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Effect of nickel deprivation on methanol degradation in a methanogenic granular sludge bioreactor

MH Zandvoort, MB Osuna, R Geerts, G Lettinga and PNL Lens

Subdepartment of Environmental Technology, Agricultural University of Wageningen, "Biotechnion"- Bomenweg, 2, PO Box 8129, Wageningen 6700 EV, The Netherlands

The effect of omitting nickel from the influent on methanol conversion in an Upflow Anaerobic Sludge Bed (UASB) reactor was investigated. The UASB reactor (30°C, pH 7) was operated for 261 days at a 12-h hydraulic retention time (HRT) and at organic loading rates (OLRs) ranging from 2.6 to 7.8 g COD l reactor^{-1} day^{-1}. The nickel content of the sludge decreased by 66% during the 261-day reactor run because of washout and doubling of the sludge bed volume. Nickel deprivation initially had a strong impact on the methanogenic activity of the sludge with methanol; e.g., after 89 days of operation, this activity was doubled by adding 2 μM nickel. Upon prolonged UASB reactor operation, methanol and VFA effluent concentrations decreased whereas the sludge lost its response to nickel addition in activity tests. This suggests that a less nickel-dependent methanol-converting sludge had developed in the UASB reactor.
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Introduction

In nature, methanol is released as a fungal biodegradation product of natural methoxylated aromatics [25], which are components of lignin polymers. Methanol is also produced during microbial growth on aromatic acids and pectin [8,30]. Moreover, methanol is an intermediate of formaldehyde conversion under anaerobic [10] and methane-oxidising [15] conditions. As the produced methanol is not used by aerobic, facultative, and anaerobic methanol-producing microorganisms [30], a niche exists for methylotrophs [31].

In anaerobic environments, methanogenic methylotrophs play a key role as methanol is — in addition to H_2/CO_2 and acetate — a direct substrate for methanogens [23,33]. Methanol can be converted to methane by microorganisms *via* several pathways. The most straightforward biodegradation route is its direct conversion to methane by methylotrophic methanogens [23,33]. Methanol can also be converted to acetate by acetogens [22,39], coupled to the conversion of acetate to methane by acetoclastic methanogens [17]. Another possibility is the conversion of methanol to H_2 and CO_2 [5,6], which can then be converted to methane by autotrophic methanogens [3,38]. Florencio *et al* [10] described the key role of cobalt on the methanol degradation pathway and the different trophic groups (acetogens or methanogens) involved in methanogenic methanol conversion. When the cobalt level is kept low during start-up, reactor instability due to acetate build-up by acetogenic activity can be prevented [9]. If methanogenesis dominates in the inoculum sludge, cobalt addition stimulates methane formation [9].

Correspondence: Dr Piet NL Lens, Subdepartment of Environmental Technology, Agricultural University of Wageningen, "Biotechnion"- Bomenweg, 2, PO Box 8129, Wageningen 6700 EV, The Netherlands
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Besides cobalt, nickel is also an important element in anaerobic microorganisms and plays a key role in methane formation. Methanogenic Archaea use several pathways to reduce the various carbon substrates (e.g., methanol, acetate, and H_2/CO_2), but all pathways converge on the common intermediate methyl-*S*-CoM [35]. Methyl-*S*-CoM contains a nickel-harbouring tetrapyrolic structure, coenzyme F_{430}, present in all methanogens and exclusively found in methanogens [11]. Scherer *et al* [29] found that *Methanosarcina barkeri* Fusaro cells grown on methanol contain two times more nickel than cobalt when grown in a medium containing 5 and 1 μM nickel and cobalt, respectively. Nickel is present in enzymes involved in the metabolic pathways of anaerobic microorganisms, acetogens and methanogens. In addition, many hydrogenase enzymes used to form or consume hydrogen gas possess nickel [7,20]. Carbon monoxide dehydrogenase (CODH), which possesses two nickel-containing metallocenters, is present in both acetoclastic methanogens and acetogenic microorganisms [16]. Nickel may also play a role in the stability of some methanogens, for instance in maintaining wall stability [19].

In this study, the effect of long-term nickel deprivation on the performance of an Upflow Anaerobic Sludge Bed (UASB) reactor was investigated. The methanol, volatile fatty acid, and metal removal performance of the reactor was monitored as a function of time. Using batch tests, the metabolic properties of the sludge that developed in the reactor were characterised as well.

Materials and methods

Source of biomass

Methanogenic granular sludge was obtained from a full-scale UASB reactor treating the alcohol distillery wastewater of Nedalco (Bergen op Zoom, The Netherlands). The sludge was elutriated to remove the fines. The total suspended solids (TSS) and volatile

Table 1 Composition of the trace element solution

Compound added	Concentration	
	Compound (mg l^{-1})	Element (mg l^{-1})
$FeCl_2 \cdot 4H_2O$	2000	562
$ZnCl_2$	50	24
$MnCl_2 \cdot 4H_2O$	500	139
$CuCl_2 \cdot 2H_2O$	38	14
$CoCl_2 \cdot 6H_2O$	2000	495
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$[a]	50	27
$Na_2SeO_2 \cdot 5H_2O$[a]	164	49

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

suspended solids (VSS) concentrations of the sludge were 10.0±0.2% and 9.5±0.2%, respectively.

Basal medium

The reactor was fed basal medium consisting of methanol, macronutrients, and a trace element solution dissolved in tap water. The inorganic macronutrients contained (in mg l^{-1} basal medium): NH_4Cl (280), K_2HPO_4 (250), $MgSO_4 \cdot 7H_2O$ (100), and $CaCl_2 \cdot 2H_2O$ (10). In addition, 0.1 ml of both acid and base trace element solution, from which nickel was omitted (Table 1), was added per litre of basal medium. To ensure pH stability, 2.52 g (30 mM) of $NaHCO_3$ was added per litre of basal medium.

To avoid precipitation in the storage vessels, the influent was composed of four streams: basal medium without K_2HPO_4, methanol with bicarbonate, K_2HPO_4, and dilution water. Tap water was used to prepare the influent and was used as dilution water. It contained only traces of nickel (2±4 nM), comparable to the nickel contamination present in demineralised water (3 nM). Also, the chemicals to make up the macronutrient solution contain some nickel contamination, resulting in a final nickel influent concentration of 9±5 nM (Table 2). Although tap water did not affect the nickel, iron, or cobalt influent concentration, the copper and zinc concentrations were increased considerably by copper and zinc impurities in the chemicals and the tap water. Most likely, the tap water contamination came from metal fittings and tubes used in the transport system.

UASB reactor operation

The experiment was performed in a Plexiglas cylindrical UASB reactor with a working volume of 7.25 l and an inner diameter of 0.1 m. The reactor operated in a temperature-controlled room at a temperature of 30±2°C. The UASB reactor was inoculated with 8.7 g VSS l^{-1} anaerobic granular sludge and operated at a hydraulic retention time (HRT) of 12 h. The conical bottom of the reactor was filled with glass marbles (1 cm in diameter) to distribute the influent evenly over the sludge bed. For the influent flow, peristaltic pumps (type 505S; Watson and Marlow, Falmouth, UK) were used. No effluent recycle was applied and the superficial upflow velocity was 0.01 m h^{-1}.

During start-up (period I), methanol was fed to the reactor at a concentration of 1.4 g COD l^{-1}, corresponding to an organic loading rate (OLR) of 2.6 g COD l^{-1} reactor day^{-1}. The methanol loading rate was increased on day 33 to 5.2 g l^{-1} reactor day^{-1} (period II) and on day 57 to 7.8 g l^{-1} reactor day^{-1} (period III) until the end of the experiment (Figure 1).

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

Specific maximum methanogenic activity test

Approximately 1.2 g (wet weight) of granular sludge was transferred to 120-ml serum bottles containing 60 ml of basal medium with the same composition as the reactor basal medium, supplemented with either methanol (4 g COD l^{-1}) or acetate (2 g COD l^{-1}) as the substrate. The bottles were closed with butyl rubber stoppers (Rubber, Hilversum, The Netherlands) and flushed with a N_2/CO_2 gas mixture (70/30 vol/vol). The experiments were done at 30±2°C in duplicate. The activity was determined by on-line measurement of the increase of headspace pressure as a result of methane production in the serum bottles using pressure transducers (Honeywell 26PCDFA1G, Freeport, IL). In order to measure the pressure in the serum bottle, a butyl rubber tube was connected to the outlet of the pressure transducer. At the other end of the tube, a needle was connected using a Luer-lock adapter (outer diameter of 3.1 mm; Unimed, Geneva, Switzerland). The needle was put through the butyl rubber stopper of the serum bottle. The signal of the transducer was sent to a computer with a data acquisition card (PCL-818-HD) *via* a multiplexer card (PCLD-789D) on which data were stored by the data acquisition program Visidaq 3.11 (Advantech Europe, Eindhoven, The Netherlands). Data were acquired every 30 min and were plotted in a rate *versus* time curve, using moving average trend lines with an interval of 15 data points.

Analyses

Total dissolved metal concentrations in the influent and effluent were determined by inductively coupled plasma mass spectrometry

Table 2 Average trace metal concentration in the influent and effluent during the reactor run

Metal	Influent (nM)	Influent (nM)	Effluent (nM)	Removal efficiency (%)
	Calculated	ICP	ICP	
Ni	0	9±5	23±21	–
Co	840	783±34	120±57	84
Fe	1000	2003±887	941±294	53
Mo	36	30±2	1±1	97
Se	62	142±19	16±9	88
Mn	253	287±28	58±43	80
Zn	37	526±336	303±205	42
Cu	22	3925±768	605±278	85

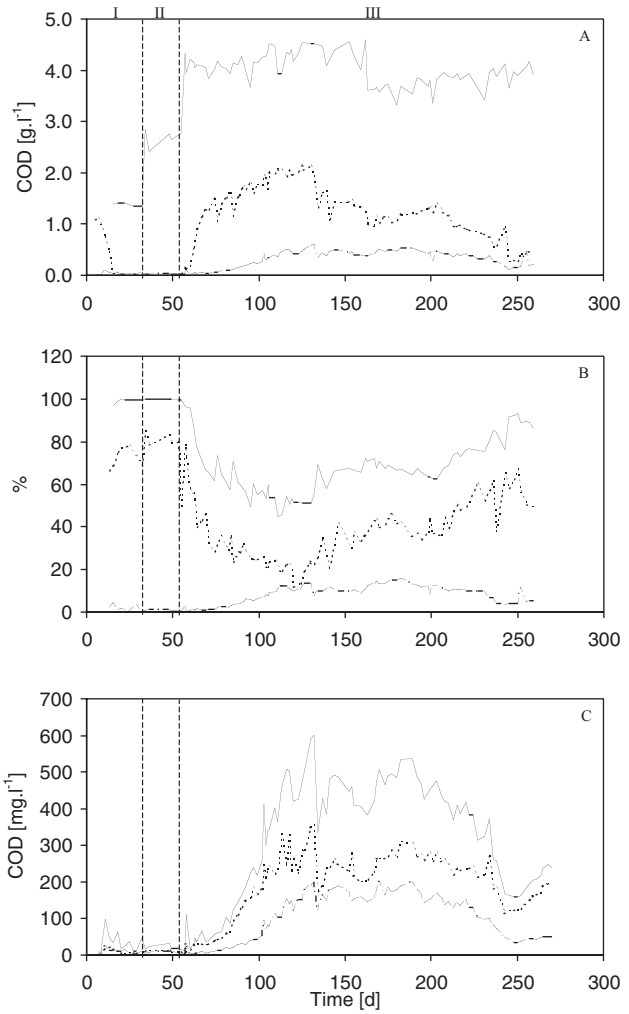

Figure 1 Reactor performance (A) methanol concentration in the influent (—), methanol concentration in the effluent (····), and the VFA concentration in the effluent (---). (B) Methanol removal efficiency (—), methanogenesis (····), and VFA accumulation (---). (C) Total VFA concentration (—), acetate concentration (····), and propionate concentration (---).

(ICP-MS; Elan 6000, Perkin-Elmer, Shelton, CT) in samples acidified with 0.1 M HNO_3. The samples were centrifuged at 10,000 rpm to remove particles from the liquid. The total metal concentration in the sludge was determined after microwave destruction (CEM 2100, Matthews, NC) of predried sludge (105°C) in a mixture of 2.5 ml of HNO_3 (65%) and 7.5 ml of HCl (37%). After digestion, the samples were paper-filtered (Schleicher and Schuell 589[1], Germany) and diluted to 100 ml; 1 ml of this solution was transferred to 9 ml of 0.1 M of HNO_3 and subsequently analysed for metal content by ICP-MS.

The concentration of methanol and VFA and the composition (CO_2, CH_4, and N_2) of the biogas were determined by gas chromatography as described by Weijma *et al* [37]. The total sulphide concentration was determined colorimetrically using the methylene blue method [36]. The TSS and VSS concentrations were determined according to *Standard Methods for Examination of Water and Wastewater* [2]. All chemicals were of analytical or biological grade and purchased from E. Merck (Darmstadt, Germany).

Results

Methanol conversion

After a start-up period of 14 days, all of the methanol was converted to methane and biomass (Figure 1A and B). Upon doubling of the loading rate on day 33, all methanol was immediately removed. The reactor could not cope with the second increase of the OLR on day 57 (period III), as the methanol started to accumulate in the effluent (Figure 1A and B). This was accompanied by an increase in the VFA concentration averaging 316±162 mg COD l^{-1} during period III (Figure 1C, Table 5). This VFA fraction consisted mainly of acetate and propionate; the concentration of these VFAs averaged 185±80 and 101±65 mg COD l^{-1}, respectively (Table 3). Period III can be divided in three subperiods. The VFA concentration in the effluent gradually increased from day 57 until day 102 after which the total VFA concentration of the effluent stabilised at 450±65 mg COD l^{-1} (256±42 and 153±34 mg COD l^{-1} for acetate and propionate, respectively). After this stable period, the VFA concentration started to decrease gradually again from day 196 onwards, to reach total VFA concentrations of ±200 mg COD l^{-1} at the end of the experiment. The methanol concentration in the effluent started to decrease at an earlier stage (from day 134 onwards). When VFA and methanol accumulation started, the reactor started to foam. The foaming lasted until day 100, after which it slowly decreased and disappeared around day 150 (data not shown). The foam partially clogged the gas outlet, but this could be prevented by installation of a collection bottle in the gas tube between the reactor and waterlock.

During period II, the percentage of CH_4 in the biogas decreased from 87.1% to 84.0% (Table 3), while the percentage of CO_2 almost doubled from 6.7% to 11.7%. In period III, as the extra load was not converted, the percentage of CH_4 remained comparable to period II. The influent contained 0.41 mM sulphate, which was reduced to sulphide. The effluent sulphide concentration was measured from days 11 to 78 and amounted to 0.17±0.03 mM.

The pH of the effluent was 7.5±0.3 in period I and 7.1±0.1 in period II. As methanol and acetate accumulated after the second

Table 3 Mean performance characteristics of the UASB reactor

Parameter	Period I	Period II	Period III
Day	0–33	34–57	58–261
Effluent composition			
pH	7.5±0.3	7.1±0.1	7.1±0.1
COD methanol influent (mg l^{-1})	1386±24	2691±148	4032±296
COD methanol effluent (mg l^{-1})	339±493	7±6	1262±528
COD VFA (mg l^{-1})[a]	33±19	19±7	316±162
COD acetate (mg l^{-1})	10±7	10±2	185±80
COD propionate (mg l^{-1})	10±7	7±4	101±65
Methanol conversion route			
Methanogenesis (%)	73.0±4.7	74.8±12.3	38.3±14.9
VFA accumulated (%)[b]	2.5±1.5	0.7±0.3	8.1±4.3
Biogas composition			
CH_4 (%)	87.1±1.7	84.0±0.3	83.3±1.6
CO_2 (%)	6.7±2.0	11.7±0.4	11.6±1.8

[a]Total amount of VFA in the effluent including butyrate and valerate.
[b]Percentage of methanol converted to VFA.

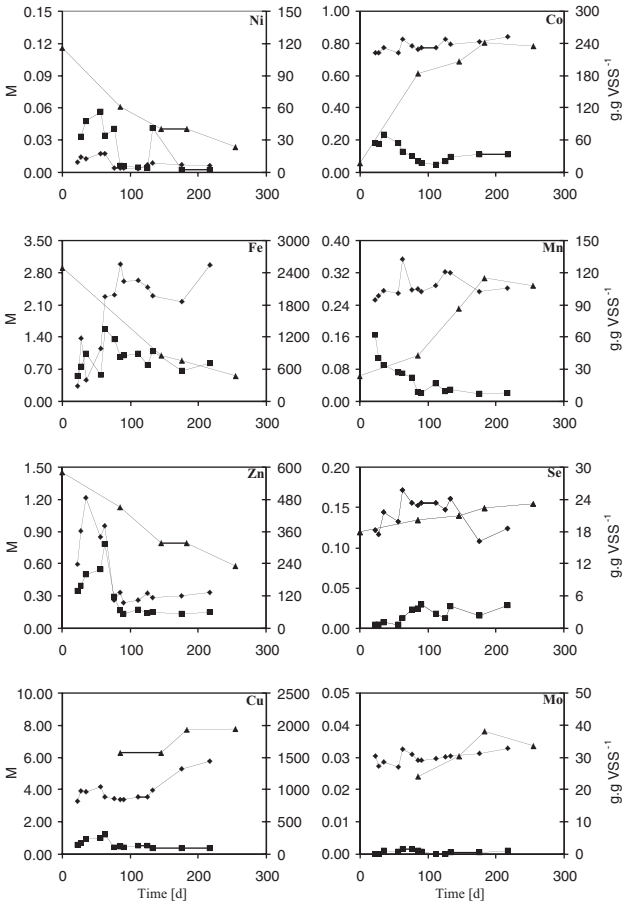

Figure 2 Evolution of the metal concentration in the influent (◆), effluent (■), and sludge (▲) as a function of time.

loading rate increase, the pH became 7.2–7.3. During period III, the pH became 7.0±0.1 as more methanol was degraded.

Metals in the influent and effluent

The reactor retained a considerable part of the metals present in the influent (Table 2, Figure 2). Zinc (42%) and iron (53%) were not well retained by the sludge, while cobalt (84%), copper (85%), manganese (80%), molybdenum (97%), and selenium (88%) were relatively well retained (Table 2, Figure 2). With time, retention of manganese increased and finally stabilised after day 85 (Figure 2). Surprisingly, the nickel concentration in the effluent (23±21 nM) was higher than the influent (9±5 nM) concentration (Figure 2), indicating that nickel leached out of the granules. During the run, approximately 1.90 mg of nickel entered the reactor with the influent, whereas 4.85 mg of nickel left the reactor *via* the effluent.

Table 4 Evolution of the metal concentration (μg g TSS^{-1}) in the sludge as a function of time

Day	Ni	Co	Fe	Mn	Mo	Se	Zn	Cu
0	116	17	2496	24	n.a.	17	582	n.a.
85	61	184	1147	47	24	20	452	1577
146	40	206	859	86	30	21	317	1567
183	40	241	756	115	38	22	316	1930
255	23	236	472	107	33	23	232	1941

n.a., not analyzed.

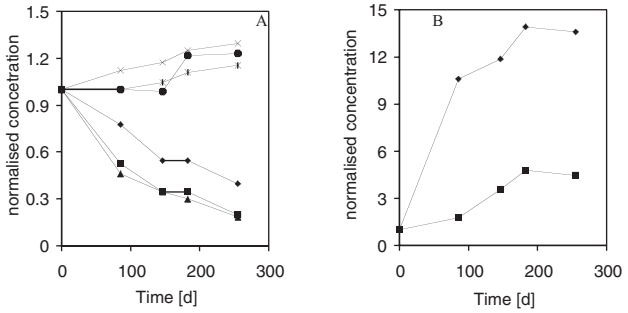

Figure 3 Normalised metal concentrations of (A) iron (▲), nickel (■), zinc (◆), selenium (×), molybdenum (⚹), and copper (●); (B) cobalt (◆) and manganese (■).

Sludge and metal concentration in the sludge

The nickel content of the inoculum (116 μg g TSS^{-1}) corresponded to 7.28 mg of nickel added to the UASB reactor with the seed sludge. The nickel, iron, and zinc concentrations in the sludge decreased over time, and at the end of the run, the nickel and iron concentrations were about five times lower than in the seed sludge (Table 4, Figure 3). In addition to washout with the effluent (Figure 2), a dilution due to the formation of new granules also occurred as the sludge bed increased at a more or less stable rate of 0.5 cm week^{-1} throughout the reactor run (data not shown) and the VSS/TSS ratio of the sludge remained constant at ±94% (data not shown). In contrast, selenium, molybdenum, and copper concentrations increased slightly in the sludge throughout the experiment (Figure 2, Table 4). Because the sludge bed volume doubled in size (data not shown), the absolute amount of selenium, molybdenum, and copper present in the reactor increased during the reactor run.

The cobalt concentration in the sludge increased to 236 μg g TS^{-1} at the end of the experiment (Table 4), which is 14 times higher than in the seed sludge. Iron depleted and cobalt accumulated while both elements were dosed in the same concentration range (1.00 and 0.84 μM, respectively), showing a distinct difference in retention of these two elements. This was reflected in the relatively high iron content of the effluent. Manganese is, next to cobalt, the only metal analysed for which the concentration increased considerably (4.5 times). The largest increase in cobalt content occurred in the first 85 days of operation (Table 4, Figure 3). Copper accumulated in the granules as well, but not as much as manganese and cobalt (Table 4, Figure 3),

Table 5 Evolution of the specific methanogenic activity (mg CH_4–COD g^{-1} VSS day^{-1}) with methanol as the substrate as a function of time and nickel concentration.

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

n.d., not determined.
[a]Methanogenic activity on acetate is presented between parentheses.

although in absolute terms, the accumulated amount of copper is considerable (363 μg Cu g TS^{-1}).

Activity tests

The methanogenic activity of the sludge with methanol as the substrate was tested at the beginning of the experiment, after 89 days of operation (when the performance of the reactor deteriorated upon the second increase in OLR) and on day 261 (the end of the run) (Table 5). The seed sludge showed a lag phase of 6 days before gas production started. A lag phase of approximately 5 h was observed with both sludges harvested after 89 and 261 days of operation. The maximum methanogenic activity (in the absence of nickel) with methanol as the substrate was similar on days 89 and 261 and amounted to 292 and 245 mg CH_4–COD g VSS^{-1} day^{-1}, respectively. These activities are 2.5–3 times higher than the activity of the seed sludge (108 mg CH_4–COD g VSS^{-1} day^{-1}).

The methanogenic activity of the seed sludge with acetate as the substrate in a medium containing nickel was 271 mg CH_4–COD g VSS^{-1} day^{-1} (Table 5). Earlier experiments with the inoculum showed that the presence or absence of the full trace element cocktail during the test did not affect the methanogenic activity on acetate [13,29]. Methanogenic activity on acetate in the absence of nickel was 111 mg CH_4–COD g VSS^{-1} day^{-1} on day 89. At the end of the run, hardly any activity on acetate was observed (21 mg CH_4–COD g VSS^{-1} day^{-1}) independent of the presence (0 or 2 μM) of nickel in the medium. At the end of the run, a considerable methanogenic activity with H_2 and CO_2 (440±27 mg CH_4–COD g VSS^{-1} day^{-1}) was observed in the absence of nickel, after a lag phase of 50 h (data not shown).

With the sludge sampled on day 89, nickel addition increased the methanogenic activity with methanol as the substrate, from 292 mg CH_4–COD g VSS^{-1} day^{-1} in the absence to 546 mg CH_4–COD g VSS^{-1} day^{-1} in the presence of 2 μM nickel (Table 5). In contrast, after 261 days of operation, only a small increase in activity with methanol due to nickel addition (2 μM) was observed from 245 to 312 CH_4–COD g VSS^{-1} day^{-1}.

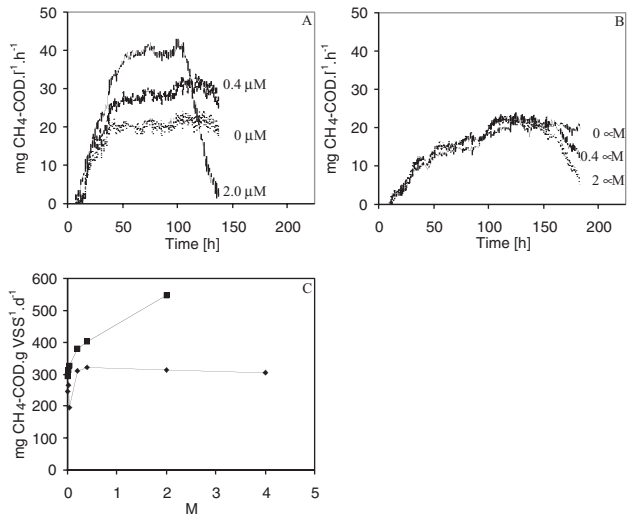

Figure 4 Methane production rate and methanogenic activity (pH 7, 30°C) with methanol as the substrate. (A) Methane production rate of the sludge after 89 days. (B) Methane production rate of the sludge after 261 days. (C) Methanogenic activity of the sludge after 89 (◆) and 261 (■) days.

Figure 4C shows that with the sludge sampled on day 89, the methanogenic activity was already stimulated by dosing nickel at concentrations as low as 20 nM and the apparent K_M for nickel dosage was 678 nM. The sludge tested on day 89 reached the maximum gas production rate after 50 h and stayed constant until the substrate was depleted (Figure 4A). The increase in methanogenic activity of the sludge by nickel addition also occurred during the first 50 h. In contrast, the rate curves are similar for all nickel concentrations tested with the sludge sampled on day 261 (Figure 4B). Remarkably, a similar period of 50 h is observed before the maximum methanogenic activity is obtained (Figure 4B).

The sludge was also tested for its response to iron (no nickel added) on days 147 and 261. Addition of iron (10 μM) to the sludge sampled on day 147 increased the activity from 244 to 315 mg CH_4–COD g VSS^{-1} day^{-1} (data not shown). On day 261, the response to iron was even larger; i.e., the addition of 10 μM iron increased the methanogenic activity from 211 to 346 mg CH_4–COD g VSS^{-1} day^{-1}.

Discussion

Effect of nickel deprivation on methanol degradation

Nickel deprivation initially had a strong impact on the methanogenic activity of the sludge (Figure 4A), but this impact decreased with operation time and was no longer present after 261 days of reactor operation (Figure 4B). Nickel leached from the sludge (4.85 mg based on effluent concentration, which is 66% of the amount present in the inoculum sludge) during reactor operation (Figure 2), despite the fact that the influent contained 9±5 nM nickel (Table 2). The latter corresponds to 1.90 mg of nickel entering the reactor during the reactor run. Assuming direct conversion of methanol, that all nickel is bioavailable, and that *M. barkeri* Fusaro (methanol-grown) has a nickel requirement of 0.135 μg g^{-1} dry cells [29], nickel added with the influent can, in theory, support the production of 14 g of dry cells. Indeed, the sludge bed volume increased to 2.3 times the initial size (data not shown), indicating that new biomass was formed.

Nickel deprivation seems to induce an adaptation of the sludge, making it less nickel-dependent (Figure 1). A change in the (enzymatic) methanol conversion pathway or growth of new less-nickel-dependent biomass may cause the absence of a response to nickel at the end of the run (Figure 4). For instance, the hydrogenotrophic archeon *Methanobacterium marburgensis* possesses two hydrogenase systems that catalyse the reduction of coenzyme F_{420}. One system consists of a nickel-containing enzyme, the other system is nickel-free. The nickel-free system is induced under nickel-limited conditions [1]. Alternatively, another trace element was limiting, e.g., either iron or zinc whose concentration in the sludge also decreased (Table 4). Indeed, iron addition (10 μM) stimulates methanogenic activity (data not shown). Zinc was not tested but it can be important, as it is present in similar concentrations as nickel in *M. barkeri* (140 μg g dry $cells^{-1}$) [29]. It is, however, rather unlikely that zinc was limiting, as the zinc concentration in the sludge was still 10 times higher than the nickel concentration.

Methanol degradation in the reactor

Overloading the reactor with methanol (period III) resulted in the accumulation of methanol and some VFA in the effluent (Figure 1).

Remarkably, not all the accumulated methanol was converted to VFAs. Florencio *et al* [10] found that the affinity (K_s) for methanol of methylotrophic methanogens (12 mg COD l^{-1}) is 60 times higher than that of acetogens (770 mg COD l^{-1}). Therefore, a low methanol concentration as prevailed in the reactor during periods I and II (Table 3) is favourable for methylotrophic methanogens. The presence of inorganic carbon is required as a cosubstrate for acetogenesis [21]. This does not explain the lack of acetogenesis observed in this study as sufficient inorganic carbon was present in the reactor, due to bicarbonate addition (30 mM) and its formation during the direct conversion of methanol to methane. The production of minor amounts of VFA (Figure 1) and the low methanogenic activity on acetate (Table 5) indicate that acetogenic bacteria were not able to outcompete methanogens under nickel-limiting conditions. This also occurs under cobalt-deprived conditions [9], using the same inoculum sludge.

Methanosarcina strain 227 grown on acetate can grow on methanol [33]. In contrast, methanol-grown cells do not degrade acetate in the presence of methanol and, in some cases, were not capable of degrading acetate at all [33]. Fukuzaki *et al* [13] determined the threshold level for acetate utilisation by *M. barkeri* and acclimatised sludge as a function of pH. Methane formation ceased at an acetate concentration of 120 mg COD l^{-1} (final pH 7.35) in the case of *M. barkeri* and 27 mg COD l^{-1} for acclimatised sludge (predominantly *Methanothrix* spp). As the predominant organisms in the sludge resemble *Methanosarcina* spp., a similar threshold in this range of 120 mg COD l^{-1} may be present, which is in agreement with effluent acetate concentrations (Figure 1C).

Some propionate accumulation was observed as well in the effluent (Figure 1C), which agrees with the lack of propionate-degrading capacity of the inoculum sludge [24]. Fukuzaki and Nishio [12] showed that propionate degradation could be reversibly inhibited due to direct toxicity of methanol. Propionate-acclimatised sludge showed 45±5% inhibition of propionate degradation at a methanol loading rate of 9.6 g COD l^{-1} day^{-1} [13]. As the methanol concentration in the effluent further decreased, the concentration of both acetate and propionate started to decrease from day 190 onwards (Figure 1). Thus, the observed initial lack of propionate degradation might be the result of methanol toxicity, absence of a propionate-degrading consortium, or a combination of the two.

Methanol degradation pathway

The rate of gas production did not constantly increase during the activity test with methanol as the substrate, but a plateau was reached (Figure 4C). Although no response to nickel was found in the second activity test at the end of the run (day 261, Figure 4B), maximum activity was reached after 50 h as well. This means that the increase in methanogenic activity took place during the first 50 h. As the nickel concentration was the only variable, it seems that uptake of the available nickel by the biomass took place in the first 50 h. Pusheva *et al* [28] showed that incorporation of ^{63}Ni by *Clostridium thermoautotrophicum*, grown in the presence of 3 μM nickel, took place in the first 20 h of growth.

The low and decreasing methanogenic activity on acetate suggests that methanol was not converted *via* acetate but directly to methane by methylotrophic methanogens. Indeed, under mesophilic conditions, direct methylotrophic methanogenesis was the main pathway [9]. In contrast, methanol is syntrophically converted to methane *via* H_2/CO_2 under thermophilic conditions (55–65°C), either in the presence [37] or absence [26] of sulfate. An explanation for the relatively high activity found with H_2/CO_2 (440 mg CH_4–COD g VSS^{-1} day^{-1}) is the fact that *M. barkeri* can use H_2/CO_2 and methanol simultaneously [18], indicating that the enzymatic pathway was present or could be induced (considering the 50-h lag phase). Alternatively, other H_2-utilising microorganisms proliferated during the lag phase. It should be noted that methanogens grown in pure cultures on methanol can produce some H_2 under mesophilic conditions, especially when cocultured with a H_2 scavenger [27].

Fate of metals in an anaerobic granular sludge reactor

The reactor retained a lot of the added metals but some washout occurred (Figure 2). Metal removal from solution is mainly achieved by precipitation of metals with carbonate, sulfide, hydroxide, and phosphate ions — the first two ions being the most important [4,39]. Also, binding to extracellular polymers and biomass contributes to the removal of heavy metals [32]. Cobalt and manganese are the only metals for which the concentration increased significantly in the sludge (Figure 3), which was also found for VFA-fed granules [24].

Nickel, cobalt, iron, copper, and zinc precipitate as sulfides as long as the total concentration of these metals does not exceed the total sulfide level [4]. The sulfate (0.41 mM) in the effluent was reduced to sulfide, which precipitates with metals in the influent. Although the concentration of sulfide in the effluent was rather low, it was still sufficient to stoichiometrically precipitate all the metals added. This contrasts with the traces of most of these elements present in the effluent (ranging from 23 nM for nickel up to 941 nM for iron). Similar findings were reported by Callendar and Barford [4], who predicted soluble metal concentrations based on chemical equilibria. Even at low sulfide concentrations (5 mg l^{-1} S), calculated concentrations were much lower than the measured concentrations, e.g., iron and cobalt concentrations were 6×10^5 and 10^7 times higher than calculated ones, respectively. As the calculations are based on free metal concentrations, differences can be expected to be related to the speciation of metals. Steffen [34] analysed the effluent cobalt concentration by adsorptive stripping voltammetry (AdSV) on day 147 using a ligand dimethylglyoxime to complex the cobalt [34]. No cobalt was detected in the AdSV-emended effluent, which contained 73 nM cobalt when determined by ICP-MS analysis. This indicates that cobalt is present in the effluent as very strongly bound chemical species (conditional stability constant, K', 9.74×10^{-3}) [34]. Note that these are not metal sulfides, which have a lower K', but metalloorganic compounds, e.g., cobalamin. This shows that further research is needed to determine the cobalt and nickel species present in the effluent, as well as their influence on the bioavailable fractions to further optimise trace element dosage to methanol-degrading UASB reactors.

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