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Enrichment of denitrifying methanotrophic bacteria for application after direct low-temperature anaerobic sewage treatment

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HIGHLIGHTS

- A new concept for low-temperature anaerobic sewage treatment is proposed.
- In this concept, denitrification and methane oxidation are performed by *Methylomirabilis oxyfera*.
- The bacteria were enriched from fresh water sediment using sequencing fed-batch reactors.
- The volumetric consumption rate has to be increased by an order of magnitude for practical application.
- Further research should focus on systems with improved biomass retention.

GRAPHICAL ABSTRACT

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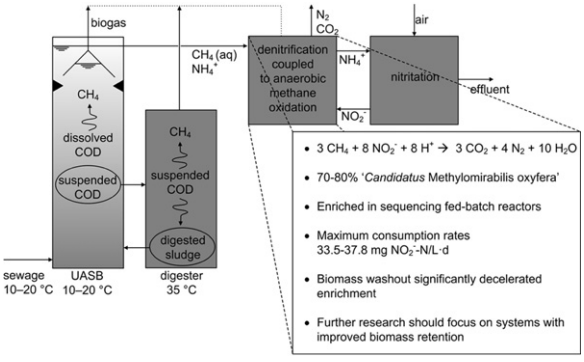

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ABSTRACT

Despite many advantages of anaerobic sewage treatment over conventional activated sludge treatment, it has not yet been applied in temperate zones. This is especially because effluent from low-temperature anaerobic treatment contains nitrogen and dissolved methane. The presence of nitrogen and methane offers the opportunity to develop a reactor in which methane is used as electron donor for denitrification. Such a reactor could be used in a new concept for low-temperature anaerobic sewage treatment, consisting of a UASB-digester system, a reactor for denitrification coupled to anaerobic methane oxidation, and a nitritation reactor. In the present study denitrifying methanotrophic bacteria similar to '*Candidatus* Methylomirabilis oxyfera' were enriched. Maximum volumetric nitrite consumption rates were 33.5 mg NO_2^--N/L d (using synthetic medium) and 37.8 mg NO_2^--N/L d (using medium containing effluent from a sewage treatment plant), which are similar to the maximum rate reported so far. Though the goal was to increase the rates, in both reactors, after reaching these maximum rates, volumetric nitrite consumption rates decreased in time. Results indicate biomass washout may have significantly decelerated enrichment. Therefore, to obtain higher volumetric consumption rates, further research should focus on systems with complete biomass retention.

1. Introduction

Anaerobic sewage treatment has many advantages over conventional activated sludge treatment. These include energy recovery as biogas instead of energy consumption, reduced sludge production and a smaller footprint (e.g. [1,2]). Despite these advantages

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and successful application of anaerobic sewage treatment in tropical regions it has not yet been applied in temperate zones [3]. For lower temperatures reactor systems with solid retention times (SRT) long enough for hydrolysis and growth of methanogens, but still relatively short hydraulic retention times (HRT) are required. Also, to comply with discharge standards, effluent from anaerobic treatment requires further treatment. This is required for remaining chemical oxygen demand (COD), but especially for nitrogen and phosphorus, which are largely conserved during anaerobic treatment. In addition, the effluent from a low-temperature anaerobic sewage treatment system contains a considerable amount of dissolved methane [4,5]. The concentration of dissolved methane can be 20 mg/L assuming Henry's law (calculated for atmospheric pressure, 10 °C and 70% methane in the biogas), and frequently methane supersaturation occurs [4,5]. If effluent containing dissolved methane would be discharged, methane would be released to the atmosphere. As it is a gas with a high global warming potential, dissolved methane has to be removed to reduce the greenhouse gas emissions of low-temperature anaerobic sewage treatment compared with conventional treatment.

Pilot-scale application of the combination of an upflow anaerobic sludge bed (UASB) reactor and a sludge digester, referred to as UASB-digester system, was successful for anaerobic sewage treatment at low temperatures [6–8]. In the UASB reactor (at 10–20 °C) dissolved COD is converted; solids are entrapped in the flocculent sludge bed and transported to the digester. In the digester (at 35 °C) suspended COD is hydrolyzed and the sludge is enriched in methanogens. The sludge is recirculated to the UASB reactor to provide methanogenic activity. With this system a total COD removal efficiency of 66% was achieved at a temperature of 15 °C and an HRT of 6 h while a long SRT of 21 d was maintained in the digester [7]. Although conventional technologies can be applied to remove remaining COD and phosphorus from the effluent, conventional nitrogen removal is not a preferred option. Effluent from an anaerobic system contains ammonium, which is usually removed by a sequence of nitrification to nitrate and heterotrophic denitrification. However, during anaerobic treatment the readily available carbon sources are removed and addition of an external electron donor, e.g. methanol, would be required to sustain heterotrophic denitrification. Anaerobic ammonium oxidation, an autotrophic process, would be an alternative [9]. However, this process will not remove dissolved methane. Instead, a new treatment concept is proposed, in which dissolved methane is used as electron donor for denitrification via nitrite. Such a system would solve two problems, viz. removal of nitrogen and dissolved methane. To provide nitrite a nitritation reactor is required. 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. Combined, the UASB-digester, a reactor for denitrification coupled to anaerobic methane oxidation, and a nitritation reactor, to supply nitrite required for the denitrifying methanotrophic bacteria, offer a new opportunity for energy-efficient wastewater treatment with a reduced carbon footprint (Fig. 1).

Though denitrification coupled to aerobic methane oxidation was studied extensively (reviewed by [10]), the progress on denitrification coupled to anaerobic methane oxidation is slow due to a limited number of enrichment cultures [11]. However, denitrification coupled to anaerobic methane oxidation (Eq. (1)), would have several advantages over aerobic processes. These include that no oxygen is required for partial methane oxidation and methane is used more efficiently. This implies that more nitrogen can be removed using the methane dissolved in the effluent from UASB-digester systems.

$$3CH_4 + 8NO_2^- + 8H^+ \rightarrow 3CO_2 + 4N_2 + 10H_2O \quad (1)$$

A few years ago a denitrifying methanotrophic culture consisting of a bacterium and an archaeon was obtained under anaerobic conditions [12]. Further research has shown that the process also proceeds without the archaea, indicating that the dominant bacterium, '*Candidatus* Methylomirabilis oxyfera' (*M. oxyfera* hereafter) can catalyze the methane oxidation on its own [13,14], expressing a unique intra-aerobic pathway [15].

Typically effluent from anaerobic sewage treatment plants contains 50 mg N/L. Using the 20 mg/L of dissolved methane, 47 mg N/L could be removed according to the stoichiometry presented in Eq. (1). The maximum volumetric nitrite consumption rate of enrichment cultures coupling denitrification to anaerobic methane oxidation reported is 36 mg NO_2^--N/L d [14]. This rate would translate to an HRT of 1.4 d. Conventional denitrification typically has an HRT of 3–4 h. Thus, for a practical application of denitrification coupled to anaerobic methane oxidation for sewage treatment, volumetric nitrite consumption rate needs to be increased by an order of magnitude. However, a stagnating rate was observed in two enrichment cultures [13,14]. It was hypothesized this could be due to production of an inhibiting compound, or absence of an unknown growth factor. Since a completely stirred tank reactor with external settler and a sequencing batch reactor were applied, inefficient biomass retention may also have been a cause for the stagnating conversion rates.

The objectives of this study were (1) to enrich denitrifying methanotrophic cultures and (2) to increase the volumetric conversion rates of the enrichment cultures, so eventually the process can be integrated in the proposed concept for anaerobic sewage treatment at low temperatures.

Denitrifying methanotrophic bacteria were enriched for a period of 651 d in two sequencing fed-batch reactors. To increase maximum volumetric conversion rates, a long settling time was applied to improve biomass retention and effluent from a sewage treatment plant was fed to one of the reactors as a source of potential growth factors. The reactors were mixed by gas recirculation, providing sufficient transfer of methane. In both reactors, nitrite consumption rates were followed in time and whole culture batch tests were performed to measure denitrifying methanotrophic activity. Washout of biomass with the effluent was quantified to evaluate biomass retention of the systems. The practical applicability of a process with denitrifying methanotrophic bacteria for nitrogen and methane removal after direct low-temperature anaerobic sewage treatment is discussed.

2. Materials and methods

2.1. Inoculum

Two sequencing fed-batch reactors (SFBRs) were inoculated with sediment (3.7 ± 0.6 g protein each) from ditches in Ooijpolder, The Netherlands, similar to [14]. Prior to inoculation the sediment was sieved (1.0 mm) and diluted with ditch water to obtain a homogeneous slurry.

2.2. Medium

Medium contained (g/L) 0.1–1.0 $KHCO_3$, 0.05 KH_2PO_4, 0.30 $CaCl_2{\cdot}2H_2O$, 0.22 $MgSO_4{\cdot}7H_2O$, 0.069–4.83 $NaNO_2$ (0.014–0.980 NO_2^{-1}–N), 0.085–0.765 $NaNO_3$ (0.014–0.126 NO_3^{-1}–N), 0.6 mM HCl, 0.5 mL acidic trace element solution and 0.2 mL alkaline trace element solution (adapted from Ettwig et al. [14]). The acidic trace element solution contained (g/L) 2.085 $FeSO_4{\cdot}7H_2O$, 0.068 $ZnCl_2$, 0.12 $CoCl_2{\cdot}6H_2O$, 0.5 $MnCl_2{\cdot}4H_2O$, 0.32 $CuSO_4$, 0.048 $NiCl_2{\cdot}6H_2O$ and 100 mM HCl. The alkaline trace element solution contained (g/L) 0.067 SeO_2, 0.05 $Na_2WO_4{\cdot}2H_2O$, 0.284 $Na_2MoO_4{\cdot}2H_2O$ and 10 mM NaOH.

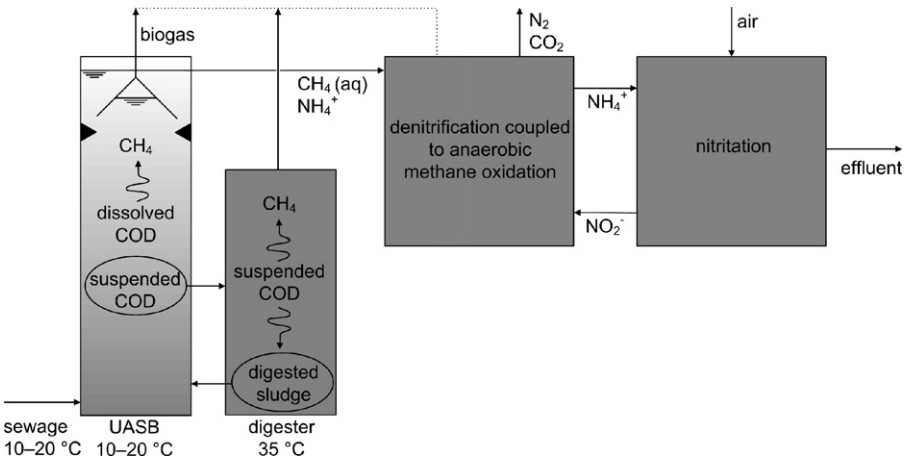

Fig. 1. New concept for sewage treatment at low temperatures, consisting of anaerobic sewage treatment for removal of organic matter, complemented with denitrification coupled to anaerobic methane oxidation and nitritation for nitrogen and dissolved methane removal.

One reactor, referred to as SFBR−, was fed with this synthetic medium. The other reactor, referred to as SFBR+, was fed with medium containing 10% (v/v) filtered effluent from the aerobic sewage treatment Bennekom, The Netherlands, as a source of potential growth factors. Effluent from aerobic treatment, i.e. low in residual COD, was selected to prevent enrichment of heterotrophic denitrifying bacteria, which might compete with, and thereby hamper, enrichment of denitrifying methanotrophic bacteria. 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-N/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.

2.3. Setup of sequencing fed-batch reactors

The enrichments were performed in two anaerobically operated SFBRs, in a setup as shown in Fig. 2. The SFBRs each had a volume of 10 L, with a working volume of 5.3–6.7 L, and 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 (effluent removed at 20–25 mL/min). 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 gas was recirculated to provide mixing and sufficient gas transfer. Gas, both supplied gas and recirculated gas, was added from the bottom of the reactor, through a glass diffuser producing small bubbles. During decanting 25 mL/min CH_4/CO_2 was supplied to counteract the effluent removal and to prevent air from entering the reactor. After 623 d in SFBR− an ultrafiltration membrane (VFU-250, Memos Membranes Modules Systems GmbH) was placed and liquid 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. Though higher than applied in sewage treatment in temperate zones, this temperature was selected for faster enrichment of denitrifying methanotrophic bacteria.

2.4. Operation of sequencing fed-batch reactors

During the reported 651 d of enrichment, the nitrite loading rate (NLR; calculated as the daily nitrite addition per maximum reactor volume, viz. 6.7 L) was controlled to match the consumption rate.

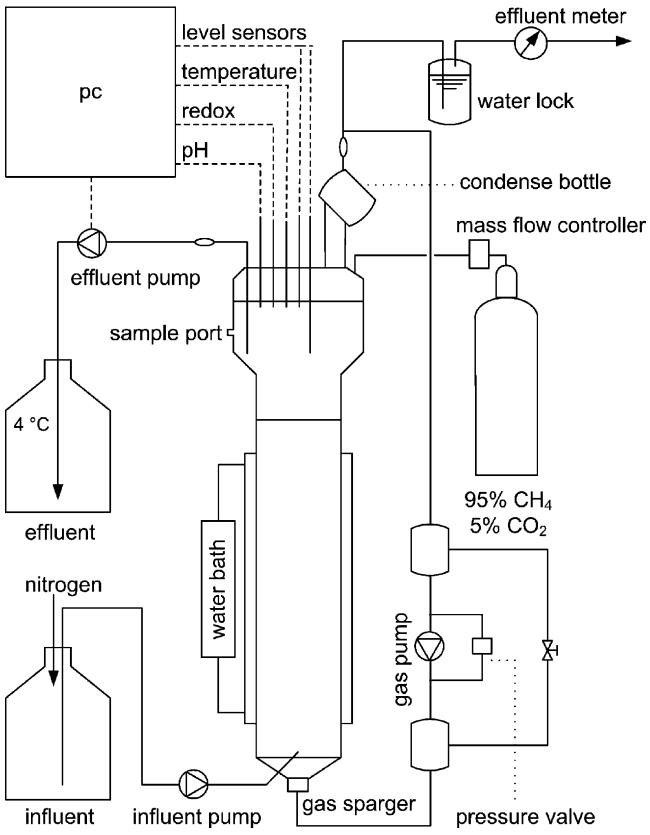

Fig. 2. Setup of sequencing fed-batch reactors.

The nitrite concentration, which was estimated 3–5 times per week, was maintained at 3–30 mg NO_2^--N/L. When the nitrite concentration was <3 mg NO_2^--N/L the nitrite loading rate was increased. When the nitrite concentration exceeded 30 mg NO_2^--N/L medium supply was stopped until concentration decreased to <15 NO_2^--N/L. NLR was adjusted by adjusting cycle duration (1.0–11.5 d) or medium concentration (0.014–0.980 g NO_2^--N/L). The nitrite concentration in the medium was increased in time as the nitrite consumption rates increased. 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.

Every 7–20 d and when changes were made to reactor operation, nitrite and nitrate concentrations and gas composition (methane, nitrogen, carbon dioxide and oxygen) were measured. Activity measurements were performed regularly to measure biomass activity. Protein concentration was measured to estimate biomass concentrations in inoculum and effluent (in SFBR− from day 556 to day 621 of the enrichment, in SFBR+ from day 551 to day 621 of the enrichment). Molecular analyses were performed to determine the microbial composition and monitor the enrichment of denitrifying methanotrophic bacteria in time.

2.5. Activity measurements

To measure the nitrite and nitrate consumption rate of the biomass in the reactors, medium supply was stopped and nitrite and nitrate concentrations were measured 5–8 times during 1–2 d. In three additional tests on each reactor, methane consumption rate and nitrogen gas production rate were measured. Gas supply was stopped, and the gas phase of the reactors was flushed with nitrogen. Methane concentration was then adjusted to 5–10% and carbon dioxide concentration was adjusted to 3–5%. Gas composition was measured, in duplicate, simultaneously with nitrite and nitrate concentration. Before gas measurements started an equilibration time of 2 h was deployed.

2.6. Analytical methods

Nitrite and nitrate concentrations were estimated using test strips (Merckoquant, Merck chemicals) and measured according to APHA standard method 4110 B [16] using ion chromatography (Metrohm IC Compact 761). The mobile phase was an aqueous solution of 3.2 mM sodium carbonate, 1 mM sodium bicarbonate and 1% (v/v) acetone. The chemical suppressor was regenerated using 50 mM sulfuric acid and 1% (v/v) acetone.

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 × 0.53 mm; 10 μm, Varian, part no. CP7355) and Molsieve 5A (25 m × 0.53 mm; 50 μm; Varian; Part.no. CP7538) connected in parallel. Standards and samples (50 μl) 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 9300 g, samples >2 mL at 5000 g) and supernatant was removed. The pellets were resuspended in 0.5 mL 1.0 M sodium hydroxide and the cells were hydrolyzed for 30 min at 50 °C. After hydrolysis, samples were neutralized with 0.5 mL 1.0 M hydrochloric acid. Next, protein concentration was measured according to the Hartree–Lowry method [17].

2.7. Molecular analyses

Inoculum and reactors were sampled (2 mL) for molecular analyses. After centrifugation (5 min at 9300 g) the supernatant was discarded and the pellets were stored at −18 °C for DNA isolation. DNA was isolated according to Ettwig et al. [14]. The isolated DNA was used as a template for polymerase chain reaction (PCR) for amplification of the 16S rRNA gene using a combination of primer 202F [14] and the general bacterial primer 1545R [18]. The obtained amplicons were used as a template for nested PCR using 'NC10' specific primers qP1F and qP2R [14]. Thermal cycling, for both PCRs, was carried out with an initial denaturation step of 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 65 °C for 1 min, and elongation at 72 °C for 3 min; cycling was completed by a final elongation step at 72 °C for 10 min. Cloning of the PCR products and sequence analysis was performed as described by Ettwig et al. [14]. ChromasLITE (version 2.01) was used to check the quality of the obtained sequences. BLAST search analysis was performed to identify newly obtained sequences and to obtain related sequences from GenBank (http://www.ncbi.nlm.nih.gov/GenBank/). Sequences were aligned in MEGA4 software using CLUSTALW [19]. Phylogenetic analysis was performed in MEGA4 using the neighbor-joining method with pairwise deletion of gaps. The tree topology was tested by bootstrap analysis (1000 replicates). Representative sequences were submitted to GenBank (accession numbers JF803475–JF803482, JQ362447 and JQ362448).

Fluorescence in situ hybridization (FISH) was performed as described by Ettwig et al. [13], however samples were stored at −18 °C and the hybridization buffer contained 50% formamide. The probes used were S-*-DBACT-0193-a-A-18 (DBACT193) and S-*-DBACT-1027-a-A-18 (DBACT1027), targeting bacteria affiliated with the 'NC10' phylum [12], the EUB mix for almost all bacteria, EUB338, EUB338II, EUB338III [20] and the DNA stain DAPI.

3. Results and discussion

3.1. Nitrite loading and consumption rates

The operation of the SFBRs was controlled based on the microbial activity. The activity was represented by the volumetric nitrite consumption rate; i.e. an increase in microbial activity was characterized by a higher consumption rate. Activity tests performed throughout reactor operation (Fig. 3) confirmed that the NLR corresponded well with the nitrite consumption rate and was therefore a good measure of microbial activity.

The nitrite consumption rates in both reactors increased in time (Fig. 3). The NLR applied to SFBR− was exponentially increased to 25.1 mg NO_2^--N/L d on day 364 (phase I in Fig. 3a) and to a maximum of 33.5 mg NO_2^--N/L d on day 457. Prior to day 361 almost all supplied nitrite was consumed (reactor concentrations averaged 2.1 mg NO_2^--N/L, ranging from 0.0 to 7.8 mg NO_2^--N/L). The period thereafter (phase II in Fig. 3a) the reactor appeared to be overloaded (up to 66.4 mg NO_2^--N/L d at day 364) and operational problems (influent pump failure; problems with level sensor resulting in undesired settling) occurred. Consequently, a lower NLR of 12.0 mg NO_2^--N/L d was applied. As a result, the nitrite concentration in the reactor decreased and was below the detection limit from day 406 onwards. Since then, the NLR was increased to an eventual new maximum of 33.5 mg NO_2^--N/L d. Starting on day 457 (phase III in Fig. 3a), the nitrite consumption rate decreased to a lower NLR of about 10 mg NO_2^--N/L d from day 609 to 623. In an attempt to increase the NLR again, a membrane was used from day 623 onwards to remove effluent and achieve complete biomass retention. In the following 30 d this led to an increase in NLR (phase IV in Fig. 3a).

The NLR in SFBR+ also increased exponentially (phase I in Fig. 3b) to a maximum of 37.8 mg NO_2^--N/L d on day 372. Subsequent operational problems (too high influent flow rate, influent pump failure, problems with level sensor resulting in undesired settling) in phase II (Fig. 3b), interrupted further NLR increase. Up until the start of phase III (day 374), nearly all supplied nitrite was consumed (reactor concentrations averaged 1.3 mg NO_2^--N/L, ranging from 0.0 to 7.4 mg NO_2^--N/L, except for a short increase from day 85 to day 121 caused by overloading, resulting in a continuous accumulation of nitrite to 18.1 mg NO_2^--N/L d at day 107). From the start of phase III, the nitrite consumption rate decreased and NLR had to be adjusted frequently (phase III in Fig. 3b). This was followed by an increase in NLR (phase IV) and finally a stabilization of the NLR around 16 mg N/L d in phase V.

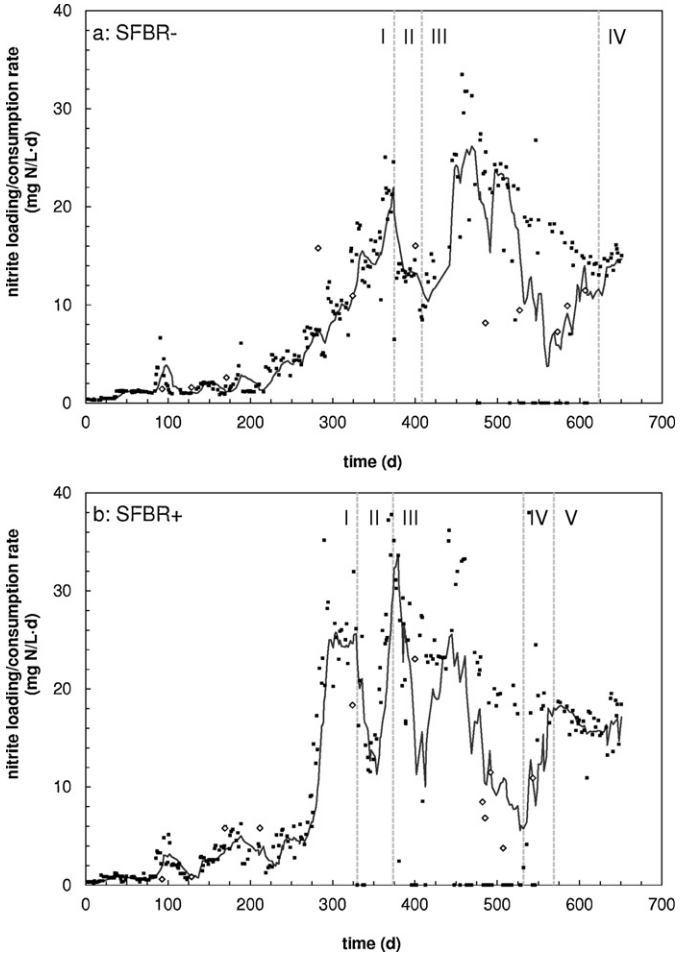

Fig. 3. Nitrite loading rate (NLR; ■), weighted average over 10 measurements of NLR (–) and nitrite consumption rate determined in activity tests (◊) in reactors (a) SFBR− and (b) SFBR+ in time. Latin numbers indicate (I) exponential increase in NLR, (II) operational problems, (III) decreasing NLR, (IV) membrane placed in SFBR−, increase in NLR in SFBR+ and (V) stabilization of NLR. An NLR of zero was set to avoid nitrite accumulation or caused by technical problems (such as failing pumps).

The nitrate consumption rates in both reactors were much lower than the nitrite consumption rates. After 4 months of enrichment the nitrate consumption rate decreased to below 2 mg NO_3^--N/L d (data not shown) and the nitrate concentration in the medium was set to 14 mg NO_3^--N/L.

The maximum nitrite consumption rates that were achieved (SFBR− 33.5 mg NO_2^--N/L d; SFBR+ 37.8 mg NO_2^--N/L d) were slightly higher than the maximum nitrite consumption rates reported by most other researchers [11–13] and similar to the maximum rate reported by Ettwig et al. [14], using similar inoculum and operational conditions. After reaching a maximum, in both reactors the consumption rates decreased and eventually stabilized at lower nitrite consumption rates. Placement of the membrane in SFBR− seemed to stop or even reverse the trend of decreasing nitrite consumption rates, suggesting the importance of efficient biomass retention (see Section 3.3).

Effluent from a sewage treatment plant was added to SFBR+ as a source of potential growth factors, which may previously have limited further increases in NLR. The maximum nitrite consumption rate in SFBR+ was 11% higher than that for SFBR−. Although it seems addition of effluent did not hamper the enrichment and might even have had a positive effect, several operational aspects (such as described operational problems and changes made to NLR) may have affected enrichment. Therefore it remains to be investigated

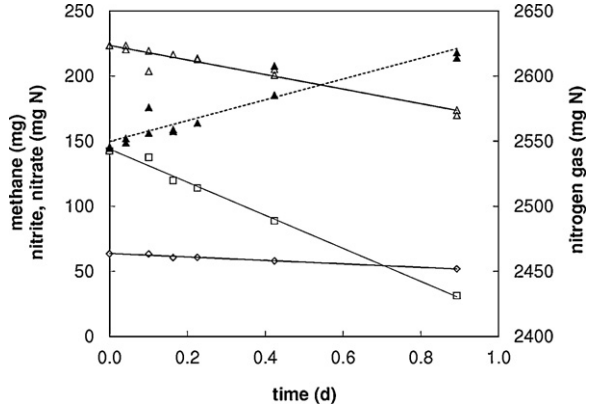

Fig. 4. Results from a whole culture batch test performed with SFBR+ after 324 d of enrichment. Nitrite (□) and nitrate (◊) on primary *y*-axis in mg N, methane (▲, measured in duplicate) on primary *y* axis in mg, nitrogen gas on secondary *y*-axis (△, measured in duplicate) in mg N. Molar conversion ratio of CH_4:NO_2^-:N_2 was 3:7.9:4.5.

if the higher NLR that could be applied to SFBR+ was because effluent from the sewage treatment plant contained a missing growth factor. The stagnation and later on decrease of volumetric nitrite consumption rates in SFBR+ could indicate that additional impediment exists, be it an inhibiting compound produced in the reactor or the absence of nutrients or unknown growth factors. The effect of effluent addition may be more pronounced once other limitations have been resolved.

3.2. Coupling nitrite and methane consumption

On each reactor, three activity tests were performed to establish, in addition to nitrite consumption, nitrate and methane consumption and nitrogen gas production. Simultaneous nitrite and methane consumption, with concomitant nitrogen gas production could be confirmed. For example, the results of an activity test with SFBR+ after 324 d of enrichment are shown in Fig. 4. Nitrite, a small amount of nitrate, and methane were consumed and nitrogen gas was produced. The molar conversion ratio of CH_4:NO_2^-:N_2 was 3.0:7.9:4.5, which is in good agreement with the stoichiometric ratio of 3:8:4 (Eq. (1)). Also in SFBR− after 324, 400 and 485 d of enrichment and in SFBR+ after 400 and 485 d of enrichment ratios close to expected stoichiometric ratios were measured (Table 1). This indicated nitrite and methane removal according to Eq. (1) was the dominant process in the reactors.

3.3. Biomass growth and washout

Biomass in the reactors was present both in suspension and attached to the walls. Consequently, representative biomass samples could not be taken and the total amount of biomass in the reactors could not be quantified. Therefore, the increase in NLR

Table 1
Molar conversion ratios (methane:nitrite:nitrogen gas; theoretical ratio 3:8:4) of SFBR− and SFBR+ in time.

Time (days)	Ratio methane:nitrite:nitrogen gas
SFBR−	
324	3.0:7.4:4.4
400	3.0:7.6:4.4
485	3.0:9.0:5.1
SFBR+	
324	3.0:7.9:4.5
400	3.0:5.0:3.8
485	3.0:11.9:4.7

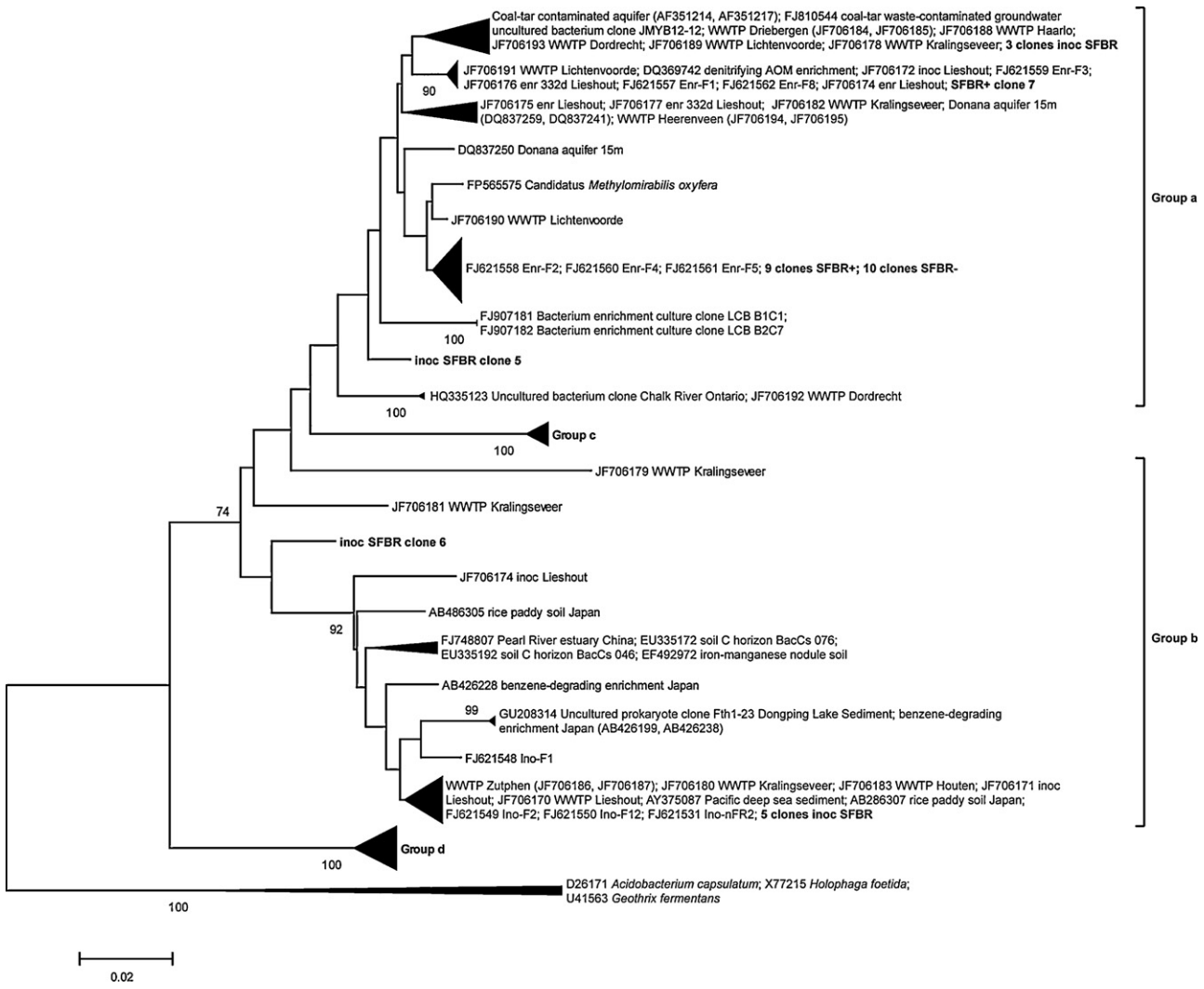

Fig. 5. Phylogenetic tree of 16S rRNA sequences of the 'NC10' phylum with *Acidobacteria* as the outgroup. Sequences obtained in this study are shown in boldface. The tree was constructed with MEGA4 software using the neighbour-joining method and pairwise deletion of gaps. The tree topology was tested by bootstrap analysis (1000 replicates).

applied to the reactors in time (phases I in Fig. 3) was used to estimate a doubling time for the amount of bacteria in the reactor. The net doubling time in SFBR− was estimated to be 1.9 months and the doubling time in SFBR+ to be 1.7 months. It remains to be investigated whether the somewhat shorter doubling time in SFBR+ was because effluent from the sewage treatment plant, fed to SFBR+, contained a missing growth factor.

To estimate if a substantial portion of the new cells was lost from the reactors, and if this loss could have contributed to the stagnation and decrease in nitrite consumption rates, biomass washout from each reactor was quantified over a period of three months. The daily growth, based on nitrite consumption, and expected biomass yield were compared. This provides an estimate of how much of the (produced) biomass washed out.

Total protein washout from SBFR− was 0.10 g between day 556 and 621 (distributed over 6 cycles). In this period, about 4.4 g NO_2^--N was consumed, thus 0.022 g protein washed out per g NO_2^--N consumed. Total protein washout from SFBR+ was 0.18 g between day 551 and 621 (distributed over 11 cycles). In this period about 7.0 g NO_2^--N was consumed, thus 0.026 g protein washed out per g NO_2^--N consumed. The growth yield of *M. oxyfera* is unknown, but assuming it is similar to the growth yield of the anaerobic nitrite consuming Anammox bacteria, viz. 0.054 g protein/g NO_2^--N [21], it can be estimated that 41–48% of the produced biomass washed out from the reactors. This indicates that, even though in this enrichment study a long settling time of 2 h was applied, compared to only 15 min applied by Raghoebarsing et al. [12] and 1–2 h applied by Ettwig et al. [14], biomass washout may have significantly decelerated enrichment. In the periods when stagnating or decreasing nitrite consumption rates were observed, biomass washout was not quantified, but it seems likely, these can mainly be attributed to (temporarily higher) biomass washout. After placement of the membrane in SFBR− the nitrite consumption rate stabilized or even increased suggesting the importance of efficient biomass retention. Prolonged reactor operation is required to see the effect on the long term.

Biomass that washed out was also examined under the microscope (results not shown). In the effluent from both reactors single cells and small flocs of up to 60 μm were observed, which indicated this biomass had poor settling characteristics.

3.4. Microbiological composition

The presence and abundance of *M. oxyfera* bacteria in the reactors was assessed by sequence analysis and FISH. Sequence analysis of 16S rRNA clones obtained from biomass from the reactors and subsequent phylogenetic analyses confirmed the presence of *M. oxyfera* bacteria in both the inoculum and after 5 months of

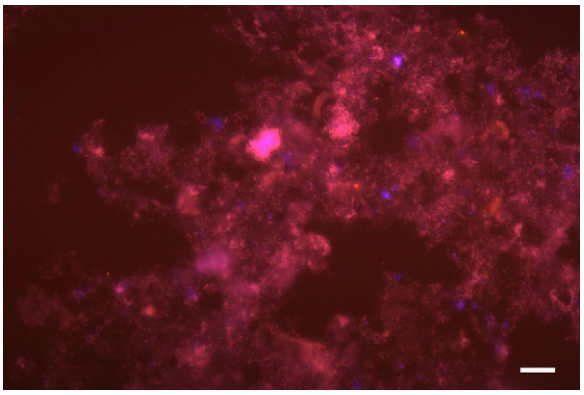

Fig. 6. Fluorescence in situ hybridization of biomass from SFBR+ after 13 months of enrichment. Fluorescence micrograph after hybridization with probes DBACT1027 (Cy3; red) specific for 'NC10' bacteria; and EUB mix (probes EUB338 I–III; Cy5; dark blue), detecting nearly all eubacteria. Due to co-hybridization with the specific and general probes, the *M. oxyfera* bacteria appear pink. The scale bar indicates 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

enrichment. The 'NC10' sequences obtained in this study were similar to sequences found in previous studies in which also *M. oxyfera* bacteria were enriched or detected (Fig. 5) [11–14]. In addition, microscopic analysis using FISH of biomass from the reactors showed an increase of *M. oxyfera* bacteria over the course of the enrichment. The amount of *M. oxyfera* bacteria in the inoculum was too low to be detected by FISH. After 8 months of enrichment, the bacteria were observed in both reactors and after 13 months of enrichment *M. oxyfera* bacteria dominated both reactors (70–80% of the population; SFBR+ after 13 months of enrichment is represented in Fig. 6), confirming the results from Section 3.2 that denitrification of nitrite coupled to anaerobic methane oxidation was the dominant process in both reactors.

3.5. Outlook

The coupled removal of nitrogen and dissolved methane makes a process with denitrifying methanotrophic bacteria a promising treatment for effluents from direct low-temperature anaerobic sewage treatment. Emission of dissolved methane present in the effluent would lead to greenhouse gas emissions. Using the dissolved methane for denitrification decreases the potential greenhouse gas emissions from direct low-temperature anaerobic wastewater treatment and the dissolved methane (20 mg NO_2^--N/L d at 10 °C) is enough to remove nearly all nitrogen (47 mg NO_2^--N/L d) in the effluent (typically containing 50 mg NO_2^--N/L d), thus avoids the need for an external carbon source for denitrification. Autotrophic nitrogen removal with Anammox could also be applied for treatment of effluent from anaerobic wastewater treatment, but this would still require removal of dissolved methane [9].

The volumetric nitrite consumption rates of both enrichment reactors are low compared to other denitrifying systems. For the treatment of effluent from anaerobic sewage treatment plants, containing 50 mg N/L, the present results would dictate a long hydraulic retention time of 1.3 (SFBR+)–1.5 d (SFBR−). The volumetric rates have to be increased by an order of magnitude. The low growth rates of denitrifying methanotrophic bacteria necessitate efficient biomass retention. Preliminary results with applying a membrane for complete biomass retention in SFBR− suggested that the decreasing trend in NLR could be stopped or even reversed in a short period of 30 d in which the membrane was applied for effluent collection. Therefore, further research should focus on using systems with better biomass retention, such as membrane bioreactors, reactors with granular sludge or biofilms to increase the volumetric conversion rates to the desired values.

In the proposed concept for sewage treatment at low temperatures, the reactor for denitrification coupled to anaerobic methane oxidation is fed with the effluent from anaerobic sewage treatment, containing ammonium, dissolved CH_4 and residual COD; and with a recycle flow from the nitritation reactor, containing nitrite and traces of dissolved oxygen (Fig. 1). These conditions could trigger processes other than denitrification coupled to anaerobic methane oxidation such as Anammox and heterotrophic denitrification, competing for nitrite with denitrifying methanotrophic bacteria. Recently, it was shown that under ammonium limitation, but with nitrite and methane supplied in excess, Anammox and *M. oxyfera* bacteria could co-exist [22]. Also traces of oxygen present in the effluent from the nitritation reactor could have an effect on the denitrifying methanotrophs. Luesken et al. [23] showed that addition of 2% and 8% of oxygen to *M. oxyfera* enriched cultures resulted in a direct decrease of nitrite and methane consumption rates and changes in gene expression showed *M. oxyfera* was under oxidative stress. Therefore, in addition to improved biomass retention, further research topics should include the performance of the proposed concept at wastewater temperatures, competition for nitrite and effects of traces of oxygen.

4. Conclusions

- Denitrifying methanotrophic bacteria offer a possible solution to treatment of effluent from low-temperature anaerobic sewage treatment plants, such as a UASB-digester system.
- Maximum volumetric consumption rates of enrichment cultures of *M. oxyfera* (70–80%) were 33.5 mg NO_2^--N/L d (using synthetic medium) and 37.8 mg NO_2^--N/L d (using medium containing effluent from a sewage treatment plant) were achieved. These denitrification rates need to be increased an order of magnitude before the process could be considered for practical applications.
- Biomass washout occurred throughout the enrichment and significantly decelerated enrichment. Therefore, further research should focus on systems with better biomass retention, such as membrane bioreactors, reactors with granular sludge or biofilms to increase the volumetric consumption rates to the desired values.

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