

Cobalt toxicity in anaerobic granular sludge: influence of chemical speciation

Jan Bartacek · Fernando G. Feroso ·
Amalia M. Baldó-Urrutia · Eric D. van Hullebusch ·
Piet N. L. Lens

Received: 24 March 2008 / Accepted: 30 July 2008 / Published online: 22 August 2008
© The Author(s) 2008. This article is published with open access at Springerlink.com

Abstract The influence of cobalt speciation on the toxicity of cobalt to methylotrophic methanogenesis in anaerobic granular sludge was investigated. The cobalt speciation was studied with three different media that contained varying concentrations of complexing ligands [carbonates, phosphates and ethylenediaminetetraacetic acid (EDTA)]. Three fractions (nominal added, dissolved and free) of cobalt were determined in the liquid media and were correlated with data from batch toxicity experiments. The average concentration of cobalt that was required for 50% inhibition of methanogenic activity (IC_{50}) for free Co^{2+} in the three sets of measurements was 13 $\mu\text{mol/L}$ with a standard deviation of 22% and a similarity of 72% between the data obtained in the three different media for the range of cobalt concentrations investigated. The standard deviation of the IC_{50} for the other two fractions was much higher, i.e. 85 and 144% for the added cobalt and dissolved cobalt, respectively, and the similarity was almost 0% for both fractions. Complexation (and precipitation) with EDTA, phosphates and carbonates was shown to decrease the toxicity of cobalt

on methylotrophic methanogenesis. The free cobalt concentration is proposed to be the key parameter to correlate with cobalt toxicity. Thus, the toxicity of cobalt to granular sludge can be estimated based on the equilibrium-free cobalt concentration.

Keywords Trace metals · Cobalt toxicity · Chemical speciation · Methanogenic granular sludge · Donnan membrane technique

Introduction

Trace metals play a key role in the anaerobic conversion of methanol to methane in upflow anaerobic sludge bed (UASB) reactors [25]. Cobalt, nickel, iron, copper, zinc and manganese are considered as the most important trace metals for methanogens [9, 12, 32, 36]. Especially cobalt was many times reported as a very important micronutrient, as the cobalt-containing cofactors (vitamin B_{12}) play a key role in the methylotrophic methanogenic pathway [10, 11, 47]. It has often been reported as a reason for failure of lab-scale reactors [10, 46] and as a limiting factor in full-scale UASB reactors [47]. Trace metals are dosed in excess in practice in industrial wastewater treatment plants to overcome problems associated with the lack of micronutrients. Although this method is effective, it is a waste of natural resources and it results in an unnecessary release of heavy metals in the environment, which leads to an increase of the operation costs [5]. Moreover, dosing trace metals at a too high concentration can cause inhibition of the methanogenic process [33]. Although heavy metal toxicity in UASB reactors has been studied abundantly, reported values of toxic concentrations vary considerably between different authors [7, 22, 26]. This is due to the fact that

J. Bartacek · F. G. Feroso · A. M. Baldó-Urrutia ·
P. N. L. Lens (✉)
Sub-department of Environmental Technology,
Wageningen University, Biotechnion, Bomenweg 2,
P. O. Box 8129, 6700 EV Wageningen, The Netherlands
e-mail: piet.lens@wur.nl

E. D. van Hullebusch
Laboratoire Géomatériaux et Géologie de l'Ingénieur (G2I),
Université Paris-Est, EA4119, 5, Bd Descartes,
Champs sur Marne, 77454 Marne La Vallée Cedex 2, France

P. N. L. Lens
Pollution Prevention and Control Core, UNESCO-IHE,
P. O. Box 3015, 2601 DA Delft, The Netherlands

most authors report the total cobalt concentration in the liquid phase, not considering cobalt speciation. Therefore, the observed toxic concentrations are affected by differences in medium composition, which causes changes in metal speciation [45].

The free metal ion has been most often used as the base of models describing metal uptake by microorganisms or higher organisms [16], such as the free ion activity model (FIAM) and the biotic ligand model [15]. Although the free metal concentration strongly depends on precipitation [19], sorption [41] and speciation [43], the free metal ion is the most important chemical species for bacterial uptake [15] and therefore is also responsible for metal toxicity [13]. The presence of organic complexing ligands such as citrate (that decrease the free metal concentration) clearly influences the cobalt and nickel toxicity to bacteria, as is the case with *Pseudomonas aeruginosa* [6] or *Bacillus subtilis* [23]. These toxicity models were, however, established and verified for natural waters and higher organisms such as freshwater algae *Chlorella kesslerii* [15], urchin larvae [27] or mussels (*Mytilus galloprovincialis*) [2]. Thus far, this type of models has never been applied to the anaerobic consortia present in biofilms or granules from wastewater treatment reactors. Most of the work confirming the FIAM has been performed in relatively simple matrixes (e.g. surface water), with biomass in suspension (e.g. algae [16]). No studies have aimed on a liquid phase matrix of a relatively high salinity and with a biofilm grown in granules as present in UASB reactors. It is therefore interesting to verify if the FIAM can be applied to such a complicated system with many possible chemical–physical processes and restricted metal transport.

The present study focused on cobalt speciation and its influence on the specific methanogenic activity of granular sludge. Cobalt was chosen as a model for essential trace metals for methanogenesis. The borders between its nutritional requirement [3, 46] and toxicity is very narrow; although so far no toxic concentrations have been reported for anaerobic granular sludge. Therefore, the present study aimed to determine cobalt toxicity data for methanol fed anaerobic granular sludge based on changes in specific methanogenic activity (SMA) upon exposure to various cobalt concentrations. The influence of cobalt speciation was evaluated as well by adding various concentrations of complexing ligands [ethylenediaminetetraacetic acid (EDTA), phosphate and carbonate] to the medium. The effect of phosphate and carbonate was studied because these inorganic ligands are widely present in methanogenic reactors and their concentrations may vary substantially in practice. The effect of Co-EDTA²⁻ addition is also of practical importance, as it is a potential candidate for cobalt dosing to UASB reactors, despite it is not present in normal bioreactors. EDTA has often been reported to keep metals in

solution and thus bioavailable [14]. However, it has also been shown that EDTA, as complexing agent, can substantially decrease heavy metal toxicity [13]. The Donnan Membrane Technique (DMT) experiments were performed in the presence of granular sludge to ensure metal speciation in the presence of natural ligands such as extracellular polymers.

Materials and methods

Medium composition

To evaluate the role of the free cobalt (Co²⁺) ion in toxicity, three sets of measurements of the SMA with different basal medium composition were performed (Table 1). Table 2 lists the chemical composition of the medium, prepared with ultra-pure water (resistivity of 18 MΩ·cm, Millipore Corporation, Billerica, MA, USA) as the dilution water. Although EDTA is usually not present in the UASB

Table 1 Sets of experiments used in the present study

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

^a Co:EDTA molar ratio of 1:1.5

Table 2 Composition of the basal medium used in the three measurement series

Macronutrients	Sets I and II		Set III	
	mg/L	mmol/L	mg/L	mmol/L
NH ₄ Cl	280	5.24	280	5.24
K ₂ HPO ₄	250	1.44	25	0.144
MgSO ₄ ·7H ₂ O	100	0.406	100	0.406
CaCl ₂ ·2H ₂ O	10	0.068	14.7	0.100
NaHCO ₃	2,520	30	–	–
MOPS (pH buffer)	–	–	2,091	10
Micronutrients	mg/L	μmol/L	mg/L	μmol/L
CuCl ₂ ·H ₂ O	0.862	5	0.862	5
ZnCl ₂	0.681	5	0.681	5
MnCl ₂ ·4H ₂ O	0.988	5	0.988	5
NiCl ₂ ·6H ₂ O	1.173	5	1.173	5
(NH ₄) ₆ Mo ₇ O ₂₄ ·4H ₂ O	0.888	5	0.888	5
Na ₂ SeO ₃ ·5H ₂ O	1.321	5	1.321	5
Na ₂ WO ₄ ·2H ₂ O	1.649	5	1.649	5
FeCl ₂ ·4H ₂ O	9.937	50	9.937	50

– not supplied

reactor influent, this ligand was chosen as an example of an extremely strong, but poorly degradable ligand [31]. The other compounds (chloride, phosphate and carbonate) were chosen as weak ligands naturally present in the UASB reactors. Cobalt chloride was used as the cobalt source in sets I and III. Cobalt-EDTA (prepared from CoCl₂ and Na₂H₂EDTA; molar ratio 1:1.5) was used in set II. Carbonates and phosphates were added to the medium in sets I and II. The pH of the medium was set to 7.0 with sodium bicarbonate (sets I and II) or with MOPS (3-(*N*-Morpholino)-propanesulfonic acid) (set III). The end pH values in activity tests in set III (6.4–7.0) were slightly lower than in the other sets (6.7–7.3) which was caused by differences in the buffering capacity of the media (due to manipulating the complexation ligands concentrations). It should be noted that the free cobalt concentration is affected insignificantly (±5%—confirmed by Visual MINTEQ Ver. 2.51, KTH Research Project Database, Stockholm, Sweden) in this range of pH changes neither is the methanogenic activity [28].

Source of biomass

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

Specific maximum methanogenic activity tests

The SMA with methanol as the substrate was determined in duplicate at 30 (±2) °C using on-line gas production measurements as described by Zandvoort et al. [46]. Granular sludge specimens (approximately 1 g wet wt) were transferred to 120 mL serum bottles containing 50 mL basal medium with 4 g chemical oxygen demand (COD)/L of methanol, along with different cobalt concentrations. The SMA data were plotted in a rate versus time curve, using moving average trend lines with an interval of 15 data points.

SMA inhibition

Inhibition of specific methanogenic activity (*I*) is defined as the decrease in SMA under certain cobalt concentration (SMA_{*i*}) in comparison to the SMA under optimal conditions (SMA_{max}):

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

In order to obtain the main characteristics of the inhibition curves (SMA_{max}, IC₅₀), the Hormesis model was used to fit the experimental data. The parameters of the curve were calculated in accordance with Schabenberger et al. [34]:

$$E[y|x] = \delta + \frac{\alpha - \delta + \gamma x}{1 + \theta \exp[\beta \ln(x)]} \tag{2}$$

where E[y|x] represents the average response at dosage, β, γ and θ are parameters and x is the metal concentration; α was considered as a constant that gives the SMA value when x goes to zero; δ was considered as a constant that gives the SMA value at x going to infinity (∞); γ is responsible for the Hormesis effect, and β and θ are responsible for the slope of increase and decrease of the curve, respectively. The parameters were calculated using nonlinear regression in Microsoft Office Excel® (Thames Valley Park, Reading Berkshire, UK).

Statistics

The obtained toxicity data were related to the cobalt concentration. For each set, a single toxicity curve for each cobalt fraction (nominal added, dissolved, free) was plotted. To decide whether the three curves obtained for a certain fraction are similar among all three sets of experiments, the *F* test was used. The method was based on comparison of a full model (three curves for three sets) with a reduced model (one common curve for all three sets). The null hypothesis (H₀) that all the data points obtained for a certain cobalt fraction in all the three experimental sets can be fitted with one common curve was compared to the alternate hypothesis (H₁) that at least one curve deviates:

$$H_0 : \beta_I = \beta_{II} = \beta_{III} = \beta_r \quad \text{and} \quad \gamma_I = \gamma_{II} = \gamma_{III} = \gamma_r \tag{3}$$

$$\text{and} \quad \theta_I = \theta_{II} = \theta_{III} = \theta_r$$

$$H_1 : H_0 \text{ is not valid.} \tag{4}$$

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

Dissolved cobalt concentration

The dissolved cobalt concentration was measured in filtrated (pore size of 0.45 μm) samples taken from the system (granular sludge—liquid medium) after 4 days of incubation (no substrate added, 30 (±2) °C, anaerobic

conditions, shaking at 75 rpm). This incubation time is required to reach chemical equilibrium [30], which was proved experimentally (data not shown).

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

The free cobalt (Co^{2+}) concentrations related to particular cobalt concentrations in the given system (measurement sets I, II, and III) were measured under the same conditions as in the SMA test, but without substrate (Fig. 1). The free cobalt concentration was determined directly in the granular sludge/medium mixture under anaerobic conditions at $30 (\pm 2)^\circ\text{C}$ using the DMT, as described by Temminghoff et al. [37] (Fig. 2). The DMT has been most often used for measurement of the free species of various metals in synthetic media [21, 37, 44], surface water [20], soil solutions [19] and manure [32].

Prior to the DMT measurements, the granular sludge/medium mixture was shaken (75 rpm) for 4 days to reach equilibrium. The donor solution had the same composition as the medium used for activity tests (Table 2). As the

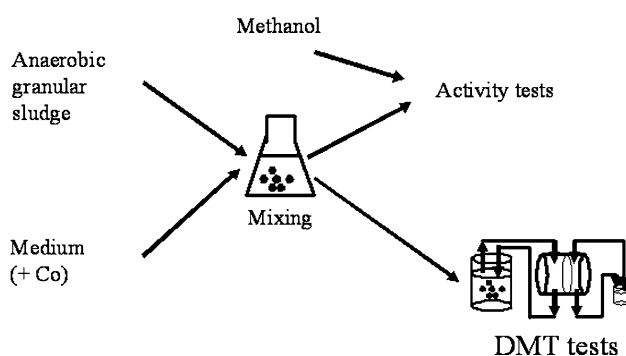


Fig. 1 Experimental design used in the present study to link the specific methanogenic activity measurement to solid phase and liquid phase speciation *DMT* Donnan membrane technique

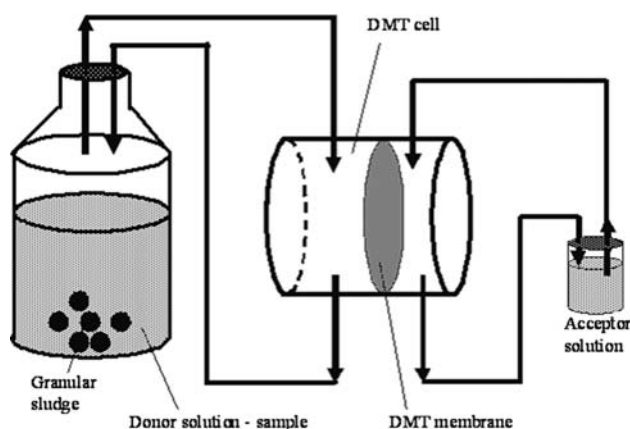


Fig. 2 Experimental set-up of the Donnan membrane technique (*DMT*) to measure the free metal concentration

DMT technique requires equilibrium conditions, no substrate was added to the medium to avoid methanogenic activity during the *DMT* test. The acceptor solution (before the start of experiments) contained 1 mmol/L CaCl_2 and 10 mmol/L MOPS as pH buffer.

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

When the Donnan equilibrium is reached, samples are taken from both the donor and the acceptor. By measuring potassium, a correction can be made for salt concentration differences. This correction is based on the theory of the Donnan equilibrium, which implies that the charge corrected ratios of all cationic activities in the solutions on both sides of the membranes are equal [17]:

$$\left(\frac{C_{\text{don},i}}{C_{\text{acc},i}}\right)^{1/z_i} = \left(\frac{C_{\text{don},j}}{C_{\text{acc},j}}\right)^{1/z_j} \quad (5)$$

where $C_{\text{don},i}$ and $C_{\text{acc},i}$ mean concentration of the targeted cation in donor and acceptor solution, respectively, and z_i is its charge. The $C_{\text{don},j}$, $C_{\text{acc},j}$ and z_j are the same quantities for the reference cation. Potassium is chosen to correct for differences in ionic strength, because it hardly forms any complexes, and thus exists almost completely as free ions [43].

Chemical analyses

The cobalt concentration was determined with inductively coupled plasma with optical emission spectrometry (Varian, Palo Alto, CA, USA) as described by Zandvoort et al. [46]. The total suspended solids and VSS concentrations were determined according to [1]. All chemicals were of analytical or biological grade and purchased from Merck (Darmstadt, Germany).

Results

Validation of *DMT*

To assess the accuracy of the *DMT* for free cobalt concentration measurements, three experiments in a synthetic medium (ultra-pure water, CaCl_2 1 mmol/L, MOPS 10 mmol/L) with three different ligands (chloride, carbonate and citrate) were performed. The results were compared with the data calculated by equilibrium modeling software (Visual MINTEQ). The experimental results were

Table 3 Validation of Donnan membrane technique (DMT) for free cobalt (Co²⁺) ion to theoretical calculations

	Ligand present		
	Cl ⁻	CO ₃ ²⁻	Citrate
Total cobalt concentration (μmol/L)	5.02	3.94	5.35
Measured Co ²⁺ concentration (μmol/L)	4.68	3.09	1.60
Calculated Co ²⁺ concentration (μmol/L)	5.00	2.95	1.66

in a good agreement (maximum 6% deviation) with the calculated data (Table 3). Therefore, DMT was chosen to be applicable for the free cobalt concentration measurements in the matrixes under investigation.

Influence of cobalt on methanogenic activity

Methanol (0.2 g COD in each bottle) was completely transformed to methane in most cases (Fig. 3). Incomplete methanol to methane conversion occurred only when higher amounts of cobalt were added (almost 100% inhibition): above 2,000 μmol/L for sets I and II and above 200 μmol/L for set III.

Cobalt limitation occurred when cobalt was added at sub-optimum amounts (Table 4). The observed decrease of the SMA at suboptimal cobalt concentrations were 39% (5 μmol/L cobalt addition), 18% (5 μmol/L cobalt addition), and 58% (0 μmol/L cobalt addition) in sets I, II, and III, respectively, in comparison with the SMA_{max}.

There is a difference between the SMA_{max} in sets I and II [2.28 and 2.06 g COD/(g VSS day), respectively] on the one hand, and set III [0.60 g COD/(g VSS day)] on the other hand (Fig. 3b). This difference was accompanied by lower end pH values in set III (6.5–7.0), which was probably caused by the different pH buffering system required for the experimental setup (Table 2). The difference in SMA_{max} could be also caused by the storage of the granular sludge between performing the set III experiments and sets I and II experiments.

The toxicity curves showed different shapes depending on the media composition in the three sets and differences between IC₅₀ values were large (Table 5, Fig. 4a). The highest IC₅₀ (478 μmol/L in set II) was almost seven times higher than the lowest one (71 μmol/L in set III). Moreover, the *P* value of the statistical test was almost zero (Table 5) and therefore the hypothesis that all three

Fig. 3 Methane production per bottle (a) and specific methanogenic activity (SMA) (b) for various cobalt concentrations in the experiments of sets I through III. The curves show methane production and activities with the following cobalt concentrations (in μmol/L); (sets I and II) a 5, b 75, c 100, d 300, e 600, f 1,200, g 2,000, h 3,200, i 5,000, and (set III) j 5, k 25, l 50, m 75, n 100, o 200, p 500, q 1,000

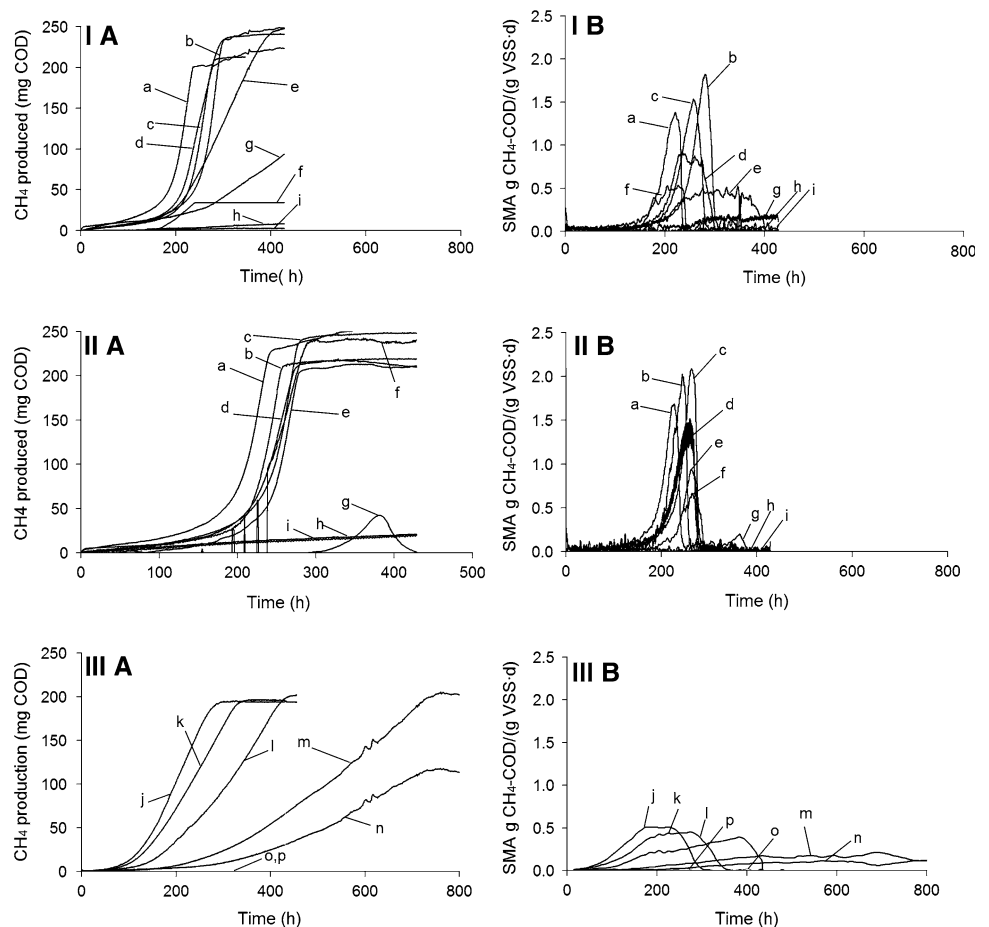


Table 4 Inhibition values obtained at different cobalt concentrations

Addition of cobalt ($\mu\text{mol/L}$)	Inhibition of SMA		
	Set I (%)	Set II (%)	Set III (%)
0	–	–	58
5	39	18	15
25	–	–	24
50	–	–	36
75	20	2	34
100	33	–2	65
200	–	–	79
300	60	33	–
500	–	–	99
600	77	54	–
1,000	–	–	96
1,200	77	70	–
2,000	87	90	–
3,000	94	98	–
5,000	96	100	–
SMA_{max} [g $\text{CH}_4\text{-COD}/(\text{g VSS day})$]	2.28	2.06	0.60

– not determined

SMA specific methanogenic activity, SMA_{max} specific methanogenic activity at optimal cobalt concentration, VSS volatile suspended solids, COD chemical oxygen demand

data sets can be fitted with one common curve was rejected. The applied cobalt concentration affected also the time when the maximal SMA occurred (t_{max}). The t_{max} was similar (220–260 h) in most of the experiments with Co-EDTA^{2-} and it was slightly increasing with higher CoCl_2 addition in set I (from 200 to 350 h). In contrast, a steep increase of t_{max} was observed in set III (from 200 to 600 h).

Table 5 The maximal specific methanogenic activity and IC_{50} values (concentration of cobalt that was required for 50% inhibition of methanogenic activity) obtained from the batch-experiments. The measured data are supplemented with standard deviation of IC_{50} between the three sets (for each cobalt fraction) and the probability that the three toxicity curves are identical

Set	IC_{50} ($\mu\text{mol/L}$)	Average IC_{50} ($\mu\text{mol/L}$)	Standard deviation IC_{50} (%)	P value of the statistical test
Nominal added cobalt				
I (CoCl_2 + carbonate and phosphate)	186	245	85	<0.001
II (CoEDTA^{2-})	478			
III (CoCl_2)	71			
Dissolved cobalt				
I (CoCl_2 + carbonate and phosphate)	36	166	144	<0.001
II (CoEDTA^{2-})	442			
III (CoCl_2)	19			
Free cobalt				
I (CoCl_2 + carbonate and phosphate)	15	13	22	0.716
II (CoEDTA^{2-})	14			
III (CoCl_2)	10			

Dissolved cobalt and toxicity

The toxicity curves obtained for dissolved cobalt also varied significantly among different measurement sets (Fig. 4b, Table 5). While the IC_{50} values for sets I and III were similar (IC_{50} of 36 and 19 $\mu\text{mol/L}$, respectively), the IC_{50} for set II (Co-EDTA) was more than ten times higher (442 $\mu\text{mol/L}$). The IC_{50} value for set II obtained for dissolved cobalt was as high as the IC_{50} for the nominal cobalt addition in set II (478 $\mu\text{mol/L}$). The P value of the statistical test was almost zero (Table 5) and therefore the hypothesis that all three data sets can be fitted with one common curve was rejected.

Figure 5 shows the differences in the cobalt distribution between the solid and liquid phase at steady state within the three systems as measured during the DMT experiments. Indeed, almost all (more than 90%) cobalt was present in the liquid phase when it was added as Co-EDTA (set II—except the cobalt concentration of 100 $\mu\text{mol/L}$). In contrast, a much lower amount of cobalt stayed in the liquid phase when it was supplied as CoCl_2 . If carbonate and phosphate were present in the system in high concentrations (set I), approximately 70% of the cobalt precipitated (or sorbed) and only approximately 30% of it remained in the liquid phase. Even more (90–100%) cobalt precipitated/sorbed at lower amounts of cobalt added (5 and 75 $\mu\text{mol/L}$). When phosphates and carbonates were not present in the system (set III), a substantial part of the cobalt was also present in the solid phase. In contrast with set I, the distribution between the solid and liquid phase in set III depended on the amount cobalt added (Fig. 5). Figure 6 shows that the equilibrium concentration in the bulk solution upon finishing an activity test was equal to that measured after a DMT experiment.

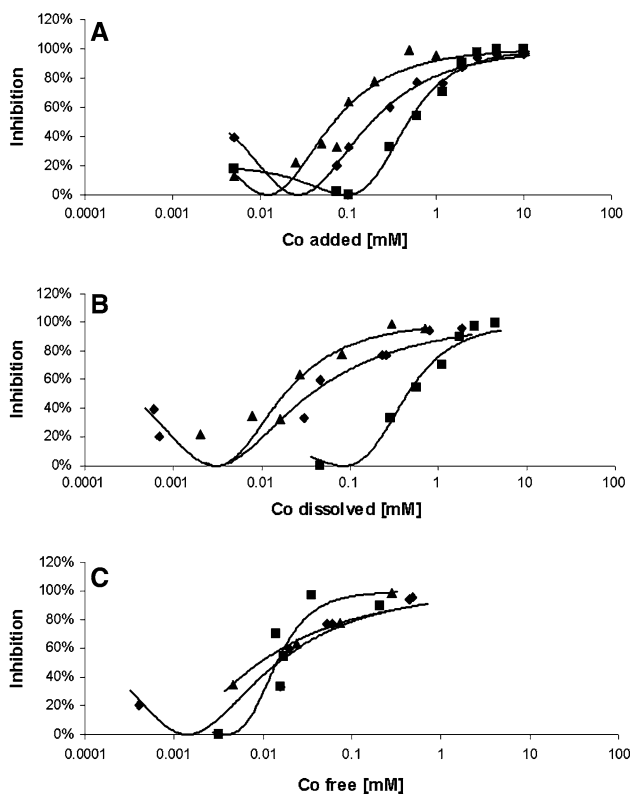


Fig. 4 Specific methanogenic activity (SMA) inhibition with various total cobalt additions (a), dissolved concentrations (b) and free cobalt (Co^{2+}) concentrations (c). Cobalt was added in the form of CoCl_2 —set I (filled diamond), Cobalt bound to ethylenediaminetetraacetate—set II (filled square), and CoCl_2 without the presence of carbonates and with low concentration of phosphates—set III (filled triangle). The curves were calculated using Eqs 1 and 2 and nonlinear regression

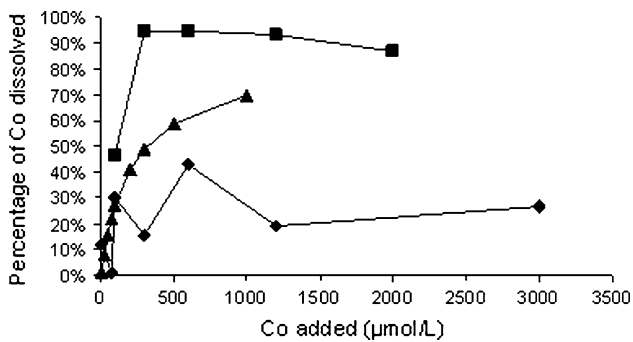


Fig. 5 The amount of cobalt (in %) that was present in the solution after addition of cobalt in various concentrations in sets I (filled diamond), II (filled square), and III (filled triangle)

Free metal content and toxicity

The inhibition curves are very similar for the three systems when the free cobalt concentration is plotted versus inhibition (Fig. 4c). The IC_{50} values for all three systems ranged from 10 to 15 $\mu\text{mol/L}$, with a standard deviation of

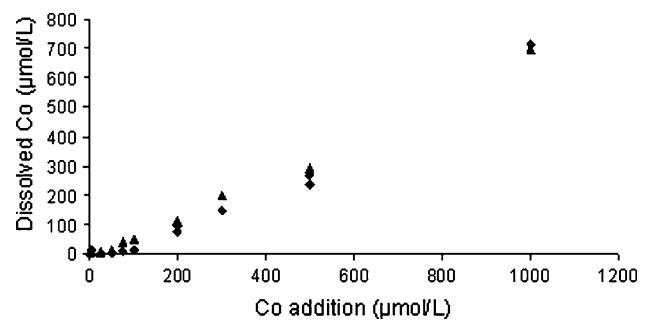


Fig. 6 Dissolved cobalt concentrations in set III obtained at equilibrium after activity tests (filled diamond) and after Donnan membrane technique (DMT) measurements (filled triangle)

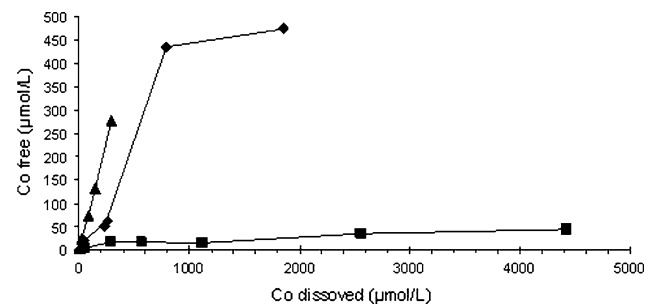


Fig. 7 Free cobalt concentrations in sets I (filled diamond), II (filled square), and III (filled triangle) at various dissolved concentrations

22% (Table 5). The P value of the statistical test was 0.72 (Table 5), confirming the hypothesis that all three data sets can be fitted with one common curve.

Figure 7 shows free cobalt concentrations at various dissolved cobalt concentrations in the three sets. There was a steep increase in the free cobalt concentration with increasing dissolved cobalt concentrations in set III and a slightly slower increase in set I. The free cobalt concentration reached typically 30–50, 10 and 90% of the total dissolved cobalt concentration in sets I, II and III, respectively.

Discussion

Role of speciation in cobalt toxicity

The present study shows that the toxicity of cobalt cannot be evaluated solely based on the total cobalt concentration in the liquid phase, but requires also the determination of the amount of cobalt precipitated/sorbed in methanogenic granular sludge and the speciation of cobalt present in the bulk solution (Fig. 4c). Moreover, the results suggest that cobalt toxicity can be assessed based on the free cobalt concentration. The latter statement is only valid when all important conditions such as pH and concentration of

competing metals (namely calcium or magnesium [15]) are kept constant. Although not only the free cobalt species can be transported into a microbial cell (e.g. vitamin B₁₂ [8], Co-citrate [23] and siderophores [45] are directly taken up by the methanogenic cell), similar IC₅₀ values for all three measurement sets (standard deviation of only 22%) were obtained when taking only the free cobalt species (Co²⁺) concentration into account (Table 5). This is due to the fact that when equilibrium conditions are established, metal toxicity can be predicted based on any metal species [45].

Although the IC₅₀ values for the dissolved cobalt fraction obtained for sets I and III are similar, the value from set II (Co-EDTA) is more than ten times higher (Table 5, Fig. 4b). It means that although the presence of ligands (EDTA, but also phosphates and carbonates) keeps cobalt in solution (Fig. 7), the bioavailability of cobalt is decreased. These results are in agreement with the conclusions of Chen et al. [6], who observed a significant decrease of cobalt toxicity to *Pseudomonas aeruginosa* in the presence of citrate and carbonate, indicating a decrease of the metal bioavailability of cobalt due to complexation. Several authors also reported EDTA to decrease nickel and cadmium toxicity to nitrificants [18] or copper, nickel and zinc toxicity to *Thiothrix* sp. [35]. Similarly, Kungolos [24] demonstrated a decrease of bioavailability (and therefore also toxicity) of heavy metals in the presence of increasing amounts of humic acids. For dissolved cobalt, the IC₅₀ values reported in the present study (Table 5) increased with the stability of the complexes formed, which increases (approximately at pH 7) in the following order: CoCO₃ (aqueous) (pK 4.28) < CoHCO₃⁺ (pK 12.22) < CoHPO₄ (aqueous) (pK 15.43) < CoEDTA²⁻ (pK 18.16) (Visual MINTEQ thermodynamic database).

Bhattacharya et al. [4] studied cobalt toxicity to anaerobic sludge in semi-continuous reactors under different experimental conditions: suspended enrichment culture, different medium composition (higher concentrations of inorganic salts) and different substrates (acetate and glucose). They observed an approximately 50 times higher IC₅₀ value for the free cobalt ion (approximately 750 and 950 μmol/L for glucose and acetate, respectively) compared to the present study. Also the IC₅₀ values obtained for cobalt addition (both approximately 14,500 μmol/L) were substantially higher than in the present study. The higher value for the supplied cobalt is probably due to higher salt concentrations (e.g. 60 mM HCO₃⁻) applied by Bhattacharya et al. [4], which lead to more cobalt precipitation and complexation. The high free cobalt IC₅₀ value is likely due to toxicity prevention by the presence of higher calcium and magnesium concentration (0.17 mM Ca²⁺, 5.25 mM Mg²⁺), because calcium as well as magnesium competes with cobalt for metal transporters [15]. As cobalt toxicity is mainly due to interaction with divalent

physiological ions (the ions having physiological functions), mainly Fe²⁺ [29], the higher iron concentration (0.2 mmol/L) used by Bhattacharya et al. [4] probably also decreased the free cobalt toxicity. The different methods for free cobalt concentration measurement (a combination of dialysis and ion exchange methods) used by Bhattacharya et al. [4] could also affect the conclusions.

Table 5 and Fig. 4 show that there is only a small difference between the optimal and toxic free cobalt concentration: the optimal concentration was shown to be approximately 7 μmol/L with an apparent K_m value of 0.9 μmol/L (based on total cobalt addition) [46], whereas a free cobalt concentration of only approximately 18 μmol/L is already significantly toxic (50% inhibition). This means that in case only a small amount of complexing agents is present in the medium, a relatively low total cobalt concentration can cause operational problems with anaerobic reactors.

Zinc, chromium, nickel, cadmium and copper are usually reported as the most toxic heavy metals that may occur in UASB reactors [7, 22, 26, 38]. Their reported toxic concentrations (based on nominal metal added) are, however, substantially higher (order of magnitude of mmol/L) than the IC₅₀ values documented in the present study for cobalt (Table 5). Also Chen et al. [6] showed that the effective concentration of metallic ion to provoke 50% response for the aerobic bacterium *P. aeruginosa* for cobalt was more than 100 times lower than the 50% effect concentration measured with cadmium and manganese. Therefore, a higher attention should be paid to induce cobalt toxicity.

Role of precipitation/sorption

Figure 5 shows that there are significant differences in the solubility of cobalt in the three systems studied. Set I (cobalt in the presence of inorganic ligands) shows the highest accumulation of cobalt in the solid phase (granules). This accumulation can be caused by or adsorption or absorption on/in the granules or by cobalt precipitation with the inorganic ions contained in the liquid medium [40]. Figure 5 shows a similar level of cobalt precipitation/sorption at the lower cobalt concentrations in sets I and III. However, in the case of set I (in contrast to set III), the portion of dissolved cobalt cannot be increased by addition of more cobalt. Since the only difference between the conditions of these two sets was the presence of carbonates and phosphates in set I, the difference between cobalt solubility in both systems was due to CoCO₃ and Co₃(PO₄)₂ precipitation. The formation of these precipitates under the applied conditions was also predicted by calculations with Visual MINTEQ (data not shown). Those precipitates are known to be present in granular sludge in significant amounts and have to be taken into account for

interpretation of data when the solid-state speciation is investigated in granular sludge [42].

The cobalt distribution between the solid and liquid phase in set III seems to be a typically adsorption-like type (as shown by van Hullebusch et al. [40]). Thus, cobalt adsorption plays a key role in the cobalt distribution in this system. However, it is almost impossible to distinguish between precipitation and adsorption in the granular sludge matrix [42]. Therefore, precipitation of part of the cobalt added (e.g. as CoS) cannot be excluded.

In the system studied in set II, there seems to be almost no cobalt transport into the solid phase. As Fig. 5 shows, almost all cobalt was always (except at concentrations as low as 100 $\mu\text{mol/L}$) present in the liquid phase. This is caused by the high affinity of EDTA to cobalt, the strong Co-EDTA²⁻ complex causes the poor precipitation/sorption of cobalt in this system.

Comparison of DMT with other available methods

Up to now, DMT has been most often used for measurement of the free species of various metals in synthetic media [21, 37, 44], surface water [20], soil solutions [43] and manure [39]. In the present study, DMT was successfully used for measurement of the free cobalt (Co²⁺) concentration in anaerobic granular sludge. Prior to the measurements on real samples, the suitability of this technique for cobalt was determined (Table 3). Similarly, Temminghoff et al. [37] obtained a good agreement between calculated and measured (with the DMT) free metal concentrations for copper and cadmium. The only problem is the high time required to obtain experimental data (4 days for equilibrium establishment, 5 days for DMT measurement), which disables DMT for measurements under dynamic conditions of bioreactors.

Acknowledgments The authors would like to thank to Eric Boer for his help with statistical treatment of experimental data. The present study was funded by the Marie Curie Excellence Grant Novel biogeological engineering processes for heavy metal removal and recovery (MEXT-CT-2003-509567) and it was partially performed during a research visit within the European Science Foundation Network Biomass fermentation towards usage in fuel cells.

Open Access This article is distributed under the terms of the Creative Commons Attribution Noncommercial License which permits any noncommercial use, distribution, and reproduction in any medium, provided the original author(s) and source are credited.

References

1. APHA/AWWA (1998) Standard methods for the examination of water and wastewater, 20th edn. American Public Health Association, Washington DC, USA
2. Beiras R, Bellas J, Fernandez N, Lorenzo JI, Cobelo-Garcia A (2003) Assessment of coastal marine pollution in Galicia (NW Iberian Peninsula); metal concentrations in seawater, sediments and mussels (*Mytilus galloprovincialis*) versus embryo-larval bioassays using *Paracentrotus lividus* and *Ciona intestinalis*. Mar Environ Res 56:531–553
3. Bhattacharya SK, Qu M, Madura RL (1996) Effects of nitrobenzene and zinc on acetate utilizing methanogens. Water Res 30:3099–3105
4. Bhattacharya SK, Uberoi V, Madura RL, Haghghi-Podeh MR (1995) Effect of cobalt on methanogenesis. Environ Technol 16:271–278
5. Burgess JE, Quarmby J, Stephenson T (1999) Micronutrient supplements for optimisation of the treatment of industrial wastewater using activated sludge. Water Res 33:3707–3714
6. Chen B-Y, Wu C-H, Chang J-S (2006) An assessment of the toxicity of metals to *Pseudomonas aeruginosa* PU21 (Rip64). Bioresour Technol 97:1880–1886
7. Fang HHP (1997) Inhibition of bioactivity of UASB biogranules by electroplating metals. Pure Appl Chem 69:2425–2429
8. Ferguson AD, Deisenhofer J (2004) Metal import through microbial membranes. Cell 116:15–24
9. Feroso FG, Collins G, Bartacek J, Lens PNL (2008) Zinc deprivation of methanol fed anaerobic granular sludge bioreactors. J Ind Microbiol Biotechnol 35(6):543–557. doi:10.1007/s10295-008-0315-z
10. Feroso FG, Collins G, Bartacek J, O'Flaherty V, Lens P (2008) Acidification of methanol-fed anaerobic granular sludge bioreactors by cobalt deprivation: Induction and microbial community dynamics. Biotechnol Bioeng 99:49–58
11. Florencio L, Field JA, Lettinga G (1994) Importance of cobalt for individual trophic groups in an anaerobic methanol-degrading consortium. Appl Environ Microbiol 60:227–234
12. Florencio L, Jenicek P, Field JA, Lettinga G (1993) Effect of cobalt on the anaerobic degradation of methanol. J Ferm Bioeng 75:368–374
13. Gikas P (2008) Single and combined effects of nickel (Ni(II)) and cobalt (Co(II)) ions on activated sludge and on other aerobic microorganisms: a review. J Hazard Mater (in press). doi: 10.1016/j.jhazmat.2008.02.048
14. Grcman H, Velikonja-Bolta S, Vodnik D, Kos B, Lestan D (2001) EDTA enhanced heavy metal phytoextraction: metal accumulation, leaching and toxicity. Plant Soil 235:105–114
15. Hassler CS, Slaveykova VI, Wilkinson KJ (2004) Some fundamental (and often overlooked) considerations underlying the free ion activity and biotic ligand models. Environ Toxicol Chem 23:283–291
16. Hassler CS, Wilkinson KJ (2003) Failure of the biotic ligand and free-ion activity models to explain zinc bioaccumulation by *Chlorella kesslerii*. Environ Toxicol Chem 22:620–626
17. Helfferich F (1962) Ion exchange. McGraw-Hill, New York
18. Hu Z, Chandran K, Grasso D, Smets BF (2002) Effect of nickel and cadmium speciation on nitrification inhibition. Environ Sci Technol 36:3074–3078
19. Jansen S, Gonzalez-Gil G, van Leeuwen HP (2007) The impact of Co and Ni speciation on methanogenesis in sulfidic media—biouptake versus metal dissolution. Enzyme Microb Technol 40:823–830
20. Kalis EJJ, Weng LP, Dousma F, Temminghoff EJM, Van Riemsdijk WH (2006) Measuring free metal ion concentrations in situ in natural waters using the Donnan membrane technique. Environ Sci Technol 40:955–961
21. Kalis EJJ, Weng LP, Temminghoff EJM, Van Riemsdijk WH (2007) Measuring free metal ion concentrations in multicomponent solutions using the Donnan membrane technique. Anal Chem 79:1555–1563

22. Karri S, Sierra-Alvarez R, Field JA (2006) Toxicity of copper to acetoclastic and hydrogenotrophic activities of methanogens and sulfate reducers in anaerobic sludge. *Chemosphere* 62:121–127
23. Krom BPB (2002) Impact of the Mg^{2+} -citrate transporter CitM on heavy metal toxicity in *Bacillus subtilis*. *Arch Microbiol* 178:370–375
24. Kungolos AA (2006) Bioavailability and toxicity of heavy metals in the presence of natural organic matter. *J Environ Sci Health A Environ Sci Eng Toxic Hazard Subst Control* 41:1509–1517
25. Lettinga G, Van der Geest AT, Hobma S, Laan JVD (1979) Anaerobic treatment of methanolic wastes. *Water Res* 13:725–737
26. Lin C-Y, Chen C-C (1997) Toxicity-resistance of sludge biogranules to heavy metals. *Biotechnol Lett* 19:557–560
27. Lorenzo JI, Nieto O, Beiras R (2006) Anodic stripping voltammetry measures copper bioavailability for sea urchin larvae in the presence of fulvic acids. *Environ Toxicol Chem* 25:36–44
28. Loureiro Paulo P, Villa G, Bernardus van Lier J, Lettinga G (2003) The anaerobic conversion of methanol under thermophilic conditions: pH and bicarbonate dependence. *J Biosci Bioeng* 96:213–218
29. Nies DH (1999) Microbial heavy-metal resistance. *Appl Microbiol Biotechnol* 51:730–750
30. Osuna MB, van Hullebusch ED, Zandvoort MH, Iza J, Lens PNL (2004) Effect of cobalt sorption on metal fractionation in anaerobic granular sludge. *J Environ Qual* 33:1256–1270
31. Oviedo C, Rodriguez J (2003) EDTA: the chelating agent under environmental scrutiny. *Quim Nova* 26:901–905
32. Paulo PL, Jiang B, Cysneiros D, Stams AJM, Lettinga G (2004) Effect of cobalt on the anaerobic thermophilic conversion of methanol. *Biotechnol Bioeng* 85:434–441
33. Ram MS, Singh L, Suryanarayana MVS, Alam SI (2000) Effect of iron, nickel and cobalt on bacterial activity and dynamics during anaerobic oxidation of organic matter. *Water Air Soil Pollut* 117:305–312
34. Schabenberger O, Tharp BE, Kells JJ, Penner D (1999) Statistical tests for hormesis and effective dosages in herbicide dose response. *Agron J* 91:713–721
35. Shuttleworth KL, Unz RF (1991) Influence of metals and metal speciation on the growth of filamentous bacteria. *Water Res* 25:1177–1186
36. Singh RP, Kumar S, Ojha CSP (1999) Nutrient requirement for UASB process: a review. *Biochem Eng J* 3:35–54
37. Temminghoff EJM, Plette ACC, Van Eck R, Van Riemsdijk WH (2000) Determination of the chemical speciation of trace metals in aqueous systems by the Wageningen Donnan membrane technique. *Anal Chim Acta* 417:149–157
38. Tiwari MK, Guha S, Harendranath CS, Tripathi S (2006) Influence of extrinsic factors on granulation in UASB reactor. *Appl Microbiol Biotechnol* 71:145–154
39. Van der Stelt B, Temminghoff EJM, Van Riemsdijk WH (2005) Measurement of ion speciation in animal slurries using the Donnan membrane technique. *Anal Chim Acta* 552:135–140
40. Van Hullebusch ED, Gieteling J, Zhang M, Zandvoort MH, Daele WV, Defrancq J, Lens PNL (2006) Cobalt sorption onto anaerobic granular sludge: isotherm and spatial localization analysis. *J Biotechnol* 121:227–240
41. Van Hullebusch ED, Peerbolte A, Zandvoort MH, Lens PNL (2005) Sorption of cobalt and nickel on anaerobic granular sludges: isotherms and sequential extraction. *Chemosphere* 58:493–505
42. Van Hullebusch ED, Utomo S, Zandvoort MH, Lens PNL (2005) Comparison of three sequential extraction procedures to describe metal fractionation in anaerobic granular sludges. *Talanta* 65:549–558
43. Weng L, Temminghoff EJM, Van Riemsdijk WH (2001) Determination of the free ion concentration of trace metals in soil solution using a soil column Donnan membrane technique. *Eur J Soil Sci* 52:629–637
44. Weng LP, Van Riemsdijk WH, Temminghoff EJM (2005) Kinetic aspects of Donnan membrane technique for measuring free trace cation concentration. *Anal Chem* 77:2852–2861
45. Worms I, Simon DF, Hassler CS, Wilkinson KJ (2006) Bioavailability of trace metals to aquatic microorganisms: importance of chemical, biological and physical processes on biouptake. *Biochimie* 88:1721–1731
46. Zandvoort MH, Geerts R, Lettinga G, Lens PNL (2002) Effect of long-term Cobalt deprivation on methanol degradation in a methanogenic granular sludge bioreactor. *Biotechnol Prog* 18: 1233–1239
47. Zandvoort MM, Van Hullebusch ED, Fiermoso FG, Lens P (2006) Trace metals in anaerobic granular sludge reactors: bioavailability and dosing strategies. *Eng Life Sci* 6:293–301