Denitrification with dissolved methane for energy efficient wastewater treatment

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Abstract
Despite many advantages of anaerobic wastewater treatment over conventional activated sludge treatment, it has not yet been applied in temperate zones. This is mainly because effluent from anaerobic treatment still contains nitrogen and dissolved methane. A new concept for energy-efficient anaerobic wastewater treatment at low temperatures is proposed, consisting of a UASB-digester system and, for treatment of the anaerobic effluent, a reactor with denitrifying methanotrophic bacteria for nitrogen and dissolved methane removal and a nitritation reactor. Before application of the denitrification process, volumetric denitrification rates have to be increased. In this research denitrifying methanotrophic bacteria, '*Candidatus* Methylomirabilis oxyfera', were enriched in a membrane bioreactor, operated at 20 °C, inoculated with a mixture of wastewater treatment sludge and fed with medium containing effluent from municipal wastewater treatment as a source of potential growth factors. After a lag phase of 300 days, the volumetric consumption rate increased to 11 mg NO_2^--N/L·d at day 421. After spiking with denitrifying methanotrophic bacteria from another reactor, the rate increased to a new maximum of 36 mg NO_2^--N/L·d at day 655. These results indicate the potential applicability of the process for wastewater treatment, but still rates have to be increased by an order of magnitude.

Keywords
Anaerobic methane oxidation, anaerobic wastewater treatment, denitrification, energy balance, membrane bioreactor, '*Candidatus* Methylomirabilis oxyfera'

INTRODUCTION

In many regions municipal wastewater is treated by activated sludge processes. These require an energy input of ca. 1 kWh/m^3 wastewater, mainly used for oxidation of organic matter and ammonium nitrogen. The chemical energy contained in the wastewater (1.8 kWh/m^3 wastewater) is not recovered, though it could provide enough energy to make the treatment self-supporting. With anaerobic wastewater treatment this chemical energy could be directly recovered as the energy carrier methane.

In temperate zones (wastewater temperatures of 10-20 °C) anaerobic treatment systems are required that enable solid retention times (SRT) long enough for hydrolysis of colloidal and suspended matter and growth of methanogens, while being operated at hydraulic retention times (HRT) of about 6 h. This was previously achieved with a system consisting of an upflow anaerobic sludge bed (UASB) reactor (at 10-20 °C) and a sludge digester (at 30-35 °C). This system is referred to as UASB-digester (Mahmoud, 2008, Mahmoud *et al.*, 2004, Alvarez *et al.*, 2004).

To comply with discharge standards, the effluent from such an anaerobic treatment system requires further treatment. This is required for residual chemical oxygen demand (COD), but especially for nitrogen and phosphorus, which are largely conserved during anaerobic treatment. In addition, the effluent from low-temperature anaerobic treatment contains a considerable amount of dissolved methane (Cookney *et al.*, 2010, Uemura *et al.*, 2000). Because methane has a high global warming potential, this also needs to be removed.

Conventional technologies can be applied to remove residual COD and phosphorus from the effluent of anaerobic treatment. In contrast, nitrogen cannot be removed using the conventional sequence of nitrification and heterotrophic denitrification, because anaerobic treatment already removes the readily available carbon sources. The autotrophic process of anaerobic ammonium oxidation could be applied (Hendrickx *et al.*, 2012). However, this process does not remove dissolved methane. Instead, the recently discovered nitrite-denitrifying methanotrophic bacterium '*Candidatus* Methylomirabilis oxyfera' (hereafter *M.* oxyfera) offers the opportunity to develop a reactor that removes both nitrogen and dissolved methane, according to eq. 1.

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

For application of this process after low-temperature anaerobic wastewater treatment, ammonium from the effluent first has to be converted to nitrite in a separate nitritation reactor. According to above stoichiometry (eq. 1) a concentration of 20 mg/L dissolved methane (calculated assuming Henry's law, atmospheric pressure, 10 °C and 70 % methane in the biogas) suffices to remove 47 mg N/L, a concentration common for effluent from anaerobic wastewater treatment plants. To conserve methane for denitrification and to save on aeration energy this reactor is positioned after the reactor for denitrification coupled to anaerobic methane oxidation. The combination of UASB-digester, a reactor for denitrification coupled to anaerobic methane oxidation (DAMO), and a nitritation reactor offers a new opportunity for energy-efficient wastewater treatment (fig. 1).

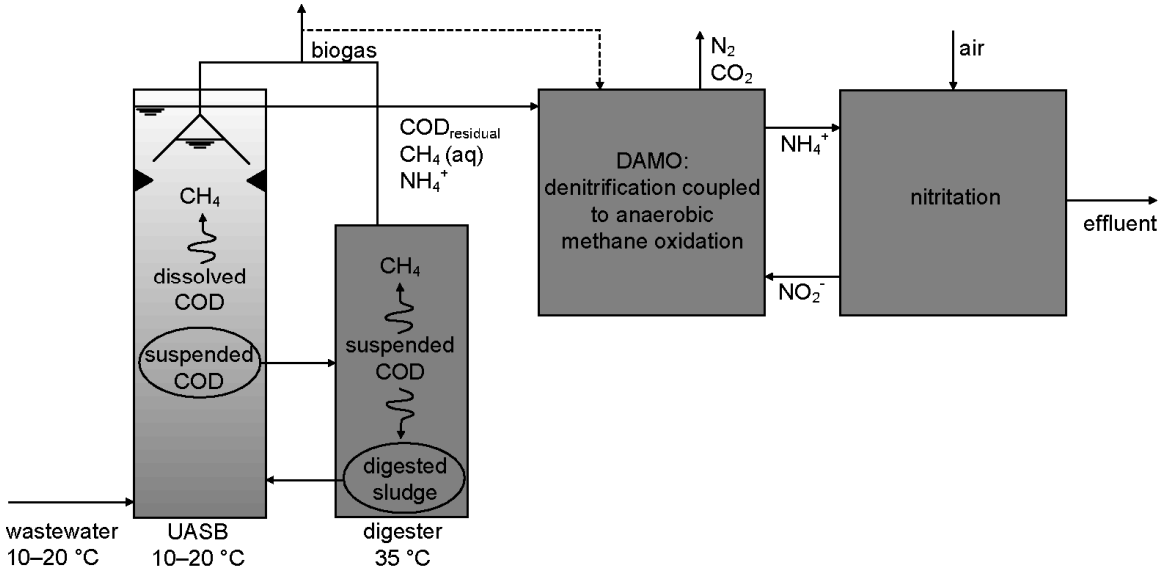

Figure 1. Proposed treatment concept for anaerobic wastewater treatment at low temperatures, consisting of a UASB-digester system, a reactor for nitrogen and methane removal by means of denitrification coupled to anaerobic methane oxidation and a nitritation reactor. Adapted from Hendrickx *et al.* (2010).

For implementation of this concept, the major challenge is to develop a reactor with denitrifying methanotrophic bacteria that can be operated at short HRT. Until now, the highest volumetric denitrifying methanotrophic activity, viz. 36 mg NO_2^--N/L·d, was obtained in a sequencing batch reactor inoculated with sediment from Ooijpolder, The Netherlands, operated at 30 °C (Ettwig *et al.*, 2009). Also in other studies, sequencing (fed-)batch reactors (Kampman *et al.*, submitted, Luesken *et al.*, 2011, Hu *et al.*, 2009, Raghoebarsing *et al.*, 2006) and a completely stirred tank reactor (Ettwig *et al.*, 2009) were used, from which possibly a significant amount of the slow-growing *M. oxyfera* washed out. In addition, a stagnating rate was observed in several enrichment cultures (Kampman *et al.*, submitted, Ettwig *et al.*, 2008, 2009). It was hypothesized this could be due to production of an inhibiting compound, or absence of an unknown growth factor.

For the treatment of effluent from anaerobic wastewater treatment plants maximum observed rates would translate to an HRT of 1.4 d. For implementation in wastewater treatment the rate needs to be increased by an order of magnitude and the process has to be operated at lower temperatures. The present study therefore focussed on enrichment of denitrifying methanotrophic bacteria from wastewater treatment sludge at 20 °C and used a membrane bioreactor (MBR) to ensure complete biomass retention. An overall energy balance for the concept will also be presented.

METHODS

Bioreactor operation

For the enrichment of denitrifying methanotrophic bacteria an MBR (working volume 4.6 L) was inoculated with 1.0 g VS/L (0.37 g protein/L) of a sludge mixture. Equal amounts (0.33 g VS/L; determined after washing) of each digested primary sludge, secondary sludge and digested secondary sludge (wastewater treatment plant Ede, The Netherlands) was added. The sludge was washed to remove dissolved organic matter because this could serve as substrate for heterotrophic denitrifying bacteria. These bacteria could compete for nitrite and thereby decelerate enrichment of denitrifying methanotrophic bacteria. Sludge was centrifuged (digested primary sludge and activated sludge 5 min, digested secondary sludge 10 min, at 2,500 g), the supernatant was discarded and the pellets were resuspended in water. This was repeated four times. Thereafter the sludge was centrifuged once more and the pellets were dissolved in a small amount of water. This concentrated, washed sludge was used to inoculate the reactor. After 421 days of operation 0.10 g protein/L and after 623 days 0.22 g protein/L of concentrated effluent from two sequencing fed-batch reactors (SFBRs) inoculated with sediment from ditches from Ooijpolder, The Netherlands (Kampman *et al.*, submitted), was added.

The MBR was continuously fed with CH_4/CO_2 (5.0-10 ml/min, 93.6-95.0 % CH_4, 5.0-6.4 % CO_2) and influent (adapted from Ettwig *et al.* (2009)). In addition it contained 10% (v/v) 0.2 µm filtered (to remove colloidal and suspended matter, such as microorganisms) effluent from municipal wastewater treatment plant Bennekom, The Netherlands, as a source of potential growth factors. At this treatment plant wastewater is treated by means of an activated sludge process, including biological nitrogen and phosphorus removal. Effluent from the 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. Reactor effluent was removed via a membrane (pore size 30-50 nm; VFU-250, Memos Membranes Modules Systems GmbH) to ensure complete biomass retention. The pH was controlled between 7.0 and 8.0 by equilibrium between CO_2 and HCO_3^-. The temperature was controlled at 20 ± 1 °C.

During the enrichment, the nitrite loading rate (NLR) was adjusted to match the consumption rate. The nitrite concentration, which was estimated 3-5 times per week, was maintained at 3-30 mg NO_2^--N/l. To prevent nitrite accumulation to toxic levels medium supply was stopped when the nitrite concentration exceeded 30 mg NO_2^--N/l. The medium supply was resumed when the concentration decreased to below 15 NO_2^--N/l. NLR was adjusted by adjusting the HRT (9-29 d) or medium concentration (0.014-0.980 g NO_2^--N/l). To control the pH between 7.0 and 8.0, the bicarbonate concentration in the medium was decreased in time (from 1.0 to 0.1 g/l), while the denitrification rate and thereby the proton consumption rate increased.

Analyses

Nitrite concentration was 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. Methane, nitrogen, carbon dioxide and oxygen were measured by gas chromatography. After

hydrolysis of the samples (Kampman *et al.*, submitted), protein concentration was determined by a modified Hartree-Lowry method (Hartree, 1972). VS concentration was determined according to Standard Method 2540 (APHA, 1998).
Fluorescence *in situ* hybridization (FISH) was performed using probes targeting bacteria affiliated with the NC10 phylum (Raghoebarsing *et al.*, 2006), the EUB mix for almost all bacteria; EUB338, EUB338II, EUB338III (Daims *et al.*, 1999) and the DNA stain DAPI (Kampman *et al.*, submitted).

Energy balance
The main assumptions for the energy balance were influent concentrations of 600 mg COD/L and 50 mg N/L; effluent concentrations of 40 mg COD/l and 10 mg N/l; an anaerobic COD conversion efficiency of 60 %; an aeration energy of 0.5 kWh/kg O_2; an energy content of 680 kJ/mol CH_4; a sludge circulation flow between UASB and digester of 2.5 % of the influent wastewater flow; and a wastewater temperature of 15 °C.

RESULTS AND DISCUSSION

Enrichment of denitrifying bacteria using a membrane bioreactor
After 300 days of low nitrite consumption (phase I, partly shown in fig. 2), the volumetric denitrification rate increased from 1.5 (day 300) to 11 mg NO_2^--N/l·d (day 421; phase II in fig. 2). After the addition of biomass from the effluent of two SFBRs enriched in *M. oxyfera* (at day 421), the nitrite consumption rate further increased to 34 mg NO_2^--N/l·d (day 623; phase III in fig. 2). In a control reactor to which no concentrated effluent was added such a sharp increase was not observed (data not shown), indicating the increase in volumetric nitrite consumption rate observed was a result of this addition. After 508 days the increase of denitrification rate slowed down. A second addition of biomass, at day 623 (start of phase IV in fig. 2), did not result in an acceleration. Instead, denitrification rate continued to increase very slowly. At day 655 a new maximum rate of 36 mg NO_2^--N/l·d was obtained.

Molecular analyses confirmed organisms of the type *M. oxyfera* were enriched and dominated the reactor (60-70 %, fig. 3) after a year of operation. Sequences obtained in this enrichment were related to sequences in other studies, in which also *M. oxyfera* was enriched or detected (Ettwig *et al.*, 2008, 2009, Raghoebarsing *et al.*, 2006, Hu *et al.*, 2009; data not shown).

The maximum volumetric denitrification rate achieved in the present study matches the highest rate reported so far. This was achieved using a membrane bioreactor for complete biomass retention, instead of a SFBR, at a lower temperature (20 °C vs. 30 °C) and with the addition of effluent from wastewater treatment, after 508 days the denitrification rate only slowly increased. Something similar was observed in previous enrichment studies (Kampman *et al.*, submitted, Ettwig *et al.* 2008, 2009). Ettwig et al. (2008) proposed this was due to production of an inhibiting compound, or absence of an unknown growth factor. Another explanation could be washout of biomass (Kampman *et al.*, submitted). However, in this enrichment study biomass retention was complete, and still denitrification rates stabilized, suggesting other limitations exist. The maximum rate in this study was reached after biomass enriched in *M. oxyfera*, originating from SFBRs inoculated with Ooijpolder sediment, was added. The maximum rate in the control reactor inoculated with sludge only stayed behind. This suggested sediment from Ooijpolder contained a growth factor lacking in a reactor inoculated with sludge or a different phylotype was enriched from the sludge than from the sediment. A second addition of biomass did not result in such a sharp increase in nitrite consumption rates as was observed before and denitrification rates stabilized, though at a higher rate. This suggests that product inhibition or lack of essential growth factors have caused the

stagnating nitrite consumption rates and prevented an increase in consumption rates even after addition of biomass. Adding potential growth factors and applying higher flow rates to washout inhibiting products are now being studied to increase the denitrification rates.

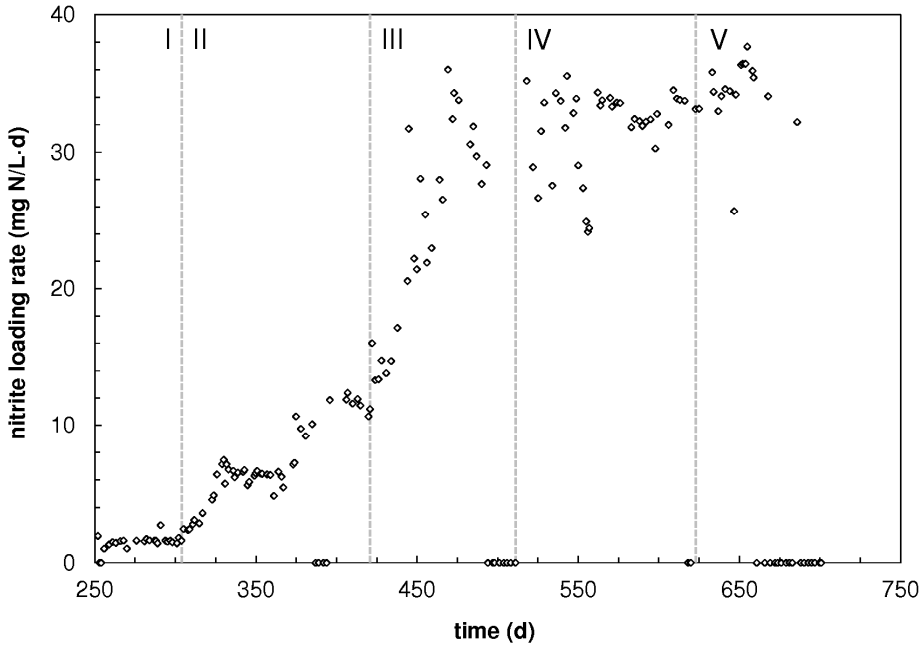

Figure 2. Nitrite loading rate applied to the reactor in time. Latin numbers indicate (I) lag phase, (II) exponential growth phase, (III) increase in nitrite loading rate after addition of concentrated effluent from two SFBRs inoculated with sediment from Ooijpolder, The Netherlands (IV) increase of nitrite loading rate slowed down and (V) addition of concentrated effluent from SFBRs. An NLR of zero was set to avoid nitrite accumulation or caused by technical problems (such as failing pumps).

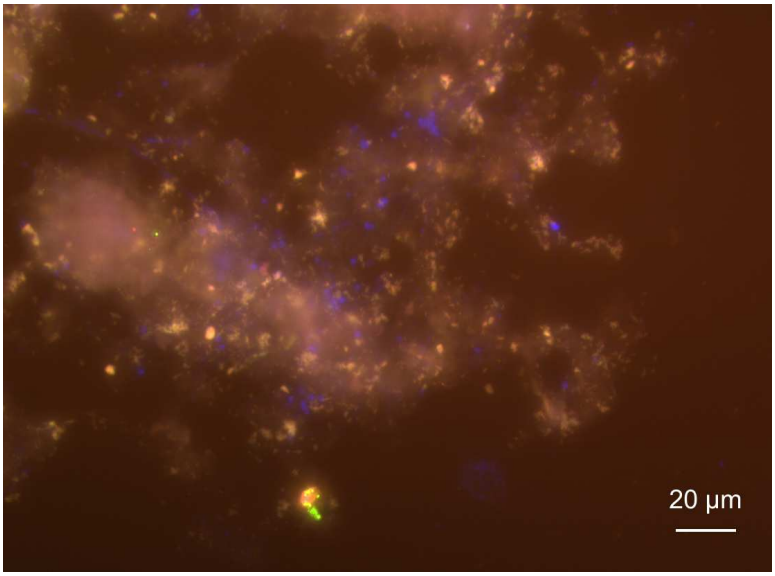

Figure 3. Fluorescence *in situ* hybridization of biomass from the membrane bioreactor after 12 months of enrichment. Fluorescence micrograph after hybridization with probes DBACT1027 (Cy3; red) specific for NC10 bacteria; and EUB mix (probes EUB338 I-III; Cy5; dark blue), detecting nearly all eubacteria. Due to co-hybridization with the specific and general probes, the *M. oxyfera* bacteria appear pink.

Energy balance

Fig. 4 shows the energy balance for direct anaerobic treatment of municipal wastewater treatment combined with the DAMO process for removal of nitrogen and dissolved methane. The energy recovered as methane (0.86 kWh/m^3 wastewater) is sufficient for heating the digester and for nitritation and oxidation of residual organic material. These calculations assumed 100% conversion efficiency of biogas to electricity and heat. Also, energy losses, occurring in e.g. heating and aeration, were not taken into account.

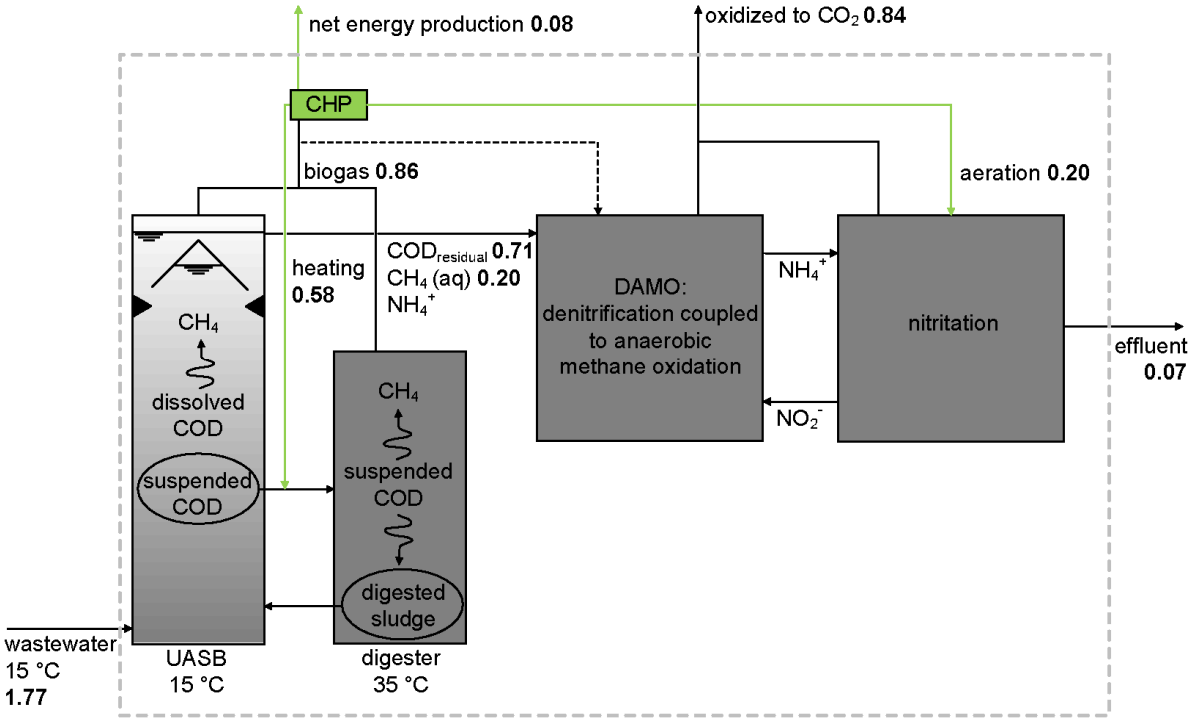

Figure 4 Energy balance for direct anaerobic treatment of municipal wastewater combined with nitritation and the DAMO process for nitrogen removal. CHP stands for combined heat and power. All numbers are in kWh/m^3 of influent wastewater. Energy recovery from digester effluent is not included. Also not included are the energy losses occurring in CHP, heating and aeration, energies for pumping, mixing, sludge processing, etc.

Some of the assumptions used in the calculations are still subject of further research. In the calculations a recirculation rate of 2.5 % of the influent flow rate was consumed. Experimental optimization should show whether this rate may be lower, whilst still achieving the necessary stabilization of the sludge from the UASB and at the same time provide sufficient methanogenic biomass that is grown in the digester. This would result in lower energy requirements for pumping sludge to the digester and for heating of the transported sludge. For the calculations an anaerobic COD conversion of 60% was assumed. Variations in the anaerobic efficiency have a large impact on the total energy balance. A lower anaerobic biodegradability may not result in net energy production. Also energy losses and energy requirements for equipment and processing will have to be taken into account. Overall, however, the proposed system will still allow a lower net energy consumption compared to conventional aerobic waste water treatment requiring an energy input of appr. 1 kWh/m^3 and not recovering chemical energy contained in the wastewater.

Implications

In this study, using a different reactor setup, viz. a MBR, a lower enrichment temperature and the addition of effluent from municipal wastewater treatment, similar bacteria were enriched and similar maximum volumetric consumption rates were obtained as in previous studies. These results indicate that it is potentially interesting to apply *M. oxyfera* in wastewater treatment, although the

volumetric denitrification rate has to be increased by an order of magnitude before practical application becomes possible. Reactor operation will be continued and effect of different growth factors and of flow rate on volumetric denitrification rate will be studied. If the rate is increased, a system with denitrifying methanotrophic bacteria could be used in a new, energy efficient, treatment concept for anaerobic sewage treatment at low temperatures. The proposed concept consists of a UASB-digester system for anaerobic wastewater treatment, a reactor for denitrification coupled to anaerobic methane oxidation for removal of nitrite and dissolved methane and a nitritation reactor for conversion of ammonium to nitrite. This concept would make use of all advantages characteristic of anaerobic treatment whilst removing effluent methane and nitrogen. The concentration of dissolved methane determines if denitrification using denitrifying methanotrophic bacteria is feasible. If not, possibly in summer periods, either biogas could be added, or denitrifying methanotrophic bacteria have to work side-by-side with anammox bacteria, as proposed by Luesken *et al.* (2011).

CONCLUSIONS

- The present research shows for the first time that *M. oxyfera* can be enriched from a mixture of sludge from municipal wastewater treatment in a membrane bioreactor at 20 °C, fed with medium containing effluent from municipal wastewater treatment.
- The maximum denitrification rate of the present enrichment (36 mg NO_2^--N/L·d) matches the highest rate in literature, despite the lower enrichment temperature (20 °C vs. 30 °C).
- The stagnating volumetric consumption rate indicates an inhibiting product is formed or a growth factor is missing.
- Maximum volumetric nitrite consumption rates have to be increased by an order of magnitude before a reactor concept with denitrifying methanotrophic bacteria can compete with conventional denitrification and can be used in wastewater treatment.
- The proposed concept of a UASB-digester and denitrification coupled to anaerobic methane oxidation offers an option for energy neutral municipal wastewater treatment.

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