Denitrification with dissolved methane from anaerobic digestion: a novel opportunity for sewage treatment

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Abstract
Despite many advantages of anaerobic sewage treatment, it is not applied at low temperatures. This is mainly because effluent from anaerobic sewage treatment contains nitrogen and, esp. at lower temperatures, dissolved methane. A new concept for anaerobic sewage treatment at low temperatures is proposed, in which a reactor system for denitrification coupled to anaerobic methane oxidation is integrated with a UASB-digester system and a nitritation reactor. For application of denitrification coupled to anaerobic methane oxidation in sewage treatment, volumetric nitrite consumption rate has to be increased significantly. This could be achieved by addition of growth factors or by improving biomass retention. Two sequencing fed-batch reactors were inoculated with freshwater sediment and operated at 30 °C. One was fed with synthetic medium, the other was fed with medium containing effluent from a sewage treatment plant as a source of potential growth factors. A maximum consumption rate of 37.8 mg NO_2^--N/L·d was obtained. Effluent from sewage treatment did not have a pronounced effect. Biomass washout was quantified and results indicate this could have significantly decelerated enrichment. Therefore, also two membrane bioreactors were operated. These were inoculated with sludge from sewage treatment, fed with medium containing effluent from sewage treatment and operated at 20 °C. The maximum volumetric consumption rate obtained in a membrane bioreactor was 35.5 mg NO_2^--N/L·d. This showed denitrifying methanotrophic bacteria can be enriched in a membrane bioreactor, from sewage sludge, and at 20 °C. Volumetric consumption rate is, however, still too low for application in sewage treatment.

Keywords
Denitrification, anaerobic methane oxidation, anaerobic sewage treatment, *Methylomirabilis oxyfera*, membrane bioreactor, sequencing fed-batch reactor

INTRODUCTION

Anaerobic sewage treatment has many advantages over activated sludge treatment, viz. energy production instead of consumption, lower sludge production, a smaller footprint, and simple construction and operation of anaerobic reactors (e.g. Lettinga, 1995, Lema and Omil, 2001). Despite these advantages and successful pilot-scale application of e.g. the UASB-digester system (Alvarez *et al.*, 2004, Mahmoud *et al.*, 2004, Mahmoud, 2008), anaerobic sewage treatment is not applied at low temperatures. This is mainly because effluent from anaerobic sewage treatment contains nitrogen and, especially at lower temperatures, a considerable amount of dissolved methane (Uemura and Harada, 2000, Cookney *et al.*, 2010). The latter is of great concern as methane becomes a greenhouse gas when released to the atmosphere.

Conventional nitrogen removal, consisting of nitrification to nitrate followed by heterotrophic denitrification, is inefficient for treatment of effluent from an anaerobic sewage treatment system. The effluent contains a low amount of readily biodegradable organic matter, therefore an external electron acceptor, such as methanol, would have to be added. Instead, the presence of nitrogen and dissolved methane offers the opportunity to develop a reactor system in which methane is used as

electron acceptor for denitrification. A new concept for anaerobic sewage treatment at low temperatures is proposed, in which such a reactor system is integrated with a UASB-digester system and a nitritation reactor, as shown in Fig. 1.

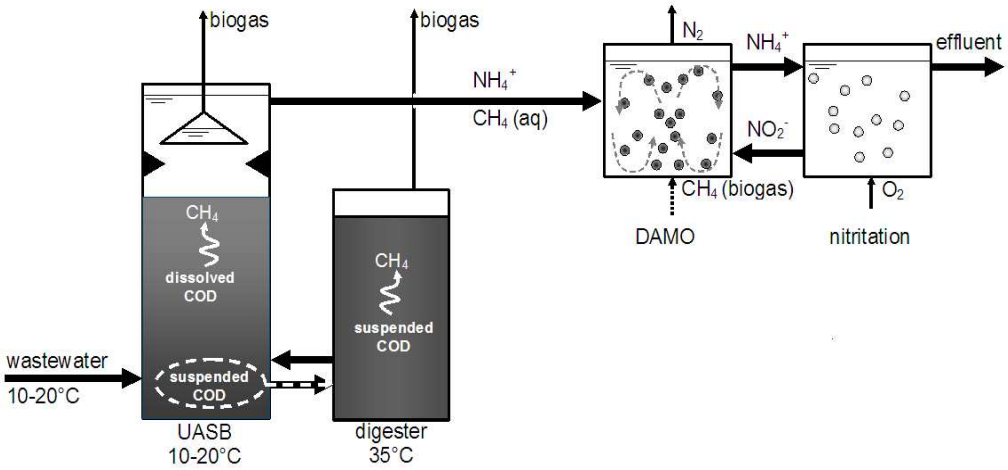

Figure 1. Proposed system configuration for anaerobic sewage treatment at low temperatures, consisting of a UASB-digester system, a reactor for nitrogen and methane removal by means of denitrification coupled to anaerobic methane oxidation (abbreviated as DAMO) and nitritation; adapted from Hendrickx *et al.* (2010).

Recently a bacterium, 'Candidatus *Methylomirabilis oxyfera*', was enriched that couples denitrification to anaerobic methane oxidation, according to Eq. 1 (Raghoebarsing *et al.*, 2006, Ettwig *et al.*, 2008).

$$3\ CH_4 + 8\ NO_2^- + 8\ H^+ \rightarrow 3\ CO_2 + 4\ N_2 + 10\ H_2O \qquad \textbf{Equation 1.}$$

The genome showed this bacterium employs a so far unknown intra-aerobic pathway. *M oxyfera* converts two molecules of nitrite, via nitric oxide, to one molecule of nitrogen gas and one molecule of oxygen. The oxygen is subsequently used for aerobic methane oxidation (Ettwig *et al.*, 2010).

Denitrifying methanotrophic bacteria have been enriched from freshwater sediments, using a completely stirred tank reactor with external settler (Raghoebarsing *et al.*, 2006) and sequencing (fed-)batch reactors (Ettwig *et al.*, 2008, Ettwig *et al.*, 2009), and from a mixture of freshwater sediment and sludge from sewage treatment, using a sequencing batch reactor (Hu *et al.*, 2009). The growth rate of denitrifying methanotrophic bacteria is extremely low and therefore enrichment times were long. Time before denitrifying methanotrophic activity was observed was shortest, viz. 5 months, and volumetric nitrite consumption rate was highest, viz. 36.1 mg NO_2^--N/L·d, in a sequencing batch reactor inoculated with sediment from Ooijpolder, The Netherlands, and operated at 30 °C (Ettwig *et al.*, 2009). For practical application, the volumetric nitrite consumption rate would have to be increased about 10 times to compete with conventional denitrification. However, at the end of reactor operation, the nitrite consumption rate stagnated (Ettwig *et al.*, 2008, Ettwig *et al.*, 2009). Stagnation was hypothesized to be due to product inhibition or growth factor deficiency (Ettwig *et al.*, 2008). With the systems that were used, not all biomass was retained and therefore stagnating activities could also have been due to an inefficient biomass retention.

In the present research denitrifying methanotrophic bacteria were enriched in two sequencing fed-batch reactors (SFBRs) inoculated with Ooijpolder sediment and operated at 30 °C. One of the reactors was fed with synthetic medium. The other was fed with medium to which effluent from sewage treatment was added as a source of potential growth factors. It was hypothesized in this reactor stagnation of nitrite consumption rate would not occur, or would occur at a higher rate.

Stagnation could also be due to inefficient biomass retention. To evaluate biomass retention of the SFBRs, biomass washout was quantified. For efficient biomass retention also two membrane bioreactors (MBRs) were operated. These were inoculated with a mixture of sewage sludge, fed with medium containing effluent from a sewage treatment plant, and operated at 20 °C to study the applicability of denitrification coupled to anaerobic methane oxidation for anaerobic sewage treatment at low temperatures. In all reactors consumption rates of nitrite, nitrate and methane were followed in time. Molecular tools were used to determine presence of denitrifying methanotrophic bacteria and follow their abundance in time. The different enrichment strategies and applicability of denitrification coupled to anaerobic methane oxidation for anaerobic sewage treatment at low temperatures were evaluated.

METHODS

Reactor setup and operation

Four reactors for enrichment of denitrifying methanotrophic bacteria were operated. An overview of the different conditions applied to each reactor is shown in Table 1.

Table 1. Overview of different conditions applied in the four enrichment reactors.

	MBR-sludge+sediment	MBR-sludge	SFBR-	SFBR+
Temperature (°C)	20	20	30	30
Liquid volume (l)	4.6	4.6	5.3 – 6.7	5.3 – 6.7
Sludge mixture [a]	+	+	-	-
Ooijpolder sediment [b]	+ [c]	-	+	+
Medium with effluent [d]	+	+	-	+
CH_4/CO_2	+	+	+	+

a. 0.33 g VS/L of digested primary sludge, digested secondary sludge and activated sludge (sewage treatment plant Ede, the Netherlands).
b. 3.0 g VS/L (based on liquid volume of 6.7 l) Ooijpolder sediment.
c. Concentrated effluent from SFBRs, inoculated with Ooijpolder sediment, added at day 421.
d. Medium contains 10% filtered (0.2 μm) effluent from sewage treatment plant Bennekom, the Netherlands.

Membrane bioreactors

Two membrane bioreactors (MBR) with a working volume of 4.6 l were continuously fed with medium and CH_4/CO_2 (5.0 – 10 ml/min, 93.6 – 95.0 % CH_4, 5.0 – 6.4 % CO_2). Effluent was pumped off via a membrane (pore size 30 – 50 nm; VFU-250, Memos Membranes Modules Systems GmbH) for complete biomass retention. The reactors were operated at 20 ± 1 °C.

Both reactors were inoculated with 1.0 g VS/L (0.37 g protein/L) of a sludge mixture, containing 0.33 g VS/L (determined after washing; described below) digested primary sludge, secondary sludge and digested secondary sludge (sewage treatment plant Ede, the Netherlands). Prior to inoculation the sludge was washed to remove dissolved organic matter which could serve as substrate for heterotrophic denitrification. Sludge was centrifuged (digested primary sludge and activated sludge 5 min, digested secondary sludge 10 min, at 2,500 g) and the pellets were resuspended in water. This was repeated four times. Thereafter sludge was centrifuged once more and the pellets were dissolved in a small amount of water. This was used to inoculate the reactors. After 421 days of operation 0.10 g protein/L of concentrated effluent from the sequencing batch reactors collected between month 12 and 15 of SFBR operation and stored at 4 °C was added.

Sequencing fed-batch reactors

Two sequencing fed-batch reactors (SFBRs) with a working volume of 5.3 – 6.7 l were operated. The reactors were operated in cycles of 1.0 – 11.5 d of continuous medium supply, followed by a

settling period of 2 h and a decanting period of 1 h. During the supply period 5.0 – 10 ml/min CH_4/CO_2 (93.6 – 95.0 % CH_4, 5.0 – 6.4 % CO_2) was supplied, and reactor contents were mixed by gas recirculation. During decanting 25 ml/min CH_4/CO_2 was supplied. After 623 days in SFBR- an ultrafiltration membrane (pore size 30 – 50 nm; VFU-250, Memos Membranes Modules Systems GmbH) was placed and since then effluent was pumped off via the membrane. Cyclic operation was controlled and data (pH and temperature) were acquired using FieldPoint modules and LabVIEW 7.0 (National Instruments). Reactor temperature was controlled at 30 ± 1 °C.
Both reactors were inoculated with 3.0 g VS/L (0.55 g protein/L; both calculated for maximum liquid volume) Ooijpolder sediment, The Netherlands.

Medium
The reactors were fed with medium adapted from (Ettwig *et al.*, 2009). The nitrite concentration in the medium and the influent flow rate were changed to adjust the nitrite loading rate (NLR) to nitrite consumption rate. The nitrate concentration was changed to have nitrate available in the reactors at all times and thereby prevent sulphate reduction. The bicarbonate concentration was changed based on pH, i.e. the concentration was decreased as the nitrite consumption rate and thereby the proton consumption rate increased. The pH was controlled between 7 and 8 by an equilibrium between bicarbonate and carbon dioxide. SFBR- was fed with completely mineral medium. SFBR+ and both MBRs were fed with medium containing 10% (v/v) filtered effluent from sewage treatment plant Bennekom, The Netherlands, as a source of potential growth factors. At this treatment plant sewage is treated by means of an activated sludge process, including biological nitrogen and phosphorus removal. Effluent from the activated sludge process is treated in a sand filter in which remaining phosphate is removed by means of iron precipitation. On average the effluent contained 1.3 mg biochemical oxygen demand/L, 24 mg COD/L, 2.1 mg Kjeldahl nitrogen/L and 3.8 mg (NO_2^-+NO_3^-)-N/L. Effluent was filtered over a 0.2 μm filter to remove colloidal and suspended matter, such as microorganisms.

Analyses
Nitrite and nitrate concentrations were estimated 3 – 5 times a week using test strips (Merckoquant, Merck chemicals) and measured once per week to once every other week by ion chromatography (Metrohm IC Compact 761), using a method adapted from APHA standard method 4110 B.
Methane, nitrogen, carbon dioxide and oxygen were measured by gas chromatography (Shimadzu GC-2010). The gas chromatograph was equipped with two columns (Porabond Q (50 m x 0.53 mm; 10 μm, Varian, part no. CP7355) and Molsieve 5A (25 m x 0.53 mm; 50 μm; Varian; Part.no. CP7538) connected in parallel. 50 μl samples were injected into an injector at 120 °C. The column was at 1.7 bar and 65 °C. Gases were detected by means of a thermal conductivity detector at 150 °C. The carrier gas was helium at 82.5 ml/min.
Samples (1 - 25 ml) for protein determination were centrifuged (5 min, 1-2 ml samples at 9,300 g, larger samples at 5,000 g) and supernatant was removed. The pellets were resuspended in 0.5 ml 1.0 M NaOH and the cells were hydrolysed for 30 min at 50 °C. After hydrolysis samples were neutralized with 0.5 ml 1.0 M HCl. Next, protein concentration was measured according to modified Hartree-Lowry method (Caprette, 1995). VS concentration was determined according to Standard Method 2540 (APHA, 1998).

RESULTS AND DISCUSSION

Nitrite loading rates
In all reactors nitrite consumption rates increased in time, but eventually stagnated and decreased. This can be seen from Fig. 2, showing the weighted average of NLR applied to the reactors.

After a lag phase of 300 days, in both MBRs (Phase I in Fig. 2A and 2B) the NLR could be increased (Phase II in Fig. 2A and 2B). At day 421 concentrated effluent from SFBRs was added to MBR-sludge+sediment and the NLR could be increased even more, though interrupted by a short period with operational problems occurring around day 510. A maximum NLR of 35.5 mg NO_2^--N/L·d was applied at day 543 (Phase III in Fig. 2A). From day 556 on, first a constant, later a decreasing NLR had to be applied (Phase IV in Fig. 2A). The decrease just started at the end of the reported period and prolonged operation is required to see if this trend continues.
The NLR applied to MBR-sludge was increased until day 476, with a maximum NLR of 26.9 mg NO_2^--N/L·d at day 464 (Phase II in Fig. 2B). From day 476 onwards, the NLR had to be decreased. For a short period of time, the NLR was increased again (a maximum NLR of 27.2 mg NO_2^--N/L·d was applied at day 518), but this resulted in too much nitrite accumulation, and therefore NLR was decreased again. At the end of the reported period, nitrite consumption rate in MBR-sludge seemed to stabilize around 8 mg NO_2^--N/L·d (Phase IV in Fig. 2B).

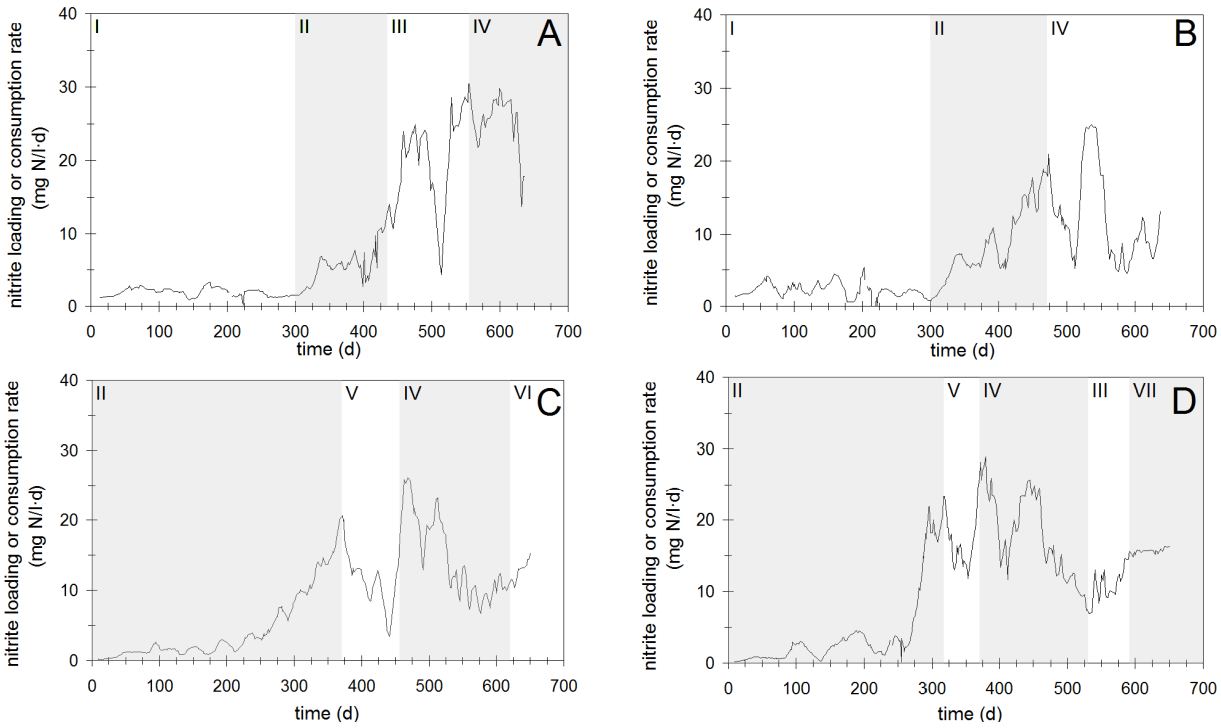

Figure 2. Weighted average (of 10 measurements) of nitrite loading rates applied to the four enrichment reactors, (A) MBR-sludge+sediment, (B) MBR-sludge, (C) SFBR- and (D) SFBR+. Areas indicated by Roman numerals indicate (I) lag phase, (II) exponential growth phase, (III) increasing nitrite consumption rates, in MBR-sludge+sediment resulting from addition of concentrated effluent from SFBRs to MBR-sludge+sediment, in SFBR+ cause remained unknown, (IV) stagnating/decreasing nitrite consumption rates, (V) operational problems, (VI) membrane placed in SFBR-, (VII) stabilizing nitrite consumption rates.

From the start, in both SFBRs the NLR could be increased. The NLR applied to SFBR- was exponentially increased to 25.1 mg NO_2^--N/L·d on day 364 (Phase II in Fig. 2C), and to a maximum of 33.5 mg NO_2^--N/L·d on day 457. Between day 361 and 406 the increasing trend was interrupted. (too much nitrite had accumulated, therefore temporarily NLR was decreased), but could eventually be increased to a new maximum of 33.5 mg NO_2^--N/L·d (Phase V in Fig. 2C). From day 457 on (Phase IV in Fig. 2C), the NLR had to be decreased because of a decreasing nitrite consumption rate. Frequently medium supply had to be paused because too much nitrite accumulated in the reactor. Finally, the NLR fluctuated around 10 mg NO_2^--N/L·d. At day 623, a membrane was placed

in the reactor for effluent extraction. Since then (Phase VI in Fig. 2C) NLR could be increased to a value of 15.3 mg N/L·d at day 651. Prolonged operation is required to see the effect on long term.
The NLR applied to SFBR+ was increased exponentially (Phase II in Fig. 2D) to a maximum of 37.8 mg NO_2^--N/L·d on day 372, though interrupted by technical problems (Phase V in Fig. 2D). After reaching this maximum, NLR had to be adjusted frequently in order not to under- or overload the reactor (Phase IV in Fig. 2D). NLR could be increased again from day 529 on (Phase III in Fig. 2B), and the nitrite consumption rate stabilized at about 16 mg NO_2^--N/L·d (Phase VII in Fig. 2D).

Until day 421, operation of MBR-sludge+sediment and MBR-sludge was similar: the same inoculum and startup procedure were used, also the NLR that could be applied to both reactors was about equal. When, at day 421, effluent from SFBRs was added to MBR-sludge+sediment, the nitrite consumption rate in this reactor increased and was higher than in MBR-sludge, with a short exception when operational problems occurred around day 510. The maximum NLR applied to MBR-sludge+sediment (35.5 mg NO_2^--N/L·d) was higher than the maximum NLR applied to MBR-sludge (27.2 mg NO_2^--N/L·d). Also, the NLR stabilized at a higher value, whereas NLR applied to MBR-sludge sharply decreased after 476 days of operation, and eventually stabilized at a much lower NLR.
The maximum NLR applied to SFBR+ (37.8 mg NO_2^--N/L·d) was somewhat higher than maximum NLR applied to SFBR- (33.5 mg NO_2^--N/L·d). Whether this was because effluent from the sewage treatment plant contained a missing growth factor remains unknown. The stagnation and later on decrease of biomass activity in SFBR+ could indicate still an inhibiting compound was produced or an (other) unknown growth factor was absent.
Despite the different reactor configuration, inoculum and lower operation temperature, the maximum NLR that could be applied to MBR-sludge+sediment was similar to the maximum NLR applied to the SFBRs. The maximum NLR was also similar to the maximum value of 36.1 mg NO_2^--N/L·d reported by Ettwig et al. (2009).
In all reactors nitrite consumption rates stagnated and/or decreased. Decreasing or stagnating consumption rates were hypothesized to be due to product inhibition, shortage of an essential growth factor and, for the SFBRs, inefficient biomass retention. In the case of the MBRs biomass retention was complete, thus probably product inhibition or shortage of essential growth factors caused stagnating/decreasing nitrite consumption rates.

Denitrifying methanotrophic activity
With the SFBRs whole culture batch tests were performed to determine the coupling between denitrification of nitrite and anaerobic methane oxidation. In these tests nitrite, a low amount of nitrate, and methane were consumed, while nitrogen gas was produced. The molar conversion ratio of CH_4 : NO_2^- : N_2 was close to the theoretical ratio (3 : 8 : 4; Eq. 1) in tests with SFBR- after 324, 400 and 485 days and SFBR+ after 324 days.

Biomass growth and washout
From the exponential increase in NLR applied to all reactors a doubling time of the amount of bacteria in the reactor was estimated. For MBR-sludge+sediment estimated doubling time was 1.5 months, for MBR-sludge 1.3 months, for SFBR- 1.9 months and for SFBR+ 1.7 months.
Throughout reactor operation biomass washed out from the SFBRs. The washout was quantified during three months and was related to nitrite consumption. In this period, from SFBR- a total of 0.10 g protein and from SFBR+ a total of 0.18 g protein washed out, while in SFBR- about 4.4 g NO_2^--N and in SBFR+ about 7.0 mg NO_2^--N was consumed. Thus, from SFBR- 0.022 g protein and from SFBR+ 0.026 g protein washed out per g NO_2^--N added. Biomass yield for *M. oxyfera* is unknown, but has been estimated to be in the range of yields observed for other methanotrophs, viz. 0.008 – 0.13 g protein/g NO_2^--N. The lower value was estimated from the extremely low biomass

yield of sulfate reduction coupled to anaerobic methane oxidation (0.008 g protein/g NO_2^--N, calculated from a biomass yield of 0.6 g cell dry weight/mol methane (Nauhaus et al., 2007), assuming 0.5 g protein/g cell dry weight and conversion of methane according to eq. 2) and a relatively high value was estimated from the biomass yield of aerobic methanotrophs (0.13 g protein/g NO_2^--N, calculated from a biomass yield of 0.6 g cell dry weight/g methane (Vary and Johnson, 1967), also assuming 0.5 g protein/g cell dry weight and conversion of methane according to eq. 2). For the current study, these growth yields would imply that between 17 and 325 % of the new biomass washed out from the SFBRs during the three months washout was quantified. This is indeed a wide range, thus it is unclear how much biomass washout affected enrichment rate, but that it significantly decelerated enrichment is evident. This was confirmed by the shorter estimated doubling times of biomass in MBRs.

Microbial composition of enrichment cultures

Clone libraries were constructed, showing sequences obtained in this sturdy were related to sequences found in previous studies, where also *M. oxyfera*-like bacteria were enriched or detected (Raghoebarsing *et al.*, 2006, Ettwig *et al.*, 2008, Ettwig *et al.*, 2009, Hu *et al.*, 2009; data not shown).

Fluoresence *in situ* hybridization showed an increase in *M. oxyfera*-like bacteria in time. The *M. oxyfera*-like bacteria were not detected in the inoculum of neither MBR nor SFBR. Also after 7 months of MBR operation *M. oxyfera*-like bacteria were hardly observed, but dominated after 12 months. After 8 months of SFBR operation the bacteria were observed in both reactors and after 13 months the bacteria dominated both reactors.

CONCLUSIONS

- The present research confirms *M. oxyfera*-like bacteria can be enriched from Ooijpolder sediment in a sequencing fed-batch reactor at 30 °C.
- The maximum volumetric consumption rate was 37.8 mg NO_2^--N/L·d, which is slightly higher than the maximum value found in previous studies.
- Addition of effluent from sewage treatment did not have a pronounced effect.
- Biomass washout could have significantly decelerated enrichment.
- The present research shows for the first time that *M. oxyfera*-like bacteria can be enriched from a mixture of sewage sludge in a membrane bioreactor at 20 °C, fed with medium containing effluent from sewage treatment.
- The maximum consumption rate obtained in this setup was 35.5 mg NO_2^--N/L·d, which is, despite the lower temperature, comparable to results obtained with Ooijpolder sediment in a sequencing fed-batch reactor.
- Estimated doubling times of the amount of bacteria in the MBRs (1.3 – 1.5 months) were shorter than in the SFBRs (1.7 – 1.9 months), indicating the MBR is a better reactor system for enrichment of slow growing bacteria.
- A new treatment concept for anaerobic sewage treatment at low temperature is proposed, using the UASB-digester system, a reactor coupling denitrification and anaerobic methane oxidation, and a nitritation reactor.
- Maximum volumetric nitrite consumption rates have to be increased about ten times before a reactor concept with denitrifying methanotrophic bacteria can compete with conventional denitrification and can be used in sewage treatment.
- Stagnating and decreasing nitrite consumption rates in all four enrichment reactors indicate an inhibiting product is formed or a growth factor is missing. By identifying such a compound activities might be increased.

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