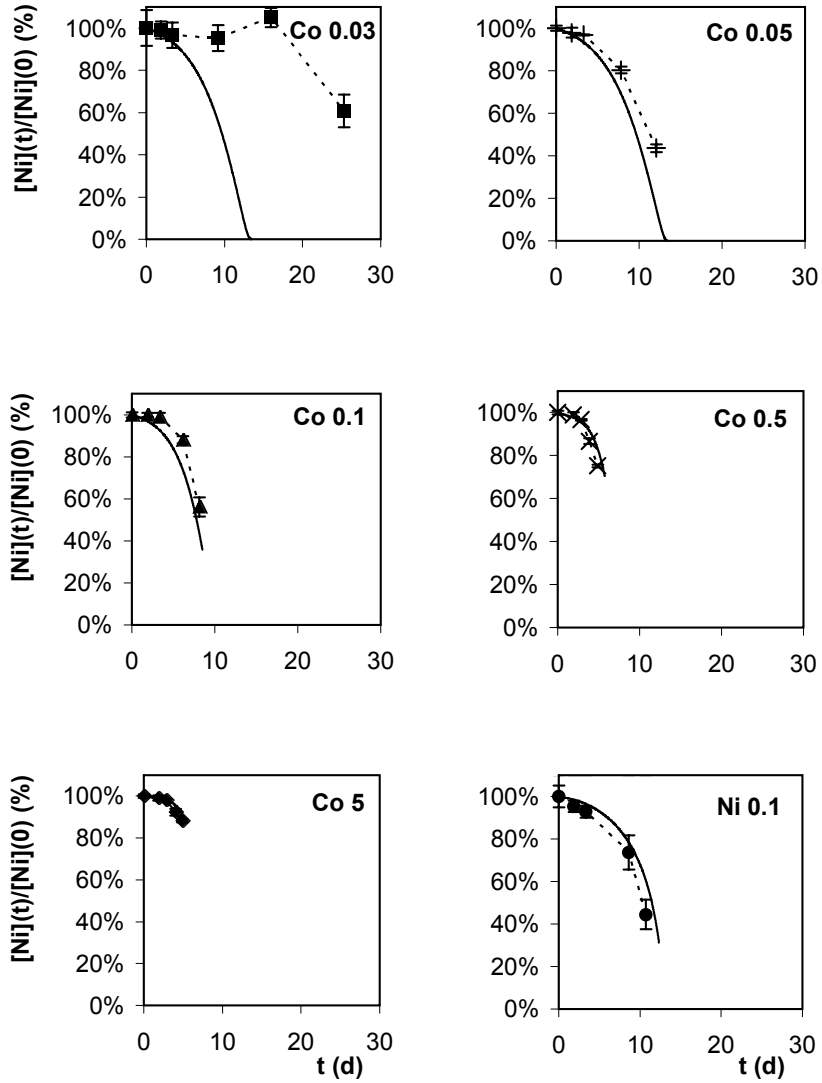


Fig. 3b. Total dissolved Ni concentration [Ni](t) in time, expressed as the percentage of the total initial concentration, [Ni](0). Experimental conditions referred to as in table 1. Dashed lines connect the data points; solid lines represent the model description.

Validation of the model and sensitivity to the key parameters

The validity of the proposed model description was tested by fitting the above mentioned experimental data. Model parameters can be found in tables 2 and 3. Table 2 gives the parameters that vary with different conditions: lag time and maintenance rate. These were calculated from the measurements as described at the end of the theory section. Table 3 gives the parameters that are constant for all conditions.

Table 2. Variable model parameters

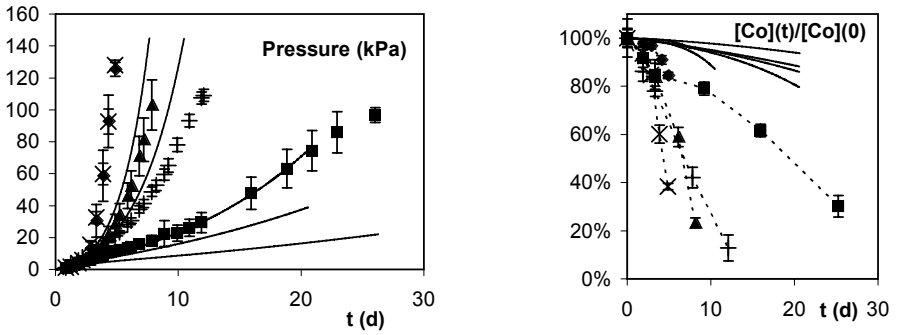
Condition	Parameter:	
	Maintenance factor *m* (mmol hr^{-1} g^{-1})	***t* lag (hr)**
Co 0.03	0.2	10
Co 0.05	0.5	12
Co 0.1	0.5	15
Co 0.5	1.2	38
Co 5	1.2	38
Ni 0.1	0.5	25

Table 3. Constant model parameters

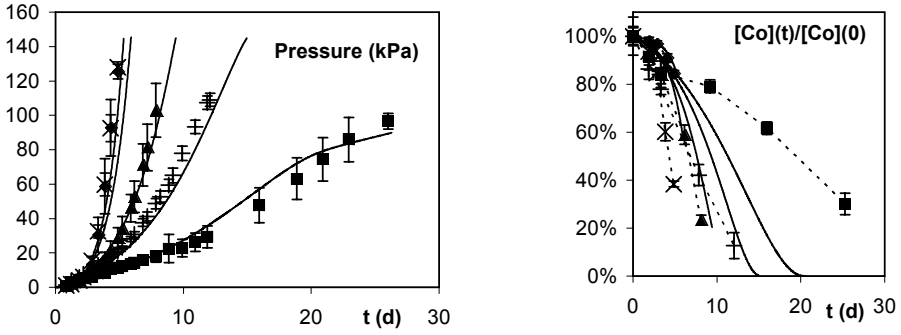
Parameter		Value	Source
μ_{max}	(hr^{-1})	0.037	Fitted to linearized maximum growth
K_{MeOH}	(mM)	0.5	Daussmann et al., 1997
$Q_{Co,min}$ $Q_{Ni,min}$	(nmol g^{-1}) (nmol g^{-1})	85 75	Fitted to growth curves and estimates from measured cell content
Y Pressure/ $c_{MeOH,consumed}$	(g l^{-1} mM^{-1}) (kPa mM^{-1})	0.0053 0.96	Calculated from measured pressure, biomass and methanol consumption.
x(t=0) c_{MeOH}(t=0)	(g l^{-1}) (mM)	0.043 151	Measured Measured
$K_{JM,Co}^{2+}$ $K_{JM,Ni}^{2+}$ $K_{JM,Co}^{2+}/K_{JM,Ni}^{2+}$ J_{max}	(nM) (nM) (-) (nmol g^{-1} hr^{-1})	$3.5 * 10^{-4}$ $3 * 10^{-6}$ $1.2 * 10^{2}$ 60	Fitted to growth and metal depletion curves

The computation was based on a scheme of discrete time steps to calculate the changes in metal content, dissolved metal concentration, biomass concentration and methanol concentration. A step size of 0.5 hr was selected having confirmed that different step sizes (0.5, 1 and 2 hrs) did not alter the result significantly. In the following paragraphs, the outcome of the model for the relevant parameters will be discussed. First, we will discuss the sensitivity of the model to the most important parameters: J_{max} (fig. 4), $K_{JM,Co}{}^{2+}/K_{JM,Ni}{}^{2+}$ (fig. 5), $Q_{Co,min}$ and $Q_{Ni,min}$ (fig. 6), $k_{eff,\ Co}$ and $k_{eff,\ Ni}$ (fig. 7) and $Q_{Co,eff\ min}$ and $Q_{Ni,eff\ min}$ (fig. 8). Variation of these parameters affects the model description in different ways, enabling independent estimation of their magnitudes. J_{max}, $Q_{Ni,min}$ and $Q_{Co,min}$ and $K_{JM,Co}{}^{2+}/K_{JM,Ni}{}^{2+}$ all affect the growth rate, albeit in different ways. Increasing J_{max} (fig. 4) increases the amount of metal taken up in all conditions, thus increasing the growth

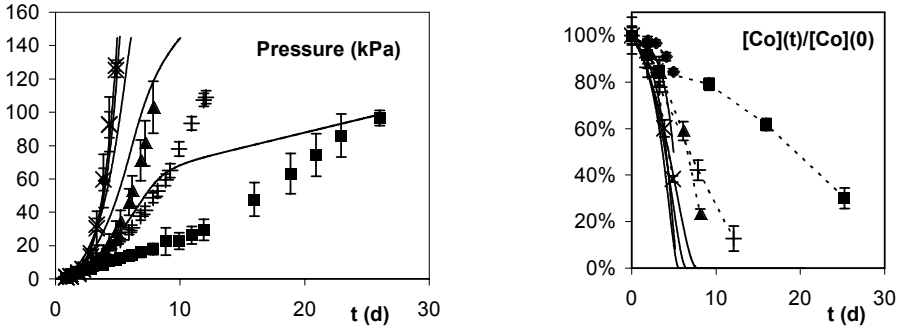
rate. Both growth rates and metal consumption are very sensitive to variation of this parameter. Variation of $Q_{Ni,min}$ and $Q_{Co,min}$ however do not influence the amount of metal taken up, but merely affect the relationship between metal content and growth rate (fig. 6). The effects of $Q_{Ni,min}$ and $Q_{Co,min}$ are independent of each other, illustrated by the fact that the former only influences the relationship between Q_{Ni} and growth rate, while the latter only influences the relationship between Q_{Co} and growth rate. The ratio between the Michaelis-Menten constants for Co and Ni ($K_{JM,Co^{2+}}/K_{JM,Ni^{2+}}$) has a huge impact on the uptake of Co compared to that of Ni, thus also influencing growth rate (fig. 5). The effects of efflux rates and threshold values for efflux are shown in figs. 7 and 8. When a threshold value is used, the efflux rates do not influence growth rates, since efflux only plays a role at metal contents where there is no further growth limitation. Only when the threshold value is lower than or in the same order as Q_{min}, growth rate is lowered. However, the effects then observed are very unrealistic, confirming the likelihood of a threshold value for excretion.



J_{max} = 10 nmol g^{-1} hr^{-1}



J_{max} = 60 nmol g^{-1} hr^{-1}



J_{max} = 200 nmol g^{-1} hr^{-1}

Fig. 4. Model sensitivity to maximum uptake flux, J_{max}.
Other model parameters kept constant at the values in table 2 and 3. Excretion not taken into account. Effects shown for growth rates (expressed as pressure increase) and Co uptake (expressed as depletion of dissolved Co) for Co 5 (◆), Co 0.5 (X), Co 0.1(▲), Co 0.05 (+) and Co 0.03 (■). With increasing J_{max}, Co uptake, Ni uptake (not shown) and growth rate increase.

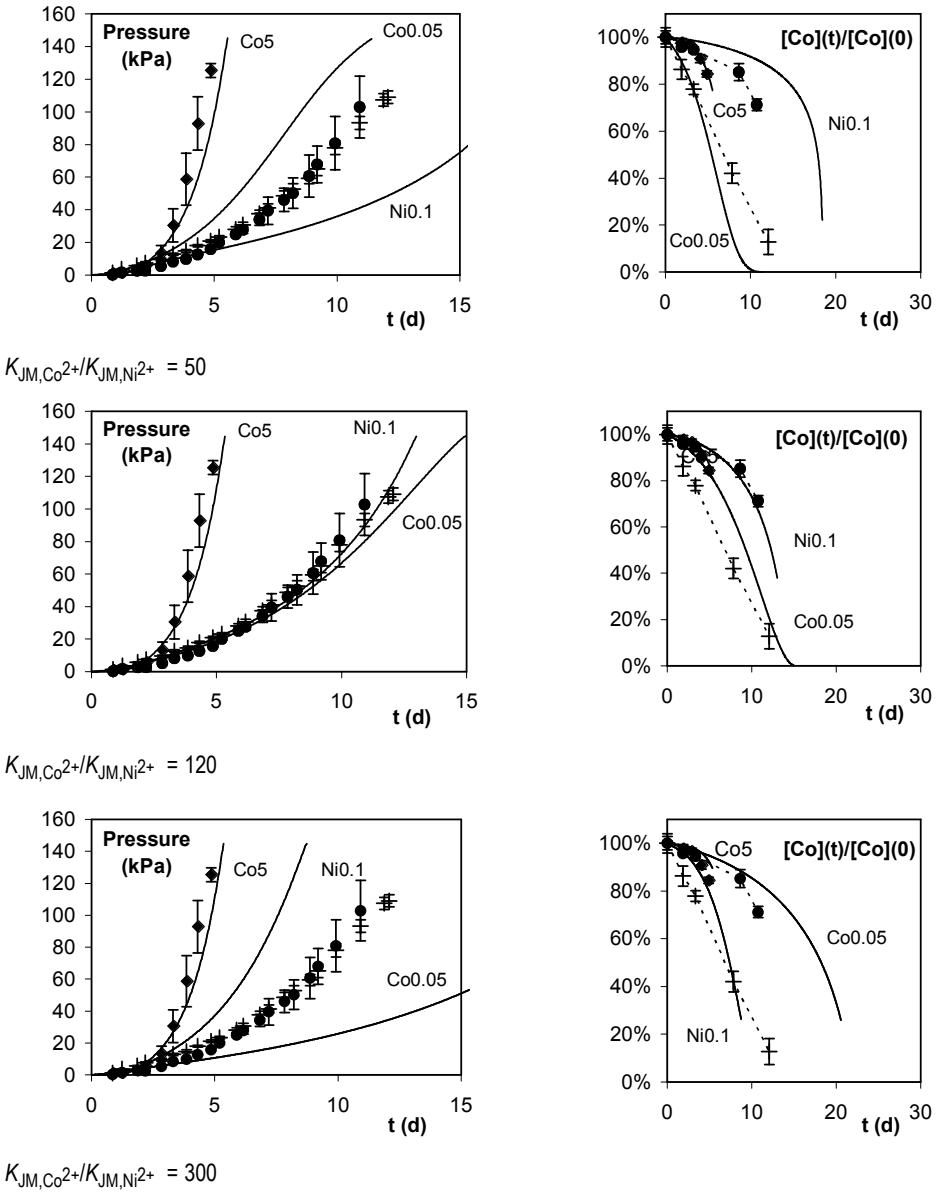


Fig. 5. Model sensitivity to the ratio between the Michaelis-Menten uptake constants for Co and for Ni, $K_{JM,Co^{2+}}/K_{JM,Ni^{2+}}$. Other model parameters kept constant at the values in table 2 and 3. Excretion not taken into account. Effects shown for growth rates (expressed as pressure increase) and Co uptake (expressed as depletion of dissolved Co) for Co 5 (◆), Co 0.05 (+) and Ni 0.1 (▲).
With increasing $K_{JM,Co^{2+}}/K_{JM,Ni^{2+}}$, the ratio between the uptake affinity for Co and that for Ni decreases, thus the sensitivity of growth to total Co increases, while that to total Ni decreases.

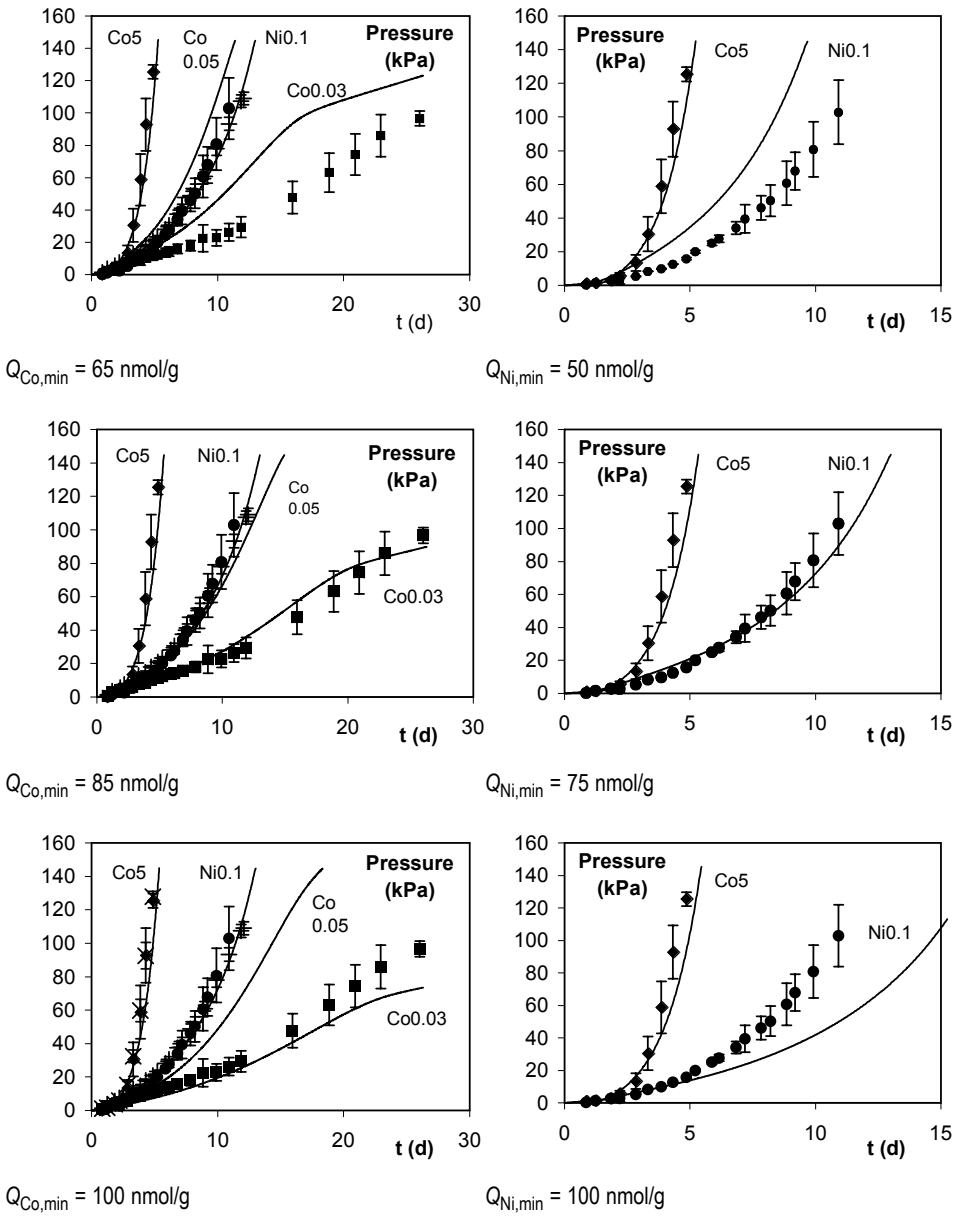


Fig. 6. Model sensitivity to minimum metal content of the cells for Co ($Q_{Co,min}$, left) and for Ni ($Q_{Ni,min}$, right). Other model parameters kept constant at the values in table 2 and 3. Excretion not taken into account. Effects shown for growth rates (expressed as pressure increase) and Co uptake (expressed as depletion of dissolved Co) for Co 5 (◆), Co 0.05 (+), Co 0.03 (■) and Ni 0.1 (▲).
Increase of the minimum metal content decreases the sensitivity of the growth rate to the total concentration.

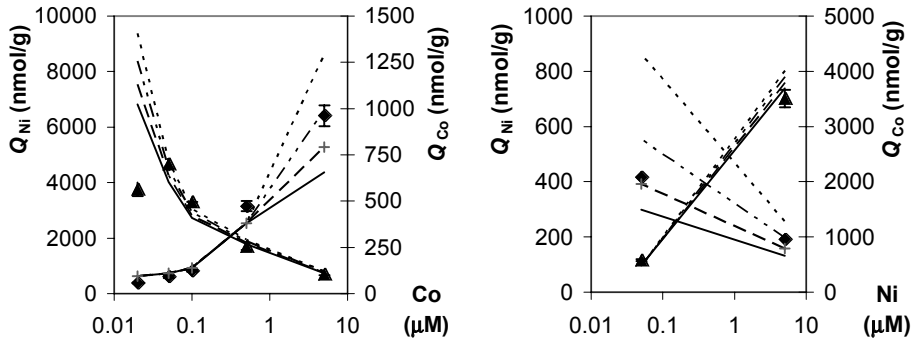


Fig. 7. Model sensitivity to efflux rate constants $k_{eff,Co}$ and $k_{eff,Ni}$.
Other model parameters kept constant at the values in table 2 and 3. Effects shown for effects on Ni and Co content at the end of growth with variation of total Co or total Ni (see fig. 2). s : Ni content (left axis); u : Co content (right axis). ·····: $k_{eff,Co}$,$k_{eff,Ni}$ = 0 hr^{-1} ; - · -: $k_{eff,Co}$ = 0.01 hr^{-1} and $k_{eff,Ni}$ = 0.001 hr^{-1} ; - -: $k_{eff,Co}$ = 0.02 hr^{-1} and $k_{eff,Ni}$ = 0.002 hr^{-1} ; — : $k_{eff,Co}$ = 0.03 hr^{-1} and $k_{eff,Ni}$ = 0.003 hr^{-1}.

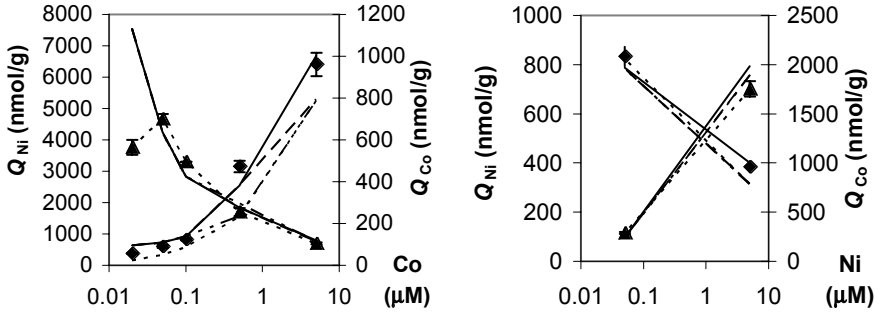


Fig. 8. Model sensitivity to threshold values for efflux, $Q_{Co\ eff,min}$ and $Q_{Ni\ eff,min}$.
Other model parameters kept constant at the values in table 2 and 3. Effects shown for effects on Ni and Co content at the end of growth with variation of total Co or total Ni (see fig. 2). s : Ni content (left axis); u : Co content (right axis). ·····: $Q_{Co\ eff,min}$, $Q_{Ni\ eff,min}$ = 0 nmol/g; - · -: $Q_{Co\ eff,min}$, $Q_{Ni\ eff,min}$ = 200 nmol/g; - -: $Q_{Co\ eff,min}$, $Q_{Ni\ eff,min}$ = 500 nmol/g; —: $Q_{Co\ eff,min}$, $Q_{Ni\ eff,min}$ = 1000 nmol/g.

Effects on growth

The maximum growth rate constant can be estimated to be around 0.04 hr^{-1}, which is somewhat lower, but of the same order as reported before (Daussmann et al., 1997).

The influence of Co and Ni is similar: a minimum metal content of 85 nmol g^{-1} for Co and 75 nmol g^{-1} for Ni fits the data best. The increase in growth rate with increasing total Co and Ni is described very well (fig. 1): indeed, the metal content is a good indicator for growth rate effects. The data and model description clearly rule out metal interreplacement, as observed for some other organisms (Timmermans et al., 2001; Sunda & Huntsman, 1995; Bertram et al., 1994): limitation by one of the metals is not alleviated at a high concentration of the other. The duration of the lag phase seems to differ with different conditions, being generally shorter for lower concentrations of Co and Ni.

Metal uptake and excretion

Taking into account competitive uptake as the reason for different Co and Ni contents at 0.5 and 5 µM Co, the estimated maximum uptake rate J_{max} is 60 nmol g^{-1} hr^{-1}. For the Michaelis-Menten constants we can only give upper limits, but the ratio between the constant for Co and that for Ni is fixed. The upper limits of the Michaelis-Menten constants are: $K_{JM,Co}{}^{2+} = 3.5 * 10^{-4}$ nM and $K_{JM,Ni}{}^{2+} = 3 * 10^{-6}$ nM. Calculating the conditional uptake parameters, $K'_{JM,Co}{}^{2+}$ and $K'_{JM,Ni}{}^{2+}$, using eq. (5) for a constant total concentration of 5 µM of the other ion (the reference concentration), we find $K'_{JM,Co}{}^{2+} = 2 * 10^{-2}$ nM and $K'_{JM,Ni}{}^{2+} = 3 * 10^{-5}$ nM. These values can also be used as reference values to compare the effect of taking into account competition or not. In fig. 9, the metal content data are compared to model fittings with or without competition. The model with competition describes the data better than the model without competition.

As was made clear from fig. 7, Co and Ni content is overestimated at high metal concentrations if efflux is not taken into account. The order of magnitude of the efflux rate constant for Co was estimated to be $k_{eff,Co} = 0.02$ hr^{-1} with a threshold value $Q_{Co,eff,min}$ of 500 nmol g^{-1}. Apparently, cofactor excretion indeed only takes place at higher metal contents. Approximate amounts of excreted cofactor correspond well with literature (Mazumder et al., 1986, 1987; Silveira et al., 1991a; Lin et al., 1989). For Ni we can also estimate an efflux rate constant ($k_{eff,Ni} = 0.002$ hr^{-1}, with a threshold value for Q_{Ni} of 500 nmol g^{-1}). These estimates are based on amounts of excreted cofactor known from literature (Mazumder et al., 1986, 1987; Silveira et al., 1991a; Lin et al., 1989). However, these values are less certain than those for Co, since fewer data are available and the model is less sensitive for Ni excretion (see fig. 7 and 8).

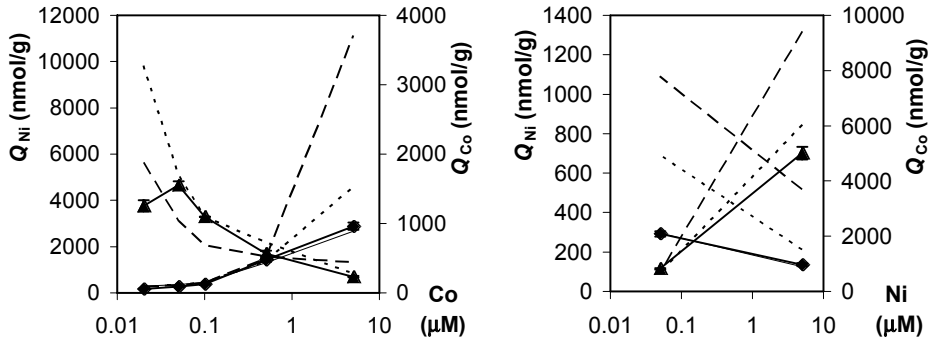


Fig. 9. Effects of taking into account competition on the model description of Co and Ni content at the end of growth (see fig. 2). Other model parameters kept constant at the values in table 2 and 3. - -: No competition; ····: Competition.

DISCUSSION

Metal uptake, cofactor contents and growth characteristics

The model presented in this paper is very well able to describe the effects of dissolved Co, Ni and methanol on the Co and Ni content of *Methanosarcina barkeri* and its growth characteristics for a broad range of conditions.

Uptake of Co and Ni by other types of microorganisms is known to take place either through unspecific Mg-transporters, which have a high maximum uptake flux (capacity) but a low affinity ($1/K_{JM}$) for Co and Ni, or via specific Co or Ni transporters with a higher affinity but a lower capacity for these metal ions (Jarrell & Sprott, 1982; Watt & Ludden, 1999a; Komeda et al., 1997; Saito et al. 2002). Compared to these data, the uptake of both Co and Ni reported in this paper has an extremely high affinity/low capacity character: Michaelis-Menten constants from the literature cover the range from 17 nM to 50 μM, while we found conditional values of the order of 10^{-5} to 10^{-2} nM, and intrinsic values that were even lower. A primary reason for this could be that in many cases (e.g. Jarrell & Sprott, 1982), speciation is not taken into account in the uptake assays. In cases that these are related to the total metal concentration instead of to the much lower free metal concentration, this leads to gross underestimation of affinities. Furthermore, the transport systems studied here might very well differ from those reported in literature since most previous studies were performed with totally different organisms and over different free metal concentration ranges. Depending on the availability in the specific environment of these organisms and the metal need of the organism itself, transport may be more or less efficient. A good example is high affinity Co transport recently found for cyanobacteria (Saito et al., 2002). These organisms live in environments typically containing only very little available cobalt. Efficient Co transporters still enable them to satisfy their considerable metal need. The extremely high affinity found in the present study provides these methanogens with a strategy to cope with the low external free metal concentrations typically encountered in sulfide-containing media and the considerable internal metal needs inside the cells.

The competition between Co and Ni for uptake was observed previously for other organisms (Jarrell & Sprott, 1982; Watt & Ludden, 1999a). Many questions remain to be answered in this respect. In the first place, the exact mechanism of competition between Co and Ni remains unknown. Secondly, it would be interesting to know whether also other ions compete with Co or Ni uptake.

The change of the amount of transporter sites was not taken into account. Many organisms respond to lower availability by an increased synthesis of the transporters (e.g.: Sunda & Huntsman, 1998). If this type of adaptation would be of importance for our data, metal uptake at low external concentrations would be underestimated by the present model. However, metal uptake under these conditions is even overestimated. Thus, it is unlikely that the organisms in the experiments reported here increase the amount of transporters in response to low metal concentrations. Still, over longer time scales the bioavailability of Co and Ni is expected to have impact, not only directly via the transporter densities, but also indirectly via effects on the overall metabolism. E.g., for granular biomass taken from respectively Co- or Ni-limited reactors, the growth response to dissolved Co

and Ni was shown to change with time of limitation (Zandvoort et al., 2002a, 2002b). It would be interesting to study these adaptation effects on a more mechanistic level.

Amounts of Co, Ni and Fe taken up lie well within the same order of magnitude as found previously by Scherer et al., 1983 and Diekert et al., 1981. However, the variation in metal contents was never studied systematically before. These data clearly demonstrate that the variation in Co and Ni content is considerable and dependent on uptake. Environmental factors strongly affect metal uptake, thus affecting biological activity. Due to the variable metal contents, the relationship between bioavailability and biological activity becomes involved. Although variation in the metal content strongly affects biological activity, it also broadens the range of metal concentration in which the organism is able to grow (Morel, 1987). Thus, it can be seen as a mechanism of efficiency in case of limited availability of these nutrients.

The incorporation of the metals into the corresponding cofactors shows some interesting patterns. For both cofactors studied, the metal/cofactor ratio is lower at low metal content, which might mean that there is a metal fraction that does not consist of cofactors, but is still indispensable for the cell. For Co, this amount is small and constant, which can be related to the fact that the main role of Co in the cells is vitamin B_{12}. For Ni however, this amount is larger and variable, which can be understood from the fact that Ni can not only serve in F_{430} but also in other Ni-containing compounds, such as hydrogenases. The observation of a large and variable metal pool present in another form than cofactors, and a low metal to cofactor ratio for Ni are consistent with data for several methanogenic archaebacteria (Diekert et al., 1981). For both Co and Ni no regulation mechanism on the level of cofactor synthesis seems to exist to prevent metal limitation.

It is known that the dynamics of cofactor synthesis, efflux and uptake are of importance for the metal distribution over cell and solution (Mazumder et al., 1986, 1987; Lin et al., 1989; Silveira et al., 1990, 1991a, Rodionov et al., 2003). In the present model, we have taken cofactor efflux into account by first order kinetics with a threshold value for the metal content. However, a more rigorous analysis would require information about the nature of the efflux, especially regarding the underlying physiological mechanisms. Unfortunately, there is a paucity of data for such processes. Apart from excretion, uptake of cofactors is also known to take place (Roth et al., 1996). In order to incorporate this into the model, more detailed knowledge is required about uptake routes and rates. Finally, cofactor synthesis kinetics is likely to be of influence. Cofactor synthesis is only expected to be of importance when it is slow compared to metal uptake and growth.

Growth rate stimulation as demonstrated by Scherer et al. (1981), Diekert et al. (1981) and Silveira et al. (1991b) is confirmed in this study. The results presented here, however, enable these effects to be linked to metal and cofactor contents directly. The variable metal contents on the one hand affect growth rates, but on the other hand broaden the range of metal concentrations suitable for growth. Metal effects on growth are successfully described by a Droop model. The variable metal contents clearly demonstrate that the frequently used Monod model is not the most suitable to describe Co and Ni limitation.

Implications for practice

An important practical application of Co and Ni limitation is in anaerobic treatment of methanol-containing wastewater. The data and model description presented in this paper enable estimation of the effects of Co and Ni on reactor performance. From the minimum metal contents and the corresponding Droop model, we can calculate optimum metal contents: 95% of maximum growth is achieved at a metal content higher than between 1 and 2 μmol g^{-1}, which for a typical yield of 0.6 g / 208 mmol methanol (=10 g COD) (e.g.: Silveira et al., 1991b) comes down to 3-6 nmol Ni and Co needed per mmol methanol.

The observation of high affinity uptake with competitive character has important implications. The high affinity uptake shows that even very low free metal concentrations encountered in the sulfide containing reactor media can still be sufficiently high to maintain reasonable metal uptake fluxes. This is demonstrated by the data in the absence of EDTA, where optimum growth was found despite the precipitation of most of the added Co and Ni. This finding is also very important in understanding processes in the natural environment. Many marine or anaerobic environments are characterized by extremely low free metal concentrations, while microorganisms which need these metals contain relatively high amounts of these metals (e.g.: Saito et al., 2003).

Competition can have important implications for the influence of different ions on the uptake of others. High concentrations of one metal ion could possibly suppress the uptake of another.

From the current modelling of metal uptake, we can further speculate on the effects at higher metal contents: i.e. toxicity. For the organisms used in the present study, we can see two defensive mechanisms against toxicity. A first possible defensive mechanism stems from the uptake characteristics: the low maximum metal uptake rate will prevent toxicity to occur over a wide range of metal concentrations. Secondly, at higher contents metals are excreted. In spite of these detoxifying mechanisms, from a certain concentration on, metals become toxic (e.g.: Jarrell et al., 1987; Gonzalez-Gil et al., 2003). Possibly metal is not only taken up via specific Ni and Co channels, but also via low affinity/high capacity transporters which are more specific towards other ions, e.g. Mg^{2+}. To illustrate this, uptake of Co was calculated through the transporter reported here (J_{max} = 60 nmol g^{-1} hr^{-1}; $K'_{JM,Co^{2+}}$ = 0.02 nM), and through a hypothetical Mg^{2+} transporter, with transport parameters J_{max} of 10 μmol g^{-1} hr^{-1} and $K'_{JM,Co^{2+}}$ of 20 μM, similar to values reported in literature (Watt & Ludden, 1999b).

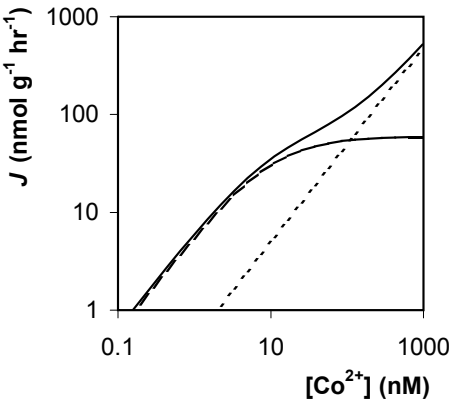


Fig. 10. Effect of combined uptake by a specific, high affinity/low capacity transporter and an aspecific, low affinity/high capacity transporter, exemplified for Co. - -: High affinity/low capacity; ····: Low affinity/high capacity; —: Sum of the two transporters.

The calculated uptake rates (fig. 10) clearly demonstrate that, even though at a low concentration uptake might be determined by a high affinity/low capacity transporter, at higher free metal concentrations transport through low affinity/high capacity transport might become dominant. This also stresses the local applicability of uptake characteristics with regard to free metal concentrations.

Finally, the excretion of cofactors can have important implications. Only few organisms are able to produce vitamin B_{12}, while many need it. In a population consisting of many different organisms, as often present in nature or in bioreactor systems, syntrophic interactions can be present in which different types of organisms derive mutual benefit from the compounds selectively produced by only a few of them.

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[7]

Bioavailability of Co and Ni for methanogens in bioreactor media: effects of speciation, precipitation and mass transfer

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ABSTRACT

In chapter 6, a Michaelis-Menten/Droop (MMD) model was developed to describe uptake of Co and Ni by methanogenic archaebacteria and its impact on microbial growth. In the present chapter, the applicability of this model under different metal speciation conditions is tested, and the effects of dissolution of metal precipitates, dissociation of metal complexes and mass transfer are studied.

First, the effects of Co and Ni on the activity of a methanogenic *Methanosarcina* sp. enrichment in the presence of cysteine were studied and analysed. Overall trends in Co and Ni uptake could be described well. Competition between uptake of Co and that of Ni is confirmed. The data provide information on the efficient uptake of vitamin B_{12}, most likely via specialized uptake routes.

The application of the model to uptake and growth in the presence of sulfide suggested different metal transport mechanisms depending on the prevailing metal speciation.

Possible kinetic factors in metal limitation (such as diffusion to the biointerface, dissolution of metal sulfide precipitates and dissociation of dissolved metal complexes) are assessed in terms of flux analysis. In most cases, the dissolution flux is not limiting, although in practical situations limitation may occur due to ageing of the precipitate and low specific surface area of the metal sulfide aggregates. The magnitude of the diffusion flux of free Ni is close to that of the biouptake flux, and therefore is a potential source of growth limitation. The rate of dissociation of dissolved complexes is not limiting.

INTRODUCTION

Trace element limitation can be important in anaerobic bioreactor performance (Lettinga et al., 1979; Zandvoort et al., 2003; Florencio et al., 1993) because of the essential biological functions of metal ions in various microorganisms (e.g. Thauer, 1998). More specifically, Co and Ni are essential for methanogenic microorganisms because of their presence in vitamin B_{12} and factor F_{430} (Roth et al., 1996; Telser, 1998). In anaerobic, sulfide-rich environments the availability of these metal ions is limited due to metal-sulfide precipitation (Callander & Barford, 1983). Thus, to optimize metal dosing in bioreactor practice, it is important to quantify the relationship between concentration and availability of the metal ions towards methanogens.

Many experimental studies on the effect of Co and Ni on methanogens have been reported (e.g., Jarrell & Sprott, 1982; Diekert et al. 1980a,b, 1981; Silveira et al., 1990, 1991a, 1991b; Nishio et al, 1992; Mazumder et al., 1986, 1987; Lin et al., 1989), yielding information on uptake, cofactor dynamics and growth. In spite of the wealth of data, a comprehensive quantitative description is lacking. In chapter 6 of this thesis, the effects of Co and Ni uptake on growth of *Methanosarcina barkeri* on methanol under conditions of well-defined speciation were described by a combination of competitive Michaelis-Menten uptake kinetics and Droop growth limitation (further also referred to as the Michaelis-Menten/Droop (or MMD) model). Growth rate limitation by Co, Ni and/or methanol was described by:

$$\mu = \mu_{max} \frac{c_{MeOH}}{c_{MeOH} + K_{MeOH}} \frac{(Q_{Co} - Q_{Co,min})}{Q_{Co}} \frac{(Q_{Ni} - Q_{Ni,min})}{Q_{Ni}} \quad (1)$$

where: μ = growth rate constant (hr^{-1}); μ_{max} = maximum growth rate constant (hr^{-1}); c_{MeOH} = methanol concentration (mol l^{-1}); K_{MeOH} = methanol Monod constant (mol l^{-1}); Q_{Co} = Co content (mol g^{-1}); $Q_{Co,min}$ = minimum Co content (mol g^{-1}); Q_{Ni} = Ni content (mol g^{-1}) and $Q_{Ni,min}$ = minimum Ni content (mol g^{-1}) (g biomass is expressed as dry weight). Limitation by Co and Ni is thus described by Droop-terms ($(Q_i-Q_{i,min})/Q_i$) instead of more common Monod-terms, thus relating the growth rate constant to internal metal concentrations instead of to external ones. This is of importance, since Co and Ni contents (also called cell quota) are known to vary considerably (as was demonstrated in chapter 6). Limitation by methanol is described by a Monod-term, since a more or less constant amount of methanol is consumed per amount of biomass formed.

The uptake fluxes J_{in} of Co and Ni were described using Michaelis-Menten equations for limited internalization rates with competition between Co and Ni, e.g. for Co uptake:

$$J_{Co,in} = J_{max} \frac{c_{Co^{2+}} / K_{JM,Co^{2+}}}{c_{Ni^{2+}} / K_{JM,Ni^{2+}} + c_{Co^{2+}} / K_{JM,Co^{2+}} + 1} \quad (2)$$

where: J_{max} = maximum uptake flux (mol g^{-1} hr^{-1}); $K_{JM,Co}{}^{2+}$ = Co Michaelis-Menten

constant (mol l^{-1}); $K_{JM,Ni^{2+}}$ = Ni Michaelis-Menten constant (mol l^{-1}) ; $c_{Co^{2+}}$ = free Co^{2+} concentration (mol l^{-1}); $c_{Ni^{2+}}$ = free Ni^{2+} concentration (mol l^{-1}).

In case of a constant concentration of the competing ion, eq. (2) can be written as:

$$J_{Co,in} = J_{max} \frac{c_{Co^{2+}}}{c_{Co^{2+}} + K'_{JM,Co^{2+}}} \quad (3)$$

where $K'_{JM,Co^{2+}}$ is a conditional Michaelis-Menten constant for Co (mol l^{-1}), defined as:

$$K'_{JM,Co^{2+}} = \frac{K_{JM,Co^{2+}}}{K_{JM,Ni^{2+}}} c_{Ni^{2+}} + K_{JM,Co^{2+}} \quad (4)$$

The efflux J_{eff} (mol g^{-1} hr^{-1}) of cell metal was approached by first order kinetics, e.g. for Co:

$$J_{Co,eff} = k_{Co,eff}(Q_{Co} - Q_{Co,eff,min}) \quad (5)$$

where $k_{Co,eff}$ = the efflux rate constant (hr^{-1}) and $Q_{Co,eff\ min}$ is a threshold value below which no efflux takes place.

Coupled with mass balances for Co, Ni, methanol and biomass, these equations enable metal effects on biomass growth in batch cultures to be described under various speciation conditions. By combination of this model with experimental data, values for key parameters describing uptake and growth were estimated. Uptake of both Co and Ni was determined by high-affinity/low-capacity transporters (J_{max} = 60 nmol g^{-1} hr^{-1}; $K_{JM,Co^{2+}}$ = 3.2 * 10^{-4} nmol l^{-1}; $K_{JM,Ni^{2+}}$ = 3 * 10^{-6} nmol l^{-1}). Evidence was found for competition between Co and Ni on the level of uptake. Growth rates were closely linked to Co and Ni contents via Droop growth limitation, with values for minimum metal contents of $Q_{Co,min}$ = 85 nmol g^{-1} and $Q_{Ni,min}$ = 75 nmol g^{-1}. Evidence for the excretion of Co and Ni at high metal contents was found. Overall, the application of the MMD model yielded a very good description of the experimental data. However, the model was only applied to data obtained over a limited metal speciation range, defined by the constant concentration of EDTA. Therefore, in this chapter we discuss the data analysis for a much broader range of experimental conditions.

First we discuss the effects of Co and Ni on the activity of a methanogenic enrichment in the presence of cysteine as the sole sulfur source. Many previous experiments were performed in such a medium, which has the advantage of the absence of metal-sulfide precipitation. New data on Co and Ni uptake and growth rates at various Co concentrations are presented and analyzed using the MMD model.

Secondly, data for effects of Co and Ni in the presence of metal sulfide precipitates are discussed. Application of the MMD model to systems with metal sulfide precipitation is

interesting for various reasons. First, we can see how uptake and growth respond to the typical solution chemistry in the presence of sulfide, which is expected to dominate metal speciation in most anaerobic environments where methanogens live. Secondly, the modelling enables us to estimate the conditions where dissolution becomes limiting. Conflicting results were presented in existing literature (e.g.: Gonzalez-Gil et al., 1999a; chapter 4 of this thesis), and it is therefore interesting to analyze these effects using the MMD model. Some initial guesses were presented previously (chapter 4); here we try to developed the model to a more comprehensive level. Thirdly, the modelling approach is different from the approach most often used to describe dissolution-limited (or otherwise mass-transfer limited) growth. The majority of examples from the literature deals with organic substrates, assuming a constant consumption of the limiting substrate per amount of biomass (e.g.: Wick et al., 2001; Harms & Bosma, 1997; Bosma et al., 1997). The difference between the use of constant or variable amounts of substrate per amount of biomass is expected to have an important impact on the growth.

Apart from Co and Ni effects in the presence of cysteine or sulfide, the effects of diffusion and dissociation will also be assessed within the framework of the MMD model.

MATERIALS AND METHODS

Biomass

For the data obtained in the presence of cysteine, a methanogenic enrichment from an anaerobic wastewater reactor consisting mainly of *Methanosarcina* sp. was used. This enrichment was also used in some previous studies on Co and Ni bioavailability (Gonzalez-Gil et al., 1999a; Gonzalez-Gil et al., 2003; chapter 4 of this thesis). The cells were grown at 30°C.

The data on effects of metal sulfide precipitates were obtained using a pure culture of *Methanosarcina barkeri* DSM 800 grown at 37°C, as also used in chapter 6 for the validation of the MMD model. This was used as a well-defined reference culture to verify effects observed previously with the above mentioned enrichment culture.

Growth and uptake in the presence of cysteine

To examine the effects of a different ligand in the absence of sulfide precipitation, the enrichment was grown in the presence of 1 mM cysteine as the sole sulfur source. In these experiments, total Co was varied (0.07; 0.3; 0.6; 2.7 μM), while total Ni and Fe were kept constant (2 and 7.5 μM resp.). Further constituents of the medium can be found in Gonzalez-Gil et al., 2003. We note that 50 nM of the total dissolved Co was present in the form of vitamin B_{12}. Before inoculation, samples were taken for determination of dissolved Ni, Co and Fe. Biomass growth was followed by measuring pressure. At different points in time, cultures were harvested. An aliquot of 5 ml of medium was taken for total metal determination. Two aliquots of approximately 35 ml were centrifuged for 10 min. at 48000 g and Co, Ni, Fe and methanol were determined in the supernatant. The cell pellet was washed with 0.02 M phosphate buffer, pH 7 and centrifuged again for 10 min. at 48000 g. One of the two cell pellets was used for dry weight determination, while the

other was used for metal content determination.

The biogas produced was measured over time as the increase in pressure (kPa) in the serum bottles as described in Zandvoort et al., 2002a. The growth curves in terms of pressure increase due to methane formation corresponded with those for dry weight increase (data not shown) confirming the validity of using pressure as a measure for growth.

Methanol concentration was measured by HPLC as described in Gonzalez-Gil et al., 1999a.

For dry weight determination, cells were resuspended after centrifugation in 2 ml H_2O and transferred to a dry aluminum cup of known weight. After drying in a stove at 103^{o}C for 24 hr, the filled cups were left to cool down in an exsiccator and weighed.

Metal concentrations (dissolved, cell-adsorbed, internalized) were determined by ICP-MS (Perkin Elmer ELAN6000). The samples for determination of dissolved and cell-adsorbed metal were acidified to 0.14 M HNO_3.

Metal content in the cells was determined after microwave digestion (CEM 2100, Matthews, NC) in a mixture of 1.5 ml HNO_3 (65%) and 4.5 ml HCl (37%). After digestion, the samples were diluted 10x before ICP-MS determination.

Growth and uptake in the presence of metal-sulfide precipitates

The experiments with the pure culture were part of a broader study (chapter 6), designed to validate the MMD model. Pure cultures of *Methanosarcina barkeri* were grown in the presence of 5 μM Ni and Co, 50 μM Fe, 20 μM EDTA and 1 mM sulfide. Under these conditions, most Ni, Co and Fe was present in the form of sulfide precipitates. Growth was not limited by Ni or Co. Data on the dissolved metal concentration, growth and cofactor content are available (chapter 6). The latter parameters enable us to estimate the metal content, independent of any metal sulfide precipitates.

RESULTS AND DISCUSSION

Growth and uptake in the presence of cysteine

The growth rate of the enrichment culture in the presence of cysteine increases with increasing total Co (fig. 1). The effect is largest between 0.07 and 0.3 μM, while there is no significant further increase between 0.6 and 2.7 μM. The effect of Co is most evident at late growth. None of the growth curves remains exponential over the whole growth period, indicating that there is another source of limitation that comes into effect at a later stage of growth.

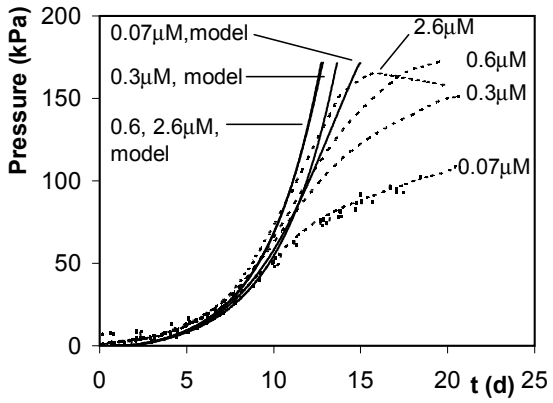

Fig. 1. Methanogenic activity of *Methanosarcina* sp. in the presence of 1 mM cysteine with various total Co concentrations. Methanogenic activity expressed as methane production (mg COD l^{-1}), calculated from the pressure change. Model parameters: see table 1.

With increasing total Co in the medium, Co content of the cells (Q_{Co}) increases (fig. 2). For each initial total Co concentration except for 2.7 µM Co, Q_{Co} decreases with time, indicating reduction of the Co content due to the ongoing growth process. Q_{Ni} as measured after 10 d decreases with increasing total Co in the medium. This is in accordance with competitive effects in the uptake step, as reported in chapter 6. The effect is no longer operational after 20 d. The iron content is 13 ± 2 µmol g^{-1} and is constant with varying total Co or time (data not shown).

No significant amounts of Ni and Co were detected in the washing buffer.

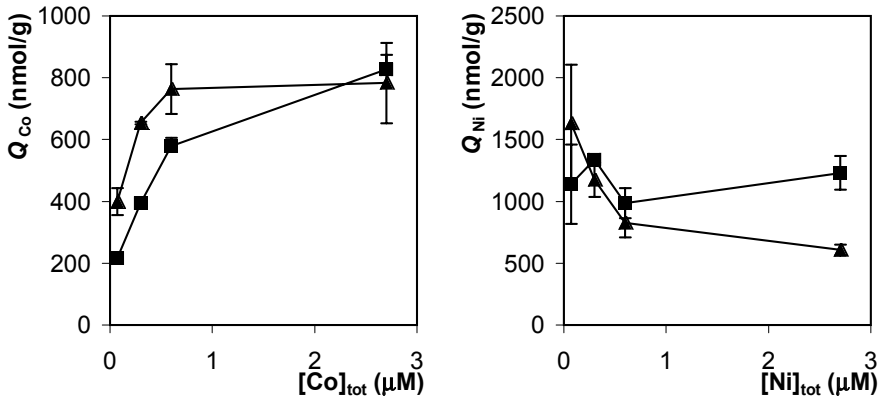

Fig. 2. Co and Ni content of *Methanosarcina* sp. grown in presence of 1 mM cysteine at various total Co concentrations. Metal contents were determined after 10 d (▲) and 20 d (■).

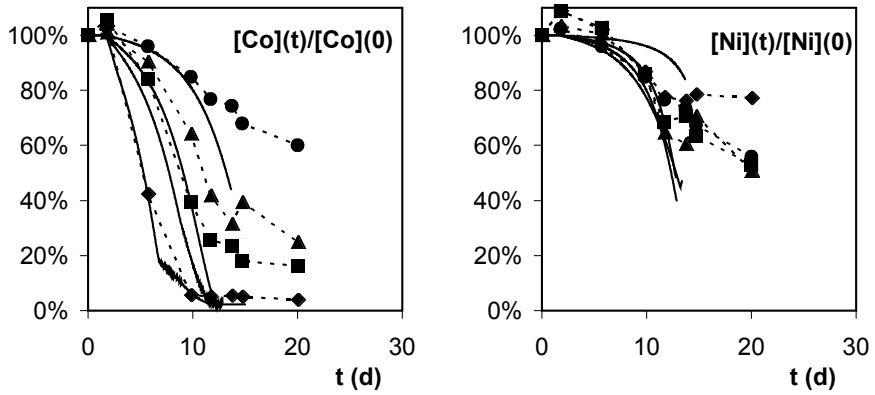

Fig. 3. Data and model description of dissolved total Co and Ni concentration in time in the presence of *Methanosarcina* sp. in presence of 1 mM cysteine at various total Co concentrations. Dissolved Co and Ni expressed as the percentage of the initial total metal concentration. Model parameters: see table 1. ◆ = 0.07 μM Co; ■ = 0.3 μM Co; ▲ = 0.6 μM Co; ● = 2.7 μM Co.

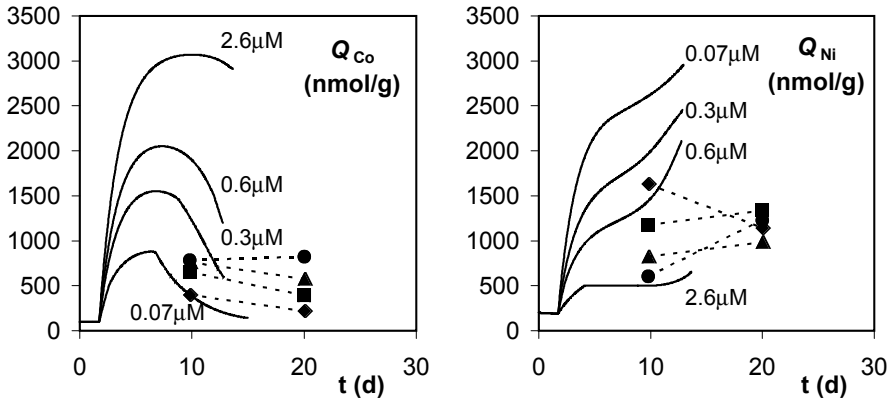

Fig. 4. Model description of Co content Q_{Co} and Ni content Q_{Ni} of *Methanosarcina* sp. in the presence of 1 mM cysteine in time at various total Co concentrations. Model parameters: see table 1. ◆ = 0.07 µM Co; ■ = 0.3 µM Co; ▲ = 0.6 µM Co; ● = 2.7 µM Co.

Table 1. Model constants to describe the Co and Ni uptake and effects in presence of 1 mM cysteine.

Parameter		Value	Remarks
(1+K') Co	(-)	$1 * 10^2$	Calculated using constants from
(1+K') Ni	(-)	$5 * 10^4$	Berthon, 1995
t_{lag} (growth)	(hr)	0	Fitted to growth curves
μ_{max}	(hr^{-1})	0.015	Fitted to non-limited growth curve
K_{MeOH}	(mM)	0.5	Daussmann et al., 1997
$Q_{Co,min}$	(nmol g^{-1})	85	See chapter 6
$Q_{Ni,min}$	(nmol g^{-1})	75	
Y	(g l^{-1} mM^{-1})	0.002	Calculated from measured pressure,
Pressure/methanol consumed	(kPa mM^{-1})	0.7	biomass and methanol consumption.
Q_{Co}(t=0)	(nmol g^{-1})	200	Estimated from Q_{Ni} and Q_{Co} measured
Q_{Ni}(t=0)	(nmol g^{-1})	200	Measured
x(t=0)	(g l^{-1})	0.01	Measured
c_{MeOH}(t=0)	(mM)	240	
J_{max}	(nmol g^{-1} hr^{-1})	60	See chapter 6
$K_{JM,Co}$	(nM)	$3.5 * 10^{-4}$	
$K_{JM,Ni}$	(nM)	$3 * 10^{-6}$	
$k_{Co,eff}$, $k_{Ni,eff}$	(hr^{-1})	0.01	Fitted
$Q_{Co,eff,min}$, $Q_{Ni,eff,min}$	(nmol g^{-1})	500	See chapter 6
$J_{max,B12}$	(nmol g^{-1} hr^{-1})	20	Fitted
$K_{JM,B12}$	(nM)	0.5	Bradbeer, 1991
t_{lag} (metal uptake)	(hr)	41	Fitted to metal uptake data

Most parameters were the same as in the previous application of the model (chapter 6). The maximum growth rate μ_{max} was estimated from the non-metal-limited growth curve. Although a lag time for growth is not needed to describe the data, we do observe that metal uptake does not start immediately (fig. 3). Therefore we allow for a lag time t_{lag} for metal uptake. The complexation capacity (1+K') can be calculated using stability constants from Berthon, 1995. Since the cultures had been grown several times without Co

and Ni added, we assumed values for $Q_{Co}(t=0)$ and $Q_{Ni}(t=0)$ of 200 nmol g^{-1}, as estimated from the minimum Co content measured in these experiments. It was assumed that cysteine remained constant over time and that metal-cysteine complexes were not bioavailable. Although some cysteine is known to be taken up (Mazumder et al., 1987), this is not expected to significantly affect the overall trends. Furthermore, the small amount of sulfide formed from cysteine by the cells was observed not to form any precipitates and can be calculated not to change metal speciation significantly (see chapter 4). The modelling is compared to the measured data in figs. 1, 3 and 4. The data can be fitted well using the same values for $Q_{Co,min}$ and $Q_{Ni,min}$ as in earlier studies with *M. barkeri* (chapter 6). The growth curves (fig. 1) are rather insensitive to the total Co concentration and limitation mostly shows up at late growth. This can be explained by the fact that the experimentally determined Co and Ni contents are high compared to $Q_{Co,min}$ and $Q_{Ni,min}$, which underlines again the importance of internal metal concentrations. Trends in uptake characteristics can be described using similar values for J_{max}, $K_{JM,Ni^{2+}}$ and $K_{JM,Co^{2+}}$ as in earlier studies with *M. barkeri* (chapter 6). The effect of speciation is very clear: although total external concentrations were similar in both cases, metal uptake in the presence of cysteine was higher than in the presence of EDTA (chapter 6). On the other hand, the decrease in the amount of Ni taken up with the increase in total Co added clearly shows the influence of competition (fig. 2 and 4). The uptake of Co is somewhat overestimated, while that of Ni is underestimated. General trends however, are described remarkably well. As observed before (chapter 6), the MMD model overestimates metal contents at high metal concentrations if excretion is not taken into account. Assuming an efflux rate constant of 0.01 hr^{-1} for both Co and Ni improves the description of dissolved metal at late growth considerably. The resulting amounts of cofactor excreted are in broad agreement with the literature data (Silveira et al., 1991b). The data on dissolved Co for an initial total Co concentration of 70 nM provide information on the uptake of vitamin B_{12} uptake. From the literature on *E. coli* it is known that vitamin B_{12} is taken up by high affinity transporters, with K_{JM} values of the order of 0.5 nM (Bradbeer, 1991; Kenley et al., 1978). Adopting this value we can successfully describe the data using a maximum uptake rate of the order of 20 nmol g^{-1} hr^{-1}, thus providing additional evidence for cofactor uptake by methanogens.

Overall, the cysteine case confirms the validity of a model consisting of competitive uptake and growth limitation by internal metal concentrations. It provides further evidence of the importance of excretion and shows that cofactor uptake is also of importance.

Growth and uptake in the presence of metal-sulfide precipitates

First, the MMD model is applied to experiments with a pure culture of *M. barkeri*, grown at 37°C in the presence of 20 µM EDTA, 5 µM Co and Ni and 1 mM S (chapter 6). No growth limitation by Co or Ni was observed, in spite of significant precipitation. The dissolved metal concentration remained constant throughout growth, indicating that there was no depletion due to uptake and thus no dissolution limitation. For these data, metal contents were estimated from the cofactor content of the cells (chapter 4). The free metal ion concentrations of Co and Ni were estimated from the solubility products for metal-sulfide precipitates from Martell and Smith, 1989. All other model parameters used were the

same as in chapter 6, and are summarized in table 2a.

Table 2. Model constants to describe effects of precipitation on Co uptake and limitation

Parameter		a: Co/Ni competition	b: Co only
(1+K') Co	(-)	$1 * 10^3$	$1 * 10^3$
(1+K') Ni	(-)	$2 * 10^2$	not used
t_{lag} (growth)	(hr)	0	0
μ_{max}	(hr^{-1})	0.037	0.037
K_{MeOH}	(mM)	0.5	0.5
$Q_{Co,min}$	(nmol g^{-1})	85	85
$Q_{Ni,min}$	(nmol g^{-1})	75	not used
Y	(g l^{-1} mM^{-1})	0.005	0.005
Pressure/methanol consumed	(kPa mM^{-1})	0.8	0.8
Q_{Co}(t=0)	(nmol g^{-1})	120	120
Q_{Ni}(t=0)	(nmol g^{-1})	150	not used
x(t=0)	(g l^{-1})	0.043	0.043
c_{MeOH}(t=0)	(mM)	115	115
J_{max}	(nmol g^{-1} hr^{-1})	60	60
$K_{JM,Co}$	(nM)	$3.5 * 10^{-4}$	0.02
$K_{JM,Ni}$	(nM)	$3 * 10^{-6}$	not used

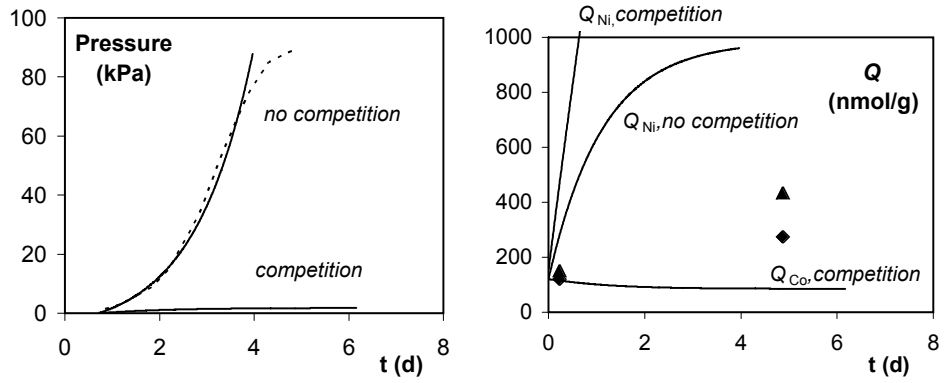

Fig. 7. Comparison between measured and modeled growth and metal uptake in presence of sulfide precipitation. Model parameters: see table 2. a) Growth as measured by pressure due to methane formed. Dashed line: measured; solid lines: model. b) Co content Q_{Co} and Ni content Q_{Ni}. Measured: Co (◆) and Ni (▲).

If competition for uptake between Ni and Co is taken into account, Ni uptake is overestimated, while Co uptake is underestimated (fig. 7). This can be understood from the metal speciation in solution. In case of sulfide precipitation, $Ni^{2+} < Co^{2+}$, whereas in case of EDTA complexation, $Ni^{2+} > Co^{2+}$. Experimentally determined amounts of the two ions taken up however are not too different for the two sets of conditions. This provides evidence that the uptake characteristics determined in the presence of EDTA are not valid over very wide concentration ranges. It is possible that, apart from the competitive Co and Ni uptake that prevails at low concentrations, other uptake channels are also active at higher metal concentrations. To correctly explain the data, we need uptake fluxes of

approximately 10 nmol g^{-1} hr^{-1} for Co and Ni. From other experiments it was concluded that in case of sulfide precipitation under similar conditions, the free metal ion concentration could be limiting uptake (chapter 4). Therefore, the value of K'_{JM} for one or both of the metal ions is expected to be close to the free metal ion concentration, in this case in the order of 0.01 nM (Co) or 1 nM (Ni). By comparison with the data for EDTA, it is most likely that the uptake of Co is limiting, because the K'_{JM}-value mentioned for Co above is indeed close to the value found for the EDTA experiments after correction for Ni competition. Thus again applying the MMD model, now only including uptake and limitation by Co (table 2b), the fit is indeed improved (fig. 7).

The effects of metal dissolution limitation can be simulated by allowing the dissolved metal concentrations to vary with the uptake and dissolution rate from a sulfide precipitate according to:

$$\frac{\partial c_{Co}(total)}{\partial t} = -\left(J_{Co,in}(t) - J_{Co,eff}(t)\right)x(t) + k'_{dissolution}(c_{Co^{2+},eq} - c_{Co^{2+}}) \qquad (6)$$

where $x(t)$ (g l^{-1}) is the biomass concentration, $k'_{dissolution}$ (hr^{-1}) is the conditional dissolution rate constant, given by the product of the surface area of sulfide precipitate (m^2 l^{-1}) and the specific dissolution rate (l m^{-2} hr^{-1}), and $c_{Co^{2+},eq}$ is the equilibrium free Co^{2+}-concentration, defined by the solubility product. As soon as the amount of metal taken up over a discrete time interval (as used in the calculation) becomes larger than the amount of Co still in solution, the amount of Co taken up is defined to be equal to the amount of Co dissolved over the pertaining time interval. Thus, from then on uptake is bound to be dissolution rate limited. For the modelling, parameters from table 2b were used. We assumed initial equilibrium between solution and precipitate. The effects of different values of $k'_{dissolution}$, and (1+K') on growth (as expressed in terms of pressure increase due to methane formation, growth rate, and the growth rate constant μ), dissolved Co and Co content of the cells are shown in fig. 8 and 9, with the aforementioned experimental data for comparison. It is clear that over a wide range of values of $k'_{dissolution}$, no metal depletion or growth limitation takes place (fig. 8). Comparison of a previously estimated value for the dissolution flux from metal sulfide precipitates of O(20) nmol l^{-1} hr^{-1}) (chapter 4) with the value in fig. 8 confirms that under these conditions metal sulfide dissolution is indeed not rate-limiting yet.

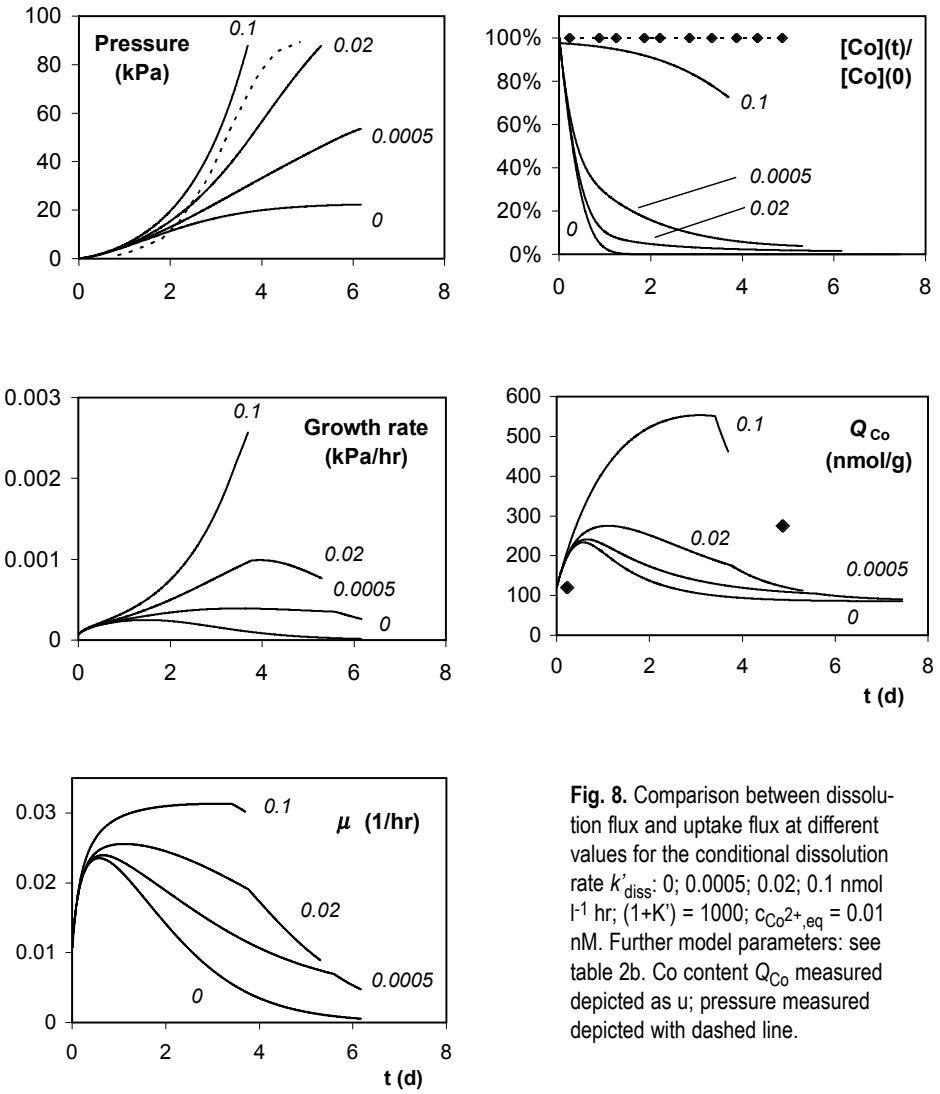

Fig. 8. Comparison between dissolution flux and uptake flux at different values for the conditional dissolution rate k'_{diss}: 0; 0.0005; 0.02; 0.1 nmol l^{-1} hr; (1+K') = 1000; $c_{Co^{2+},eq}$ = 0.01 nM. Further model parameters: see table 2b. Co content Q_{Co} measured depicted as u; pressure measured depicted with dashed line.

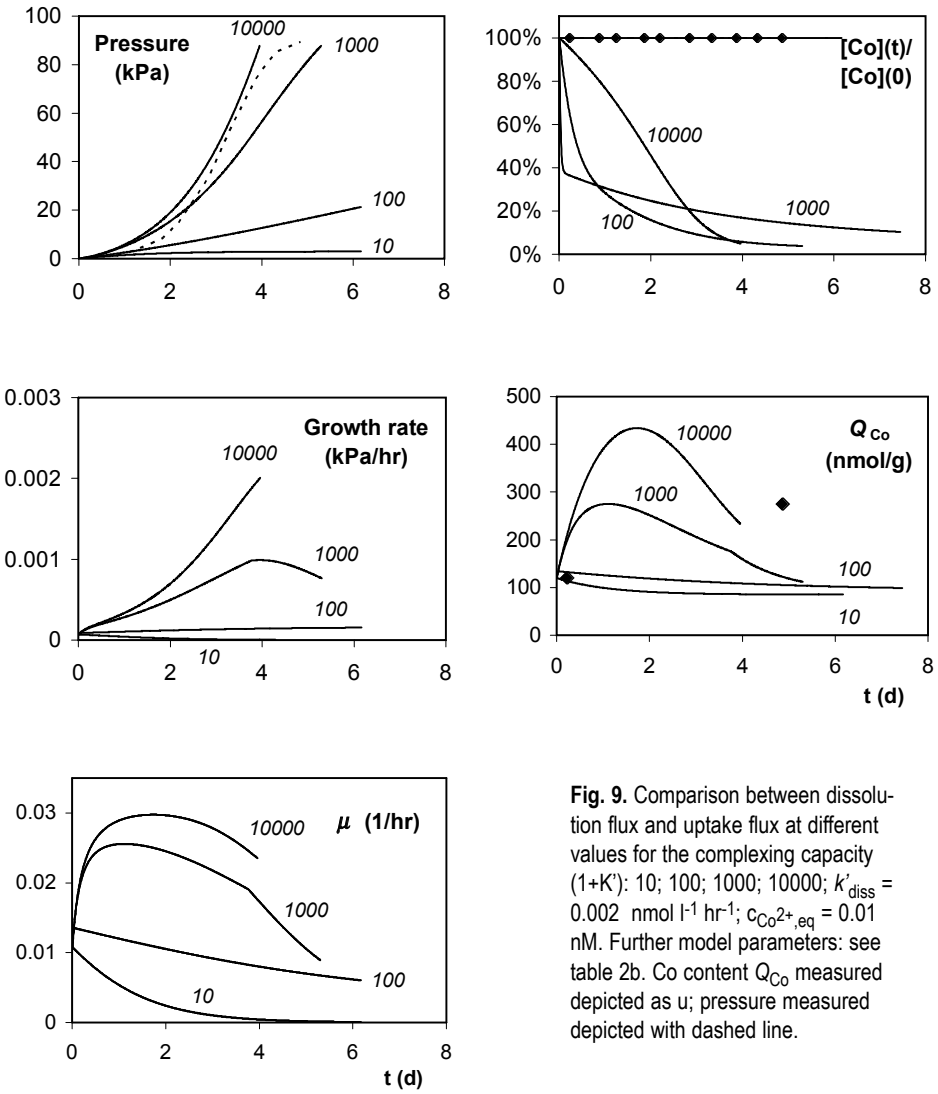

Fig. 9. Comparison between dissolution flux and uptake flux at different values for the complexing capacity (1+K'): 10; 100; 1000; 10000; k'_{diss} = 0.002 nmol l^{-1} hr^{-1}; $c_{Co^{2+},eq}$ = 0.01 nM. Further model parameters: see table 2b. Co content Q_{Co} measured depicted as u; pressure measured depicted with dashed line.

Fig. 9 (1+K') shows that metal complexation works as a metal ion buffer: at higher complexation capacity, limitation is less likely to occur because the dissolved Co concentration remains high throughout a large part of the test. Thus, the large amount of dissolved metal-sulfide complexes in the presence of sulfide (e.g. Al-Farawati & Van den Berg, 1999) has a positive effect on preventing dissolution rate limitation. For instance, for Co, in the presence of 1 mM S at pH = 7, the complexing capacity can be estimated to be of the order of 10^3 to 10^4, high enough to maintain a high dissolved metal concentration over a wide range of conditions. Secondly, these calculations confirm the findings that addition of ligands (in lower concentrations than sulfide) can alleviate metal limitations, as was shown previously for yeast extract as added ligand mixture (Gonzalez-Gil et al., 2003).

For all curves, the transition to dissolution rate limitation can be seen well from the curvature in the plots for the Co content, growth rate and growth rate constant. From this point on, these parameters decrease at a faster rate. This will be different if a constant amount of substrate per unit of cell weight is assumed, as for many cases in the literature for organic substrates (e.g.: Wick et al., 2001; Mulder et al., 2000). In those cases, after mass transfer limitation sets in, the growth rate remains constant.

Some previous experiments on Co and Ni effects in batch cell suspensions provide evidence for the absence of dissolution rate limitation (chapter 4). This is confirmed by the modeling results presented here. However, other data do suggest dissolution rate limitation. An example is Gonzalez-Gil et al., 1999a, who tested the effect of Co and Ni on a crushed anaerobic methanogenic sludge. They observed several phases in the rate of methanol consumption, which were probably due to different phases of growth rate limitation. After an initial exponential phase, a drop in the rate was observed, followed by linear increase of the growth rate with time. Interestingly, the first two phases are well described by the MMD model: during the first phase, dissolution rate is not limiting at all, whereas in the second phase, dissolution rate limitation sets in and the growth rate decreases, along with decreasing metal content. The model cannot describe the third phase: possibly, other mechanisms come into effect, such as changes in the relevant parameters as a result of adaptation.

Dissociation of dissolved metal complexes

Strong metal complexation can be present in anaerobic bioreactor media, leading to extremely low free metal concentrations. Therefore, it is worthwhile to consider whether the finite rate of dissociation of dissolved metal complexes can become limiting for biological uptake.

Let us consider for instance the medium with 5 μM Co, 200 μM EDTA and 1 mM Ca (all total concentrations) at pH = 7. This can be regarded as an extreme case with very low free metal concentration, but still considerable rates of biological uptake (chapter 6). The equilibrium of importance for the dissociation of CoEDTA is:

$$\mathrm{CoEDTA} + \mathrm{Ca}^{2+} \underset{k_a}{\overset{k_d}{\rightleftharpoons}} \mathrm{CaEDTA} + \mathrm{Co}^{2+} \qquad (7)$$

The rate constant for CoEDTA dissociation (k_d) can be calculated from the complex formation rate constant (k_a) and the equilibrium constants:

$$k_d = \frac{K_{CaEDTA}}{K_{CoEDTA}} k_a \quad (8)$$

The rates of formation and dissociation of the CaEDTA complex are fast compared to those of the CoEDTA complex; therefore, the formation rate constant of CoEDTA (k_a) is determined by the rate of loss of water from the inner hydration shell of Co^{2+} (Eigen&Wilkins,1965;Margerum etal, 1978):

$$k_a = k_{Co,\text{-}H_2O} K_{OS} \quad (9)$$

where is the inner-shell dehydration rate constant for Co^{2+} and K_{OS} is the stability constant for the outer-sphere complex.

By combination of eqs. (8) and (9) we can calculate the bulk CoEDTA dissociation rate R_{diss}, which is defined as:

$$R_{diss} = dc_{CoEDTA}/dt = k_d c_{CoEDTA} c_{Ca^{2+}} = \frac{K_{CaEDTA}}{K_{CoEDTA}} k_{Co,\text{-}H_2O} K_{OS} c_{CoEDTA} c_{Ca^{2+}} \quad (10)$$

The same type of reasoning applies for Ni and Fe.

Table 3. Comparison of orders of magnitude for dissociation and uptake rates for typical concentrations of 5 µM Co, 5 µM Ni and 50 µM Fe in presence of 200 µM EDTA, pH = 7 (K_{OS} taken to be 2 M^{-1} from Morel & Hering, 1993; $k_{\text{-}H2O}$ taken to be 2 * 10^6 s^{-1} for Co and 3 * 10^4 s^{-1} for Ni from Margerum et al., 1978).

Metal ion	Rate (nmol l^{-1} hr^{-1})		
	R_{uptake} (start of growth)	R_{uptake} (end of growth)	$R_{dissociation}$
Co^{2+}	3	30	2 * 10^8
Ni^{2+}	3	30	1 * 10^4
Fe^{2+}	10	100	4 * 10^{11}

In table 3, uptake rates (taken from chapter 6) are compared to the maximum dissociation rates. Dissociation is not limiting uptake, not even with varying metal-binding strength. The conditions discussed here are relatively extreme ones, in the sense that free metal ion concentrations are extremely low, while uptake rates are relatively high. In many other examples (e.g. sulfide, cysteine), free metal concentrations are higher while uptake is at a similar order of magnitude, so there is less reason for dissociation rate limitation.

Diffusion

In order to assess the importance of diffusion limitation, we have to compare the diffusion flux with the uptake flux. The magnitude of the maximum diffusion flux depends on the relative magnitudes of the radius of the microorganism or aggregate (r) and the steady-state diffusion layer thickness (δ):

$$J_{diff} = D_{Co^{2+}} c_{Co^{2+}} \left(\frac{1}{r} + \frac{1}{\delta} \right) \tag{11}$$

where is the diffusion coefficient of the free Co^{2+}. Eq. (11) converts to spherical diffusion for the case $r < \delta$, and to planar diffusion for the case $r > \delta$. Computed estimates of the maximum diffusion fluxes and uptake fluxes for typical values taken from experiments with EDTA (chapter 6) are shown in fig. 10. We assumed small cell aggregates (r = 3 μm), as was done in the model calculations and consistent with dispersions of individual cells, which are known to have such size (Gonzalez-Gil et al., 1999b). For Co, the uptake fluxes are still smaller than the calculated diffusion fluxes; for Ni however, uptake and diffusion fluxes are in the same order, implying that diffusion may be limiting. The cell aggregate size can affect the relationship between the uptake flux and the diffusion flux in two ways (fig. 11). First, the diffusion becomes less convergent with increasing aggregate size, the ratio between the spherical and the planar terms in eq. (11) becoming smaller. On top of that, with increasing aggregate size the effective surface area to volume ratio of the cell aggregate decreases, thus decreasing the uptake rate per unit cell mass. Overall, the chances of diffusion limitation increase with increasing cell aggregate size.

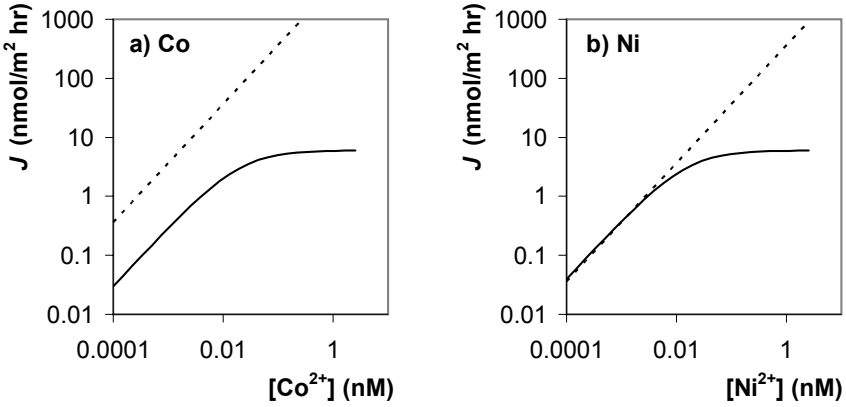

Fig. 10. Comparison between diffusion flux (- -) and uptake flux (—). a) Co; b) Ni. Model parameters used: $D(Ni^{2+}, Co^{2+}) = 10^{-9}$ m^2 s^{-1}; $\delta = 10^{-6}$ m; $c_{Co^{2+}} = 0.01$ nM; $c_{Ni^{2+}} = 1$ nM; $J_{max} = 60$ nmol g^{-1} hr^{-1}; $K_{JM,Co^{2+}} = 0.02$ nM; $K_{JM,Ni^{2+}} = 0.0015$ nM.

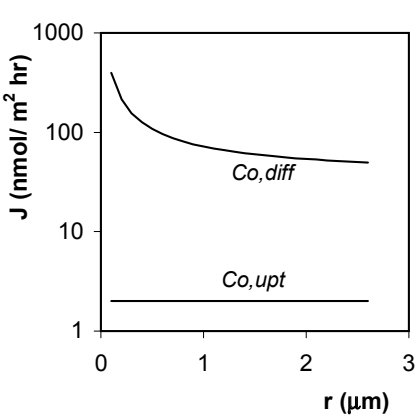

Fig. 11. Effect of radius on uptake flux and diffusion flux of Co. Model parameters used: $D(Co^{2+})$ = 10^{-9} m^2 s^{-1}; δ = 10^{-6} m; c_{Co}^{2+} = 0.01 nM; J_{max} = 60 nmol g^{-1} hr^{-1}; $K_{JM,Co}^{2+}$ = 0.02 nM.

In case of diffusion limitation, a Best type of equation (Best, 1955) for coupled uptake and diffusion is needed to describe the data:

$$J/J_{max} = \frac{(1+a+b)}{2b}\left\{1-\left[1-\frac{4b}{(1+a+b)^2}\right]^{\frac{1}{2}}\right\} \qquad (12)$$

where J is the overall flux, a is the normalized bioaffinity parameter ($a=K'_{JM,Co}/c_{Co}^{2+}$) and b is the limiting flux ratio, defined as the ratio of the maximum uptake flux over the maximum diffusion flux for a given free metal concentration ($J_{upt,max}/J_{diff}$).

The resulting growth rates under varying limiting diffusion fluxes are shown in fig. 12. Most parameters used were the same as in the case of the modelling of the effects of metal-sulfide dissolution rate limitation (table 2b). Initially, a fast change in Co content is observed, but the rate of change slows down until almost no change is observed at all. This region of changing cell quota can extend over a long period of time, enabling near-exponential growth over a wide range of diffusion fluxes. This is a big difference as compared with cases of constant cell quota, where diffusion limitation would automatically lead to a constant growth rate.

If diffusion is limiting, labile species will generally contribute to the uptake (Van Leeuwen, 1999). The strong metal-sulfide complexes are not expected to be labile, but other important inorganic complexes, such as metal carbonates, might be able to contribute to uptake under such conditions. Furthermore, dissolved complexes can contribute to uptake as long as dissociation in the bulk is sufficiently fast on the timescale of depletion as resulting from uptake.

In practice, the diffusion limitation might even be more important due to decreased effective diffusion coefficients in anaerobic biofilms, as was demonstrated by several authors. For instance, the effective diffusion coefficient (D_e) of Li^+ in anaerobic biofilms reported by Nilsson & Karlsson (1989) ranges from 22 to 33% of that in water. Similar findings were reported for diffusion coefficients in various relevant biofilms (Alphenaar et al., 1993; Lens et al., 2003; De Beer & Schramm, 1999).

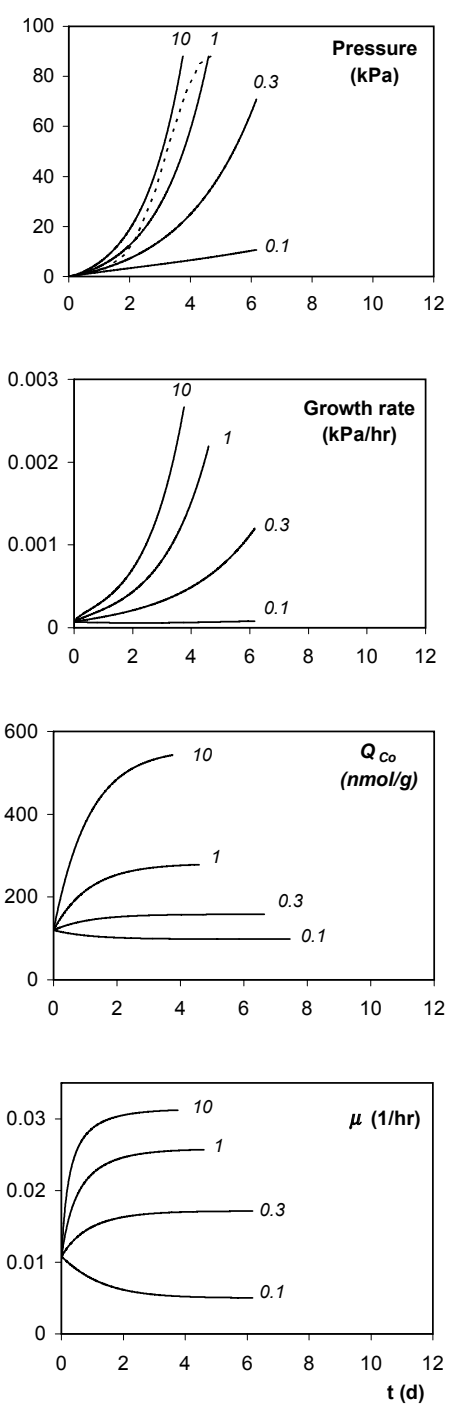

Fig. 12. Comparison between dissolution rate and uptake flux described using a Best equation using constant maximum surface flux J_{diss} : 0.1; 0.3; 1; 10 nmol m^{-2} hr^{-1}; (1+K') = 1000; $c_{Co^{2+},eq}$ = 0.01 nM. Further model parameters: see table 2b.

Implications for bioreactor practice

We conclude with some implications of the modelling for anaerobic bioreactor practice.

The high-affinity uptake characteristics for Co and Ni ensure efficient uptake even at very low free metal concentrations. The effects of competition between the two ions might complicate the uptake and growth rate limitation characteristics. It is not likely that the pertaining transporters provide the sole route of uptake: at higher metal concentrations, uptake via less specific transporters seems to come into play.

In typical bioreactor media, where metal concentrations are determined by their sulfide solubilities, Ni is not likely to be limiting, since its free ion concentration and uptake affinity are substantially higher than for Co. This is even more likely considering the fact that the usual background contamination with Ni is much higher than for Co.

For the eventual limitation of growth (and thus bioreactor performance), it is important to note the importance of the concentrations of the metals inside the cells instead of those in the external medium. In practice, this will lead to a long resistance to low external concentrations once the biomass is 'loaded' with essential nutrients (Zandvoort et al., 2004).

The microorganisms in the anaerobic bioreactors are usually caught within granular structures. This has a number of consequences for metal bioavailability.

Firstly, a great part of the granular matrix is formed by strongly metal-sorbing material, resulting in a considerable pool of metal stock in the granular matrix (e.g.: Zandvoort et al. 2003, 2004). On the one hand this matrix acts as a metal sink, immobilizing the bioavailable soluble metals into matrix-bound forms and rendering them less available for uptake. On the other hand, the matrix functions as a reservoir and slowly releases metal over longer periods of metal shortage, thus ensuring a steady metal supply to the microorganisms. Whether this balance works out in favour of the biomass or not, is highly dependent on the properties of the metal-binding matrix. In practice, several metals leach especially from the lower-binding strength fractions, while remaining relatively inert in more stable fractions (Zandvoort et al., 2004). Apart from release from the granular matrix, metal is also released by active excretion, or, more plausibly under metal-limited conditions, on death of biomass (or decay). The ensuing effects on the dissolved concentrations differ for various metal ions and influent concentrations (Zandvoort et al., 2002a,b and 2003).

Apart from its metal sorption characteristics, the physical structure of the granular biofilm matrix can be of great importance. The impact on the diffusion coefficients of the metal ions was already pointed out. Also, the dimensions of the biofilm have big impact on mass transfer conditions. Typically, the granules have a size of the order of a few mm (e.g. Alphenaar et al., 1993), and the aggregates inside are accordingly large. This means that diffusion from the medium towards the granular outer surface will definitely be planar (compare eq. (11)). Furthermore, the large size of the aggregates could even lead to mass transfer limitations such severe that part of the biomass is completely inactive (Alphenaar et al., 1993). A point of discussion is still the distinction between external and internal mass transfer limitation, and the importance of convection. A study on the importance of mass transfer in case of conversion of acetate (Gonzalez-Gil et al., 2001) led to the conclusion that mass transfer into the granule is mainly diffusive, convection being relatively

unimportant, in spite of the presence of gas bubbles and volcano-like structures (as mentioned by Henze & Harremoes, 1983). Finally, the granular structure is very heterogeneous in physicochemical parameters and microbial composition (e.g. Gonzalez-Gil et al., 2001), further complicating in mass transfer phenomena.

External hydrodynamic conditions alter with changing reactor conditions. In the UASB reactors, strong liquid flow is ensured, leading to efficient external mass transfer. The relation between the Solids Retention Time (SRT) and Hydraulic Retention Time (HRT) influences the effective metal load. Operation in case of a UASB reactor has the advantage of a high SRT over HRT ratio, thus ensuring an effective dosing of metal to the cells present in the solid phase.

For estimating effects on bioreactor performance, we can consider the reactor as a continuous system, where steady-state conditions prevail. Eventually, growth rate (which determines the reactor performance) can be estimated by the steady-state metal speciation. It seems likely that diffusion and depletion are the most important sources of limitation; dissolution could become limiting after ageing of precipitates. The use of complexing agents will probably be mostly effective on this level, since they provide a buffer of dissolved metal.

We could model possible effects of metals in an anaerobic bioreactor by assuming a constant dissolved metal concentration and limitation by mass transfer, which could be diffusion or dissolution. Taking into account the possibility of limitation by diffusion, the same picture arises as shown in fig. 12. Thus, metal contents will decrease until metal uptake and growth rate become constant. The cells are able to adapt to a broad range of metal concentrations, because of their flexibility in metal content.

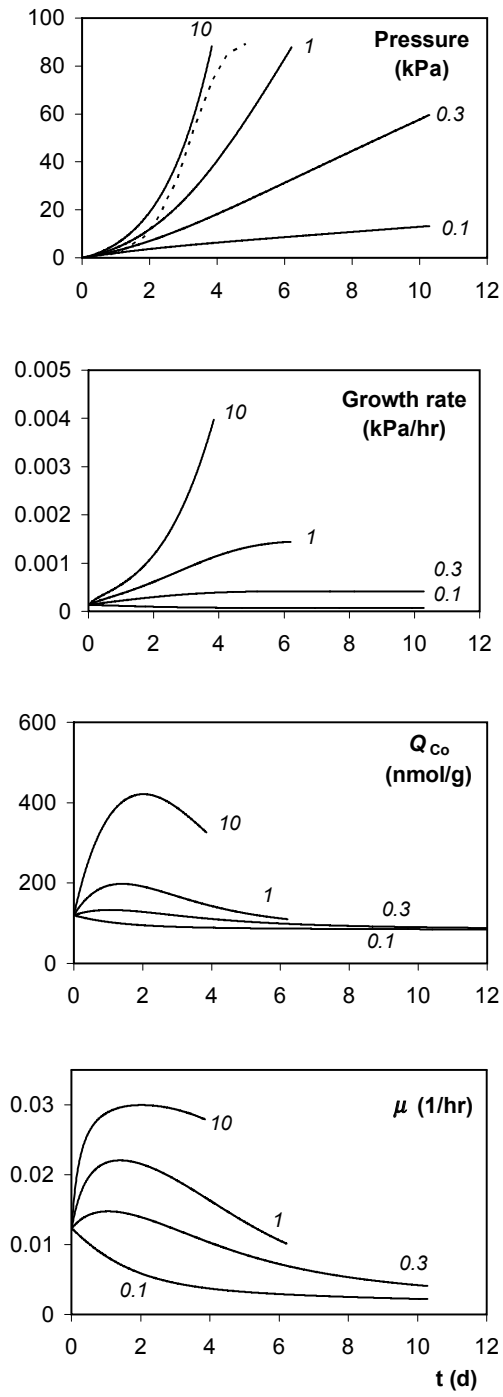

Fig. 13. Comparison between dissolution rate and uptake flux described using a Best equation using constant maximum volume dissolution rate r_{diss}: 0.1; 0.3; 1; 10 nmol l^{-1} hr^{-1}; (1+K') = 1000; $c_{Co^{2+},eq}$ = 0.01 nM. Further model parameters: see table 2b.

In case of metal dissolution rate limitation, a constant rate of supply of metal to the solution can be assumed. Hence, the limiting flux per unit surface area of cells will decrease with growth. This can be modelled using an equation similar to eq. (12), with the limiting flux ratio b varying over time with the biomass concentration x according to:

$$b(t) = b(0)\frac{x(t)}{x(0)} \tag{13}$$

The outcome (fig. 13) shows clear differences with the case of diffusion limitation (fig. 12). Due to the increasing biomass concentration and a constant supply flux, metal contents in all cases will eventually approach the minimum metal content. This leads to growth curves that are much less exponential than in case of diffusion limitation. From a certain dissolution rate onwards, the growth rate is constant.

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Summary, concluding remarks and outlook

INTRODUCTION

Anaerobic wastewater treatment is an efficient way of degrading numerous pollutants, such as methanol. A successful technique for this is the Upflow Anaerobic Sludge Bed (UASB) reactor. In this reactor, several groups of microorganisms degrade organic pollutants to methane, which can be used as biogas. In many of the bioconversions, metals are essential nutrients. For instance, Co and Ni are essential elements in the conversion of methanol to methane by methanogenic archaebacteria. Within the microorganisms, these elements are mainly needed in vitamin B_{12} and factor F_{430}, which catalyze the biochemical conversion. A lack of available Co and Ni will therefore lead to limitation of the bioconversion rate.

Generally, the availability of metals is greatly influenced by the different physicochemical forms (or *species*) in which they are present (as a whole referred to as *speciation*). Normally, free metal is more bioavailable than bound metal, but the exact relationship between speciation and bioavailability can be subtle and is the current subject of extensive research. In anaerobic wastewater a wide variety of binding and precipitation processes occur, which may affect biouptake. Until now, the cascade of chemical speciation, biouptake and growth effects of the metals was poorly understood. This rendered the strategy of dosing metal to the reactors very difficult: dosing too little results in limitation, but dosing too much is costly, could lead to toxicity for the microorganisms, and results in sludge with high heavy metal contents. Thus, the objective of this thesis was to quantify the dynamic relationships between speciation, biouptake and growth effects in anaerobic wastewater systems, focusing on the role of Co and Ni in the conversion of methanol to methane. The research was part of a multidisciplinary project at Wageningen University, in which partners from the Laboratories of Environmental Technology and Microbiology participated.

SPECIATION ANALYSIS

For analysis of Co and Ni in anaerobic wastewater systems, we need a technique that can cope with low metal concentrations and strongly binding agents, including sulfide and a variety of complexing and sorbing components. CLE-AdSV (Competitive Ligand Exchange - Adsorptive Stripping Voltammetry) was selected as a potentially suitable technique. It combines competitive ligand exchange (CLE), between a deliberately added ligand L_{ad} and the ligands L present in the sample solution, with electrochemical detection of the metal complex ML_{ad} following adsorptive accumulation onto the detector electrode surface. The ligand exchange step is the relevant one for the speciation analysis. Key parameters are the concentration of the added ligand and the stability of its complex with the target metal ion. The subsequent electrochemical measurement, with in-built preconcentration step, quantifies the amount of ML_{ad} complexes formed and provides adequate sensitivity for detection of low metal concentrations. In this thesis, some fundamental kinetic features of the technique are analysed and it is applied to anaerobic bioreactor media.

In chapter 2, the basic kinetic features of CLE-AdSV are analysed, regarding both the ligand exchange step, and the adsorptive accumulation step.

The ligand exchange step has important consequences with respect to the range of metal species contributing to the signal (also referred to as the detection window). In this chapter, the upper border of the thermodynamic detection window is defined by the requirement of equilibration with the added ligand L_{ad} before accumulation of the complex. Lability criteria for the different types of metal species are derived. Thus, it is demonstrated that any sample complexes that remain in solution after the CLE step generally are nonlabile and thus do not contribute to the signal. Any residual free metal however does contribute to the adsorptive accumulation process, but the magnitude of this effect will be practically zero because of the usually low concentration remaining after ligand exchange.

The adsorption of the surface-active complex ML_{ad} is demonstrated to be of a transient nature for only the first small fraction of the total accumulation time. For the major part of the adsorption period, the mass transport occurs via steady-state convective diffusion.

In chapter 3, AdSV measurements on anaerobic bioreactor or similar batch media are presented. The stripping voltammetric peaks for Co and Ni are well-behaved. Sulfide interference, in effect above certain concentration levels, is avoided by acidification and degassing, followed by pH normalisation. Linear calibrations are obtained for both Co and Ni down to about 1 nM.

Comparison of metal concentrations measured using AdSV with total metal concentrations measured using ICP-MS gives information about the fraction of dissolved metal in strongly bound form. In the matrices studied, up to 95% of the Co and Ni is present in strongly bound forms ($K_{MeL} \geq 2\text{-}3 * 10^{10}$ mol^{-1} l). In methanogenic media, no further binding was detected upon addition of Co and Ni. Presumably, the bound forms are the cobalt-containing vitamin B_{12} and the nickel-containing factor F_{430}, which are excreted by methanogenic archaebacteria. Effluent from sulfate reducing bioreactors however shows strong and extensive binding of added Co (c_L = 0.4 µM; $K_{CoL} \geq 5 * 10^9$ mol^{-1} l).

The effects of sulfide on Co and Ni speciation are further assessed in chapter 4.

Although free metal concentrations must be low due to metal sulfide precipitation, dissolved concentrations are 100 to 1000 times higher. This can be understood from the precipitation kinetics and the importance of dissolved metal sulfide complexes. The main dissolved metal sulfide species of Co and Ni under typical bioreactor conditions (pH = 7; $[S]_{total}$ = 1 mM) are $Co(SH)_2^0$ and $Ni(SH)_2^0$.

Overall, AdSV is a suitable technique for analysing dissolved Co and Ni speciation in anaerobic bioreactor media, dominated by strongly binding ligands. To summarize, Co and Ni speciation is determined by metal sulfide precipitation, dissolved metal sulfide complexes, and a group of other strong complexes, presumably vitamin B_{12} and factor F_{430}. Within actual bioreactors, sorption to solid matrix will probably be an extra factor of importance.

BIOLOGICAL EFFECTS

In chapters 4 to 7, biological effects of Co and Ni on methanogenesis are studied. The effects were studied in batch cultures, either of an enrichment culture of *Methanosarcina* sp., which was obtained from an anaerobic bioreactor (further referred to as the enrichment culture), or of a pure culture of the well-defined model organism *Methanosarcina barkeri*. Speciation was varied in different ways to study effects of precipitation and dissolved metal complexes.

In chapter 4, the effect of metal sulfide precipitation dynamics on the activity of the enrichment culture is investigated by varying total Co and Ni and total sulfide in systems containing metal sulfide precipitates. Methanogenic activity increases with increasing total metal or decreasing total sulfide. The stimulation of the methanogenic activity seems to be related to the free metal ion concentration. Calculations show that limitation by slow dissolution of the solid metal sulfide is unlikely, because calculated rates of dissolution are fast compared to rates of biouptake. This is confirmed by direct measurement of Co and Ni uptake by *Methanosarcina barkeri* in the presence of metal sulfide precipitates. Growth limitation therefore seems to be controlled by biouptake characteristics.

In chapter 5, yeast extract is shown to increase the bioavailability of Co and Ni, as concluded from the increase of the methanogenic activity of the enrichment culture. AdSV analysis demonstrates that Co and Ni form relatively strong organic complexes with yeast extract ($\text{Log } K_{NiYE} \geq 10$). The yeast extract is shown to increase the dissolved metal concentration in the presence of their sulfide precipitates. The growth stimulation observed could be due to the formation of dissolved bioavailable complexes, and/or to increased dissolution rates of metal sulfides.

Chapter 6 discusses the prevention of metal sulfide precipitation by adding EDTA. This enables a direct study into the relationship between the speciation and uptake of Co and Ni and its influence on cofactor content and methanogenesis from methanol by *Methanosarcina barkeri*. Separate growth limitations by Co and Ni and competition between Co and Ni uptake are demonstrated. Vitamin B_{12} and factor F_{430} content broadly follow the cell content of the metal involved, but at low metal contents the percentage of metal in the cofactor is distinctly lower.

A model consisting of Michaelis-Menten uptake kinetics and Droop growth limitation (further referred to as the Michaelis-Menten/Droop, or MMD model) successfully describes the data. Growth rate is determined by the metal content of the cells. Minimum Co and Ni contents $Q_{Co,min}$, $Q_{Ni,min}$ are found to be 85 and 75 nmol (g dry weight)$^{-1}$ respectively. The uptake is best described by competitive, high affinity/low capacity uptake of Co and Ni. Cell efflux of metals (presumably in the form of cofactors) is significant in a comprehensive description of the data.

In chapter 7, the applicability of the Michaelis-Menten/Droop (MMD) model described in chapter 6 is tested for different metal speciation conditions, and the effects of dissolution, dissociation and mass transfer are studied.

First, data on the effects of Co and Ni on the methanogenic activity of the enrichment culture in the presence of cysteine were obtained and analysed using the MMD model. Overall trends in Co and Ni uptake can be described well. Competition between uptake of Co and Ni is confirmed. The data provide information on the efficient uptake of vitamin B_{12}, most likely via specialized uptake routes.

The application of the model to uptake and growth in the presence of sulfide suggests different metal transport mechanisms depending on the prevailing metal speciation.

In most cases, the dissolution flux is not limiting, although in practical situations limitation may occur due to ageing of the precipitate and low specific surface area of the metal sulfide aggregates. The diffusion flux of free Ni is close to the biouptake flux, and therefore is a potential source of growth limitation. The dissociation of dissolved complexes was shown not to be limiting.

IMPLICATIONS FOR BIOREACTOR PRACTICE

The knowledge obtained provides a rational basis for some recommendations for metal dosing in practice. First we will summarize some relevant results.

Upon dosing, the speciation of the metals will be highly affected by sorption and complexation (chapter 3). In cases where the free metal ion concentration is controlled by precipitation equilibria, strong complexation acts as a dissolved metal buffer, preventing the systems against dissolution rate limitation or depletion limitation (chapter 7).

The characteristics of Co and Ni biouptake (high affinity, low capacity, competitive) have important consequences for the effects of dosing (chapter 6). Even very low free metal concentrations, as encountered in case of sulfide precipitation, can be sufficient to satisfy the biouptake flux requirements. Furthermore, overdosing of a single metal might not only result in toxicity of this metal, but might also cause limitation by another metal ion due to strong competition by the overdosed one. Generally, Co is more likely to be limiting than Ni. This is due to the lower sulfide solubility product, lower background concentration levels and lower biouptake affinity for Co.

The eventual biological effects are determined by the internalised metal concentration (chapter 6). Thus, a high metal content in the cells will provide the microorganisms with an effective buffer against external reduction in metal availability, and somewhat alleviates the metal requirements. However, if availability is low over sustained periods of time the internal metal stock will be depleted and external supply will become necessary.

Dynamic features within the granular micro-environment might be of importance (chapter 4, 7). Metal precipitation does not necessarily lead to bioconversion limitation: dissolution rates can be high enough to maintain sufficient bioavailability in the medium. Still, dissolution might become rate limiting, due to ageing of the precipitates. Dissociation kinetics of any type of dissolved metal complex does not limit metal uptake

and microbial growth, although complex contributions may be effective only at the level of buffering the free metal concentration in the bulk medium (prevention of depletion). Diffusion limitation might play a role, but can be minimized by forced convection in the liquid.

On the basis of the above knowledge, the following practical recommendations can be formulated:

- Free concentrations of Co and Ni should be maintained at values of minimally 0.01 nM to maintain reasonable uptake fluxes (chapter 6). Although these values are low, they are typical values in the presence of *ca*. 1 mM sulfide, an order of magnitude likely to be reached in anaerobic bioreactors. Total dissolved metal concentrations in these environments are much higher due to strong complexation (typical values: $[Me]_{total,dissolved}/[Me^{2+}]$=100-1000).
- Dosing excessive amounts of a single metal ion should be avoided. Not only does one run the risk of toxicity, but there is also a risk that uptake of another metal ion will become limiting due to competition (chapter 6).
- The minimum amount of Co and Ni needed by the cells is around 80 nmol/g dry weight (chapter 6). A (bioavailable) amount of 1 μmol of Co and Ni per g dry weight of cells will result in 90-95% of the maximum growth rate. Expressed as the amount of metal needed per amount of substrate converted, this comes down to 0.06 μmol metal per g COD methanol (assuming a cell yield of 0.6 g dry weight per 10 g COD).
- The metal ions should preferentially be dosed in complexed form. This prevents them from being immobilized by precipitation or sorption. The concentration and binding strength of the ligands used should be a compromise. One the one hand, the extent of metal binding should not be so strong that the free metal ion concentration is too drastically decreased. On the other hand, the overall metal binding should be sufficient to adequately increase the dissolved metal concentration, otherwise addition of the ligand does not have a significant effect. Even relatively strong ligands, such as EDTA, can maintain a sufficient metal uptake flux (chapter 6). Cofactors, such as vitamin B_{12} are taken up efficiently as such, and furthermore have the advantage of not being subject to precipitation or complexation (chapter 7). Another example of a ligand to be dosed is yeast extract (chapter 5). Whether or not it is profitable to use one or more of these ligands of course depends on their cost-effectiveness.
- Metal ions are best dosed continuously to avoid sorption and ageing of precipitates formed.

OUTLOOK

The information obtained provides a useful framework for understanding metal interactions and effects in anaerobic wastewater treatment systems. Still, many questions remain to be answered.

With respect to the metal speciation, metal sulfide precipitation and dissolution dynamics are very important, but difficult to quantify. Dissolved metal sulfide complexes are found to be of major importance, but there still is some debate about the exact values of their stability constants. Furthermore, metal sulfide precipitation and dissolution dynamics together with its influence on metal uptake and effects could very well vary with different operating conditions, such as dosing strategy of metals, ligands and sulfur sources.

AdSV analysis demonstrates the presence of Co and Ni complexes. It would be extremely useful to have direct information on the nature of these complexes. Furthermore, the interplay with the biology deserves further study: under what conditions are these compounds produced, and what is their role in metal bioavailability?

The MMD model provides a good description of the data, but warrants further refinement. The mechanism of uptake of Co and Ni, including the effects of their competition and the impact from other ions in the reactor medium, merits further investigation. Furthermore, it is worthwhile to characterize uptake and growth over wider metal concentration ranges, since the data suggest that various metal transporters play a role depending on the external metal concentration. The uptake and excretion of cofactors were shown to be important, but their exact characteristics are yet to be elucidated. It would also be interesting to see whether the relationship between growth responses and internal metal concentrations apply for other conversions of importance for anaerobic bioreactors. Finally, the model was only applied to well-defined batch cultures; it would be interesting to test its applicability to the real bioreactor system, and assess the effects of processes such as aggregation, sorption, etc.

The information obtained can be applied outside the scope of anaerobic wastewater treatment. In many natural anaerobic environments, processes like metal sulfide precipitation, dissolution and complexation in relation to bioavailability and eventual effects on biological activity are of great importance. It would be interesting to apply approaches used in this thesis to such a broader range of media.

Samenvatting, conclusies en perspectieven

INLEIDING

Anaërobe (=zuurstofloze) afvalwaterzuivering is een efficiënte manier om diverse soorten verontreinigingen, zoals methanol, af te breken. Een succesvolle techniek hiervoor is de *Upflow Anaerobic Sludge Bed* (UASB) reactor. In deze reactor breken verschillende groepen micro-organismen organische verontreinigingen af tot methaan, wat gebruikt kan worden als biogas. Metalen zijn essentiële voedingsstoffen in veel van de betrokken biologische omzettingen. Cobalt (Co) en nikkel (Ni) bijvoorbeeld zijn nodig voor de omzetting van methanol door methanogene (=methaanvormende) micro-organismen. Deze elementen zijn vooral nodig voor vitamine B_{12} en factor F_{430}, verbindingen die de biochemische omzetting katalyseren. Een tekort aan beschikbare Co en Ni zal daarom leiden tot een beperking van de biologische omzettingssnelheid.

In het algemeen wordt de beschikbaarheid van metalen voor een groot deel beïnvloed door de chemische vormen (of *species*) waarin ze voorkomen (als geheel *speciatie* genoemd). Normaliter is vrij metaal biologisch beter beschikbaar dan gebonden metaal, maar de exacte relatie tussen speciatie en biologische beschikbaarheid kan subtiel zijn en is dan ook het onderwerp van uitgebreid wetenschappelijk onderzoek. In anaërobe afvalwaterzuivering kunnen er allerlei bindings- en neerslagprocessen optreden, die de biologische beschikbaarheid beïnvloeden. De cascade van chemische speciatie, biologische opname en effecten op de groei was tot nu toe nog slecht begrepen. Dit maakte het moeilijk om metaal goed aan de reactoren te doseren: te lage dosering leidt tot tekorten, maar te hoge dosering is duur, kan schadelijk zijn voor de micro-organismen, en resulteert in slib met hoge gehaltes aan zware metalen. Het doel van dit proefschrift was dan ook het quantificeren van de dynamische relaties tussen speciatie, biologische opname en effecten op de groei in anaërobe afvalwaterzuiveringssystemen, toegespitst op de rol van Co and Ni in de omzetting van methanol in methaan. Het onderzoek maakte deel uit van een multidisciplinair project van Wageningen Universiteit, waaraan onderzoekers van de laboratoria voor Milieutechnologie en Microbiologie deelnamen.

SPECIATIE-ANALYSE

Voor de analyse van Co en Ni in anaërobe afvalwaterzuiveringssystemen is een techniek nodig die kan omgaan met lage metaalconcentraties en sterk metaal-bindende stoffen, waaronder sulfide en een variëteit aan andere verbindingen. CLE-AdSV (*Competitive Ligand Exchange - Adsorptive Stripping Voltammetry*, ofwel: *Competitieve Ligand Uitwisseling - Adsorptieve Strip Voltammetrie*) is geselecteerd als een mogelijk geschikte techniek. Hierbij vindt eerst uitwisseling plaats (ofwel competitive ligand exchange (CLE)) tussen een toegevoegde metaalbindende stof (ligand) L_{ad} en de liganden L die aanwezig zijn in de onderzochte oplossing. Daarna vindt adsorptieve accumulatie plaats van het metaalcomplex ML_{ad} aan het oppervlak van een electrode, waar het geadsorbeerde complex tenslotte electrochemisch gedetecteerd wordt. De ligand-uitwisselingsstap is de relevante voor de speciatieanalyse. Sleutelparameters hierbij zijn de concentratie van het toegevoegde ligand en de stabiliteit van het complex dat dit vormt met het onderzochte

metaalion. De daarna volgende electrochemische detectiemethode, inclusief preconcentratiestap, quantificeert de gevormde hoeveelheid ML_{ad}-complexen, en verschaft adequate gevoeligheid om lage metaalconcentraties te detecteren.

In hoofdstuk 2 worden de onderliggende kinetische eigenschappen van CLE-AdSV geanalyseerd met betrekking tot de ligand-uitwisselingsstap en de adsorptieve accumulatiestap. De ligand-uitwisselingsstap heeft belangrijke consequenties met betrekking tot de klasse van metaalspecies die bijdragen aan het signaal (ook wel *detection window* genoemd). In dit hoofdstuk is de bovengrens van het thermodynamische *detection window* gedefinieerd door de voorwaarde van evenwichtsinstelling met het toegevoegde ligand L_{ad} voor accumulatie van het complex. Daarnaast zijn labiliteitscriteria voor de verschillende soorten metaal species zijn afgeleid. Op deze manier is gedemonstreerd dat alle complexen die aanwezig zijn in de meetoplossing na de ligand-uitwisselingsstap over het algemeen niet labiel zijn, en dus niet bijdragen aan het meetsignaal. Overgebleven vrij metaal echter zal wel bijdragen aan het meetsignaal, maar de grootte van dit effect zal verwaarloosbaar zijn vanwege de gewoonlijk lage concentratie van het vrij metaal na ligand-uitwisseling. In hoofdstuk 2 wordt verder aangetoond dat de adsorptie van het oppervlakte-actieve complex ML_{ad} slechts in een kleine fractie van de totale accumulatietijd een *transient* karakter heeft. Voor het overgrote deel van de adsorptietijd vindt er massatransport plaats via *steady-state* convectieve diffusie.

In hoofdstuk 3 worden CLE-AdSV-metingen van anaërobe afvalwaterzuiveringsreactoren en soortgelijke groeimedia gepresenteerd. Co en Ni zijn hierin goed te meten tot een concentratieniveau van *ca*. 1 nM. Interferentie door sulfide, die optreedt boven een bepaalde concentratie, kan worden vermeden door aanzuren en ontgassen, gevolgd door normalisatie van de pH. Vergelijking van de metaalconcentraties, gemeten door middel van AdSV, met totale metaalconcentraties, gemeten door middel van ICP-MS, geeft informatie over de hoeveelheid opgelost metaal in sterk gebonden vorm. In de matrices die bestudeerd zijn, komt tot 95% van de Co en Ni voor in sterk gebonden vorm ($K_{MeL} \geq 2\text{-}3 * 10^{10}$ mol^{-1} l). In media waarin methaanproducerende micro-organismen voorkwamen, werd geen verdere binding gedetecteerd waneer Co en Ni werden toegevoegd. Het is aannemelijk dat de gebonden vormen het cobalt-bevattende vitamine B_{12} en het nikkel-bevattende factor F_{430} zijn, die uitgescheiden worden door methaanvormende archaebacteriën. Effluent uit bioreactoren waarin sulfaatreducerende bacteriën voorkomen echter, vertoont wel sterke en aanzienlijke binding van toegevoegd Co (c_L = 0.4 µM; $K_{CoL} \geq 5 * 10^9$ mol^{-1} l).

De effecten van sulfide op de speciatie van Co en Ni zijn verder onderzocht in hoofdstuk 4. Hoewel de vrije metaalconcentraties laag moeten zijn tengevolge van neerslagvorming met sulfide, worden totale opgeloste concentraties gevonden die 100 tot 1000 keer hoger zijn. Dit komt door de kinetiek van de neerslagvorming en door het belang van opgeloste metaalsulfide-complexen. De belangrijkste opgeloste metaalsulfide-species van Co en Ni onder typische bioreactorcondities (pH = 7; $[S]_{total}$ = 1 mM) zijn $Co(SH)_2^0$ en $Ni(SH)_2^0$.

Concluderend kan worden gesteld dat AdSV een geschikte techniek is om de speciatie van opgelost Co en Ni in anaërobe bioreactor media te bestuderen, die wordt gedomineerd

door sterk bindende liganden. De speciatie van Co and Ni wordt bepaald door metaalsulfide-neerslagvorming, opgeloste metaalsulfide-complexen, en een groep andere sterk gebonden complexen, waarvan het aannemelijk is dat deze wordt gevormd door vitamine B_{12} and factor F_{430}. In bioreactoren zal binding aan de vaste matrix waarschijnlijk een extra factor van belang zijn.

BIOLOGISCHE EFFECTEN

In hoofdstuk 4 tot en met 7 zijn effecten van Co en Ni op de methaanvorming door micro-organismen (ofwel methanogene activiteit) bestudeerd. De effecten zijn bestudeerd in *batch* culturen, hetzij van een ophopingsculture van *Methanosarcina* sp., die verkregen was uit een anaërobe afvalwaterzuiveringsreactor (in het vervolg aangeduid als de ophopingsculture), dan wel met een pure culture van het goed gedefinieerde modelorganisme *Methanosarcina barkeri*. De speciatie is op verschillende manieren gevariëerd om effecten van neerslagvorming en opgeloste metaalcomplexen te bestuderen.

In hoofdstuk 4 is het effect van metaalsulfide-neerslagvormingsdynamiek op de activiteit van de ophopingsculture onderzocht door de totale concentratie van Co, Ni en sulfide te variëren in systemen die metaalsulfide-neerslagen bevatten. De methanogene activiteit neemt toe met toenemende concentratie aan totaal metaal en met afnemende concentratie aan totaal sulfide. De stimulering van de methanogene activiteit lijkt te zijn gerelateerd aan de concentratie van het vrije metaalion. Berekeningen tonen aan dat beperking van de biologische activiteit door langzame oplossnelheid van het vaste metaalsulfide niet waarschijnlijk is, aangezien de berekende oplossnelheden groot zijn ten opzichte van de bio-opname-snelheden. Dit wordt bevestigd door directe meting van de opname van Co en Ni door *Methanosarcina barkeri* in de aanwezigheid van metaalsulfide-neerslagen. Beperking van de groei lijkt dan ook te worden bepaald door bio-opname-karakteristieken.

In hoofdstuk 5 wordt gedemonstreerd dat gistextract in staat is de biobeschikbaarheid van Co en Ni te vergroten, zoals kan worden geconcludeerd uit de toename van de methanogene activiteit van de ophopingsculture. AdSV-analyse laat zien dat Co en Ni vrij sterke organische complexen vormen met gistextract (Log $K_{NiYE} \geq 10$). Het gistextract blijkt verder de opgeloste metaalconcentratie in aanwezigheid van metaalsulfide-neerslagen te verhogen. De waargenomen stimulering van de groei zou veroorzaakt kunnen zijn door de vorming van opgeloste complexen en/of door een toename van de oplossnelheden van metaalsulfiden.

Hoofdstuk 6 bespreekt een experiment waarin neerslagvorming met sulfide werd voorkomen door toevoeging van de complexvormer EDTA. Dit maakt een directe studie mogelijk van de speciatie en opname van Co en Ni en de invloed daarvan op de gehaltes aan vitamine B_{12} en factor F_{430} en de methanogene activiteit op methanol door *Methanosarcina barkeri*. Het kon worden aangetoond dat tekorten aan Co en Ni in de cel de groei onafhankelijk van elkaar beïnvloeden en dat de twee metalen bij opname competitie vertonen. Gehaltes aan vitamine B_{12} en factor F_{430} volgen grofweg de totaalgehaltes van de betrokken metalen, maar bij lage metaalgehaltes is het percentage metaal in de cofactor aanzienlijk lager. Een model dat bestaat uit Michaelis-Menten-opnamekinetiek

en Droop-groeibeperking (in het vervolg aangeduid als het Michaelis-Menten/Droop-, ofwel MMD-model) beschrijft de gegevens succesvol. De groeisnelheid wordt bepaald door het metaalgehalte van de cellen. Minimum Co- en Ni-gehaltes $Q_{Co,min}$, $Q_{Ni,min}$ zijn respectievelijk 85 en 75 nmol per g drooggewicht. De opname kan het beste worden beschreven als transport met competitie tussen Co en Ni en hoge affiniteit en lage capaciteit voor deze metaalionen. Uitscheiding van de metalen, naar we aannemen in de vorm van cofactoren, is significant voor een complete beschrijving de gegevens.

In hoofdstuk 7 is de toepasbaarheid van het Michaelis-Menten/Droop(MMD)-model voor verschillende omstandigheden wat betreft de metaalspeciatie getest. Tevens zijn hierin de effecten van het oplossen vanuit neerslagen, dissociatie vanuit opgeloste complexen en massatransport bestudeerd. In de eerste plaats zijn gegevens van de effecten van Co and Ni op de methanogene activiteit van de ophopingsculture in de aanwezigheid van cysteine als zwavelbron verkregen en geanalyseerd met het MMD-model. Onder deze omstandigheden vindt geen neerslagvorming van Co en Ni plaats. De trends in de opname van Co en Ni kunnen goed beschreven worden. Competitie tussen Co- en Ni-opname wordt bevestigd. De gegevens geven aanwijzingen voor efficiënte opname van vitamine B_{12}, waarschijnlijk via gespecialiseerde opnameroutes. De toepassing van het model op opname en groei in de aanwezigheid van sulfide suggereert verschillende mechanismen van metaaltransport, afhankelijk van de heersende omstandigheden van metaal speciatie. Uit modelberekeningen blijkt dat in de meeste gevallen de oplosflux niet beperkend is, ofschoon in praktische situaties beperking op kan treden door veroudering van de neerslagen en een laag specifiek oppervlak van de metaalsulfide-aggregaten. De diffusieflux van het vrije Ni ligt in de buurt van de bio-opname-flux, en is dan ook een mogelijke bron van groeibeperking. De dissociatie vanuit opgeloste metaalcomplexen blijkt niet beperkend te zijn.

IMPLICATIES VOOR DE BIOREACTOR-PRAKTIJK

De kennis die is verkregen verschaft een rationele basis voor een aantal aanbevelingen voor metaaldosering in de praktijk. Allereerst zullen we een aantal relevante resultaten samenvatten.

Wanneer metalen gedoseerd worden, zal de speciatie van de metalen in hoge mate beïnvloed worden door sorptie en complexering (hoofdstuk 3). In gevallen waarin de concentratie van het vrije metaalion bepaald wordt door neerslagevenwichten, treedt sterke complexering op als een buffer voor opgelost metaal, en beschermt de systemen zo tegen beperking door trage oplossnelheid of depletie (hoofdstuk 7).

De karakteristieken van de bio-opname van Co en Ni (hoge affiniteit, lage capaciteit, competitie) hebben belangrijke consequenties voor de effecten van dosering (hoofdstuk 6). Zelfs zeer lage concentraties van vrij metaal, zoals kan voorkomen in geval van metaalsulfide-neerslagen, kunnen voldoende zijn voor de behoeftes van de bioopname-flux. Verder kan overdosering van een enkel metaal niet alleen leiden tot toxiciteit van het desbetreffende metaal, maar kan dit ook leiden tot beperking door een ander metaalion tengevolge van de sterke competitie door het metaal dat overgedoseerd werd. Over het algemeen is

beperking door Co waarschijnlijker dan door Ni. Dit komt door het lagere neerslagproduct met sulfide, de lagere achtergrondconcentratie en de lagere bio-opname-affiniteit voor Co.

De uiteindelijke biologische effecten worden bepaald door de geïnternaliseerde metaalconcentratie (hoofdstuk 6). Om die reden zal een hoog metaalgehalte micro-organismen een buffer verschaffen tegen een externe reductie van de metaalbeschikbaarheid, en op die manier metaalbehoeftes enigzins verlichten. Echter, als de beschikbaarheid laag is gedurende langere tijd, zal de interne metaalvoorrad uitgeput raken en zal externe aanvoer nodig zijn.

Dynamische eigenschappen binnen de micro-omgeving van de korrels (granules), waarin de micro-organismen in de bioreactor praktijk voorkomen, kunnen van belang zijn (hoofdstuk 4, 7). Neerslagvorming leidt niet noodzakelijkerwijs tot beperking van de bioconversie: oplossnelheden kunnen hoog genoeg zijn om een voldoende biobeschikbaarheid in het medium te houden. Evengoed kan oplossnelheid soms snelheidsbepalend worden door veroudering van de neerslagen. Kinetiek van de dissociatie van wat voor opgelost metaalcomplex dan ook beperkt metaalopname en microbiële groei niet, ofschoon bijdragen door het complex effectief zouden kunnen zijn in het bulk medium (voorkoming van depletie). Beperking door diffusie zou een rol kunnen spelen, maar kan geminimaliseerd worden door convectie in de vloeistof op gang te brengen.

Op basis van de hierboven beschreven kennis kunnen de volgende praktische aanbevelingen worden geformuleerd:

- Concentraties Co and Ni in de vorm van vrij metaal moeten op waarden worden gehouden van minimaal 0.01 nM om redelijke opnamefluxen te behouden (hoofdstuk 6). Ofschoon deze waarden laag zijn, zijn ze vrij normaal in de aanwezigheid van *ca*. 1 mM sulfide, een orde van grootte die gemakkelijk kan worden bereikt in anaërobe bioreactoren. Totale concentraties aan opgelost metaal in deze omgevingen zijn veel hoger door sterke complexering (typische waarden: $[Me]_{totaal,opgelost}/[Me^{2+}] = 100\text{-}1000$).
- Het doseren van excessieve hoeveelheden van een enkel metaalion moet vermeden worden. Men loopt niet alleen het risico van toxiciteit, maar er is ook een risico dat de opname van een ander metaalion beperkend wordt door competitie (hoofdstuk 6).
- De minimale hoeveelheid Co and Ni die nodig is voor de cellen ligt rond de 80 nmol per g drooggewicht (hoofdstuk 6). Een (biobeschikbare) hoeveelheid van 1 µmol Co en Ni per g drooggewicht cellen zal resulteren in 90-95% van de maximale groeisnelheid. Uitgedrukt als de hoeveelheid metaal nodig per hoeveelheid omgezet substraat, komt dit neer op 0.06 µmol metaal per g COD methanol (waarbij we een celopbrengst aannemen van 0.6 g drooggewicht per 10 g COD).
- De metaalionen moeten bij voorkeur gedoseerd worden in gecomplexeerde vorm. Dit voorkomt dat ze worden geïmmobilizeerd door neerslagvorming of sorptie. De concentratie en bindingssterkte van de liganden moet een compromis zijn. Aan de ene kant moet de mate van metaalbinding niet zo groot zijn dat de concentratie van het vrije metaal drastisch verlaagd wordt. Aan de andere kant moet de metaalbinding voldoende zijn om de opgeloste metaalconcentratie adequaat te doen toenemen, want anders heeft het toevoegen van het ligand geen significant effect. Zelfs relatief sterke liganden, zoals EDTA, kunnen een afdoende metaal-opnameflux onderhouden (hoofdstuk 6). Cofactoren, zoals vitamine

B_{12}, worden als geheel efficiënt opgenomen, en hebben verder het voordeel dat ze niet worden neergeslagen of gebonden (hoofdstuk 7). Een ander voorbeeld van een ligand dat kan worden gedoseerd is gistextract (hoofdstuk 5). Uiteraard spelen bij de vraag of en welke liganden moeten worden toegediend ook de kosten een rol.

- Metaalionen kunnen het beste continu worden gedoseerd om sorptie en veroudering van de neerslagen te voorkomen.

PERSPECTIEVEN

De informatie die verkregen is, verschaft een bruikbare basis om metaal-interacties en effecten in anaërobe afvalwaterzuiverings systemen te begrijpen. Maar er zijn nog altijd vele vragen te beantwoorden.

Met betrekking tot de metaalspeciatie, zijn metaalsulfide-neerslagvorming en oplosdynamiek erg belangrijk, maar moeilijk te quantificeren. Opgeloste metaalsulfide-complexen blijken van groot belang, maar er is nog onduidelijkheid over hun stabiliteitsconstanten. Verder kunnen metaalsulfide-precipitatie- en -oplosdynamiek, en hun effecten op metaalopname en effecten, sterk variëren met de verschillende omstandigheden waaronder de reactor werkt. Hierbij kan men denken aan de doseringstrategie van de metalen, liganden en zwavelbronnen.

AdSV analyse toont de aanwezigheid aan van Co en Ni complexen aan. Het zou erg nuttig zijn om directe informatie te hebben over de aard van deze complexen. Verder verdient de relatie met de biologie verdere aandacht: onder welke omstandigheden worden deze verbindingen geproduceerd, en wat is hun rol voor de biobeschikbaarheid van de metalen?

Het MMD-model verschaft een goede beschrijving van de gegevens, maar vereist verdere verfijning. Het mechanisme van de opname van Co en Ni, inclusief de effecten van hun competitie en de invloed van andere ionen in het reactor medium, verdient verder onderzoek. Verder is het de moeite waard om de opname en groei te karakteriseren over grotere ordes van metaal concentraties, want de gegevens suggereren dat verschillende metaaltransportsystemen een rol spelen afhankelijk van de externe metaalconcentratie. Het is aangetoond dat de opname en uitscheiding van cofactoren belangrijk is, maar hun exacte karakteristieken moeten nog worden opgehelderd. Het zou interessant zijn om te zien of de relatie tussen effecten op de groei en interne metaalconcentraties ook van toepassing zijn voor andere omzettingen die van belang zijn in anaërobe bioreactoren. Tenslotte is het model alleen toegepast op goed gedefinieerde *batch* cultures; het zou interessant zijn om de toepasbaarheid op echte bioreactorsystemen te testen, en de effecten te onderzoeken van processen zoals aggregatie, sorptie, etc.

De informatie die is verkregen kan ook worden toegepast buiten de het gebied van anaërobe afvalwaterzuivering. In veel anaërobe omgevingen zijn processen zoals het neerslaan en oplossen van metaalsulfiden, en complexering in relatie tot de biologische beschikbaarheid en uiteindelijke effecten op de biologische activiteit van groot belang. Het zou interessant zijn om de aanpak die gebruikt is in dit proefschrift ook toe te passen binnen deze bredere context.

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CURRICULUM VITAE

Stefan Jansen was born in Heemstede on January 25, 1975. In 1993 he went to study Molecular Sciences at Wageningen University. For his first graduation thesis, he did an experimental study on the adsorption of humic acid onto iron oxide at the Laboratory of Physical Chemistry and Colloid Science, under supervision of dr. Luuk Koopal and dr. Maarten van der Wielen. His second thesis was performed at the Laboratory of Soil Science, where he worked on a model for the above mentioned adsorption process under supervision of dr. Jeroen Filius and prof. Willem van Riemsdijk. He spent an internship at the Swiss Federal Institute for Environmental Science and Technology (EAWAG). Here he studied Ni speciation in freshwater and groundwater under supervision of prof. Laura Sigg and dr. HanBin Xue, and with supervision from Wageningen by prof. Herman van Leeuwen. His final graduation thesis was on the anaerobic degradation of chloroaromatic compounds by *Desulfitobacterium dehalogenans*, under supervision of dr. Bram van de Pas and prof. Fons Stams at the Laboratory of Microbiology. After graduating as an environmental chemist in Wageningen in 1998, he obtained a grant from the Institute for Encouragement of Innovation through Science and Technique in Flanders (IWT) to study the uptake of cadmium and zinc by carp at the Laboratory for Ecophysiology and Toxicology in Antwerp. In 2000 he started with the PhD project at the Laboratory of Physical Chemistry and Colloid Science from which the results are presented in this thesis. The work was performed within a multidisciplinary project involving partners from the Laboratories of Microbiology and Environmental Technology. Apart from doing research, he followed courses within the Graduation School SENSE, took part in the PhD-council of this Graduation School and assisted several practical courses.

At this moment the author works as a postdoc at the Max Planck Institute for Marine Microbiology, where he performs *in situ* studies into biogeochemical processes in Waddensea sediment.

DANKWOORD

De micro-organismen zijn inmiddels genoeg in het zonnetje gezet, tijd voor een aantal ‘hogere’ organismen, die bij dit onderzoek betrokken waren.

Allereerst mijn co-promotor Herman van Leeuwen. Van zijn kritische blik en kennis van zaken heb ik enorm veel geleerd. Daarnaast maken zijn inzet, enthousiasme, gevoel voor humor, betrokkenheid en flexibiliteit hem een enorm prettig persoon om mee samen te werken.

Meer in het algemeen heb ik genoten van mijn tijd op de leerstoelgroep Fysische Chemie en Kolloïdkunde. Dat ik hierbij geen namen noem, moet gezien worden als een groot compliment aan iedereen. De sfeer op de vakgroep is er namelijk één waarin iedereen met elkaar omgaat, en er een grote sociale en wetenschappelijk betrokkenheid is. Dat heb ik bijzonder gewaardeerd, en ik wil hun allemaal er hartelijk voor bedanken.

Een andere ‘community’ waar ik deel van uitmaakte, werd gevormd door het zware-metalen-project. An important starting-point for this was previous research by Graciela Gonzalez-Gil. She has taken part in the project for one and a half year as a postdoc. The positive effect of this cannot be underestimated. I want to thank her very much for the pleasant cooperation. Wie beweert dat ik met twee maten heb gemeten, heeft in ieder geval in één opzicht gelijk als daarmee Marcel Zandvoort en Bo Jiang bedoeld worden, de andere twee OIO’s binnen het zware-metalen-project. It is extremely pleasant to have people nearby with whom it is possible to discuss your work profoundly, and who -on the other hand- look at it from a different perspective. Marcel, ik vond het leuk om regelmatig bij je op milieutechnologie langs te komen voor langere of kortere gesprekken of samenwerking rond de reactoren. Bo, thanks for all the cooperations, help and exchange of ideas at microbiology. Het algehele project stond eerst onder leiding van Look Hulshoff-Pol en later van Piet Lens, die er mede verantwoordelijk voor zijn dat het zo goed gefunctioneerd heeft. De interesse en kritische blik van de diverse begeleiders (waaronder Gatze Lettinga en Fons Stams), Ronald Mulder (Paques) en de andere leden van de begeleidingscommissie zorgden bovendien regelmatig voor frisse feedback, wat ik erg op prijs heb gesteld.

Af en toe was ik meer elders te vinden dan op Fysko: in die tijden zagen de mensen mij steeds weer met een kratje over het universiteitsplein zeulen. Het zou kunnen dat ik dan op weg was van of naar een prachtig gebouw bovenop de berg, waar het Laboratorium voor Microbiologie zich bevindt. Een fijne plek om te gast te zijn, met goede sfeer en organisatie van het lab, en enorm hulpvaardige en vriendelijke mensen. Allen hartelijk bedankt! Het had ook gekund dat ik met dat kratje op weg was naar de zevende verdieping van een minder mooi gebouw op diezelfde berg, waar we de leerstoelgroep Milieutechnologie vinden. Daar borrelden de reactoren van Marcel, onder de deskundige handen van Roy Geerts en later Jarno Gieteling. Daar kon ik ook langsgaan om even te brainstormen met Eric Hullebusch en, aan het einde, Svetlana Golubnic. En tenslotte waren er ook hier vele andere hulpvaardige en vriendelijke mensen.

Bij het meten hebben diverse mensen mij geholpen. Allereerst was er natuurlijk mijn kamergenoot Wim Threels. Een prettige en gemoedelijke kamergenoot en een handig en onverstoorbaar meter. Daarnaast heb ik het genoegen gehad Fraukje Steffen te begeleiden

in het kader van haar opleiding Laboratoriumtechniek. Ik vond dat erg leuk om te doen, en heb er zelf ook veel van geleerd. In het laatste stadium van mijn onderzoek kreeg ik nog ondersteuning van Susan Buykx, aan wie het voorbereiden van uitgebreide experimenten goed was toevertrouwd, en met wie het daarnaast ook bijzonder prettig samenwerken was.

Regelmatig kwam ik met wat rekjes monsters bij de mensen van het Centraal Lab van Bodemkunde, waar ik met name met Peter veel contact heb gehad. Het is prettig een dergelijk goed uitgerust lab zo dichtbij te hebben, en het goede contact zorgde er nog eens extra voor dat ik er graag langsging.

Apart from the vast community in Wageningen, I had the pleasure to have contact with some very nice people from places elsewhere too. Paulo Pinheiro visited Fysko a couple of times, during which he was a very nice partner for scientific discussion and a good game of squash. Thanks a lot too for the hospitality in Faro too! Another regular guest was Raewyn Town. I am still amazed about how accurately and quickly she could read and correct manuscripts, for which I cannot thank her enough. Also I have to mention her friendliness and interest. At the beginning of my PhD I had the pleasure to visit the lab of Stan van den Berg at the Laboratory for Oceanography in Liverpool for two weeks. I want to thank him for the nice time over there.

Dan is er natuurlijk ook nog leven buiten het lab! Ik ben bijzonder blij met alle interesse, betrokkenheid, en plezier die ik aan mijn familie en vrienden te danken heb. De korte lengte van dit stukje staat dan ook in schril contrast met de grote betekenis die zij hebben. Speciale dank ook voor Joost en Frederiek voor de hulp met de vormgeving.

Julia heeft van iedereen ongetwijfeld het meest meegeleefd met alle facetten van de afgelopen tijd. Ik ben enorm blij dat zij er is!

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Cover:
Bottles containing anaerobic medium showing different appearances of metal sulfide precipitates.
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