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ORIGINAL PAPER

Anammox enrichment from reject water on blank biofilm carriers and carriers containing nitrifying biomass: operation of two moving bed biofilm reactors (MBBR)

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Abstract The anammox bacteria were enriched from reject water of anaerobic digestion of municipal wastewater sludge onto moving bed biofilm reactor (MBBR) system carriers—the ones initially containing no biomass (MBBR1) as well as the ones containing nitrifying biomass (MBBR2). Duration of start-up periods of the both reactors was similar (about 100 days), but stable total nitrogen (TN) removal efficiency occurred earlier in the system containing nitrifying biomass. Anammox TN removal efficiency of 70% was achieved by 180 days in both 20 l volume reactors at moderate temperature of 26.0°C. During the steady state phase of operation of MBBRs the average TN removal efficiencies and maximum TN removal rates in MBBR1 were 80% (1,000 g-N/m^3/day, achieved by 308 days) and in MBBR2 85% (1,100 g-N/m^3/day, achieved by 266 days). In both reactors mixed bacterial cultures were detected. Uncultured *Planctomycetales bacterium clone P4*, Candidatus *Nitrospira defluvii* and uncultured *Nitrospira* sp. clone 53 were identified by PCR-DGGE from the system initially containing blank biofilm carriers as well as from the nitrifying biofilm system; from the latter in addition to these also uncultured *ammonium oxidizing bacterium clone W1* and *Nitrospira* sp. clone *S1-62* were detected. FISH analysis revealed that anammox microorganisms were located in clusters in the biofilm. Using previously grown nitrifying biofilm matrix for anammox enrichment has some benefits over starting up the process from zero, such as less time for enrichment and protection against severe inhibitions in case of high substrate loading rates.

Keywords Anammox · Biofilm · Reject water · Moving bed biofilm reactor · Biofilm carriers

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Introduction

Supernatant from an anaerobic digester typically has a high content of (NH_4^+) while containing biodegradable organic carbon only at low concentrations. For example, reject water from anaerobic digestion of municipal wastewater sludge contains 500–850 mg N/l of NH_4^+ (Szatkowska et al. 2007a; Zekker et al. 2011) and has a biological oxygen demand (BOD_7) of around 350 mg/l (Zekker et al. 2011). Recycling of such a stream to the head of a WWTP contributes to the average increase in the total nitrogen (TN) load by about 15–20% (Szatkowska et al. 2007b) while it makes up to only 2% of the total influent flow. Nitrogen removal from reject water using conventional nitrification–denitrification technology would mean high

energy consumption for aeration (Fux and Siegfrist 2004), costs for additional carbon source (methanol) and increased production of excess sludge.

The anammox (anaerobic ammonium oxidation) process provides an alternative to the conventional nitrification–denitrification technology. The anammox reaction uses NH_4^+ as electron donor and NO_2^- as electron acceptor, converting chemically bonded nitrogen into N_2 gas.

Taking into account carbon fixation and biomass growth, the stoichiometry of the anammox process is as follows (Strous et al. 1999):

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 0.066CH_2O_{0.5}N_{0.15} + 2.03H_2O \quad (1)$$

According to Eq. 1, 55–60% of NH_4^+ present in the wastewater has to be oxidized into NO_2^- in the nitritation phase preceding to the anammox process phase. In case of conventional nitrification–denitrification (NO_3^- as the terminal electron acceptor), NH_4^+ has to be oxidized entirely (to NO_3^-) to achieve nitrogen removal. Treating substrate with a low organic carbon to inorganic nitrogen ratio (i.e. reject water) the autotrophic nitrogen removal process is energetically beneficial as there is no need for an external carbon source. Thus, biological treatment of nitrogen-rich wastewaters by the anammox process is one of the most economical processes for nitrogen removal (Fux and Siegrist 2004). However, the combined nitritation-anammox technology has its limits: up to 90% of nitrogen can be removed at most (emanating from Eq. 1), as minor amounts of NO_3^- are formed (Strous et al. 1999). For higher nitrogen removal, a mixed consortium of bacteria is needed, dominated by anammox organisms, but having a minor population of denitrifiers as well.

Most of anammox microorganisms (*Brocadiales*—affiliated to the *Planctomycetes* (Strous et al. 1999)) are strict anaerobic autotrophs. Implementation of this process is limited by low availability of anammox biomass as a result of its slow growth rate (doubling time approximately 11 days) (Strous et al. 1999). Biofilm configuration (De Clippeleir et al. 2011) and two types of suspended growth based sludge have been used for anammox start-up: flocs (Desloover et al. 2011) and granules (Vlaeminck et al. 2009). Various methods have been employed to culture and enrich anammox biomass, e.g. anammox biomass has been enriched from different types of inoculums—sludge from anaerobic (Tsushima et al. 2007a) or nitrifying/denitrifying reactors (Jeanningros et al. 2010; Desloover et al. 2011; Gaul et al. 2005; Tang et al. 2010b). To our knowledge enrichment of anammox microorganisms directly from wastewater on biofilm carrier elements without using seeding sludge has not been reported in previous studies.

In the properly designed and operated anammox systems the growth of microorganisms can be accelerated if operated with real municipal anaerobic digestion reject water. To achieve a stable combined nitritation–anammox process, inorganic carbon to NH_4^+–N ratio of 1:1 should be maintained (Gali et al. 2007). In the upflow anaerobic sludge blanket process the TN removal rate of 45,240 g-N/m^3/day has been achieved (Tang et al. 2010a). In anammox biofilm reactors TN rates have been 24,000 g-N/m^3/day (Tsushima et al. 2007b). However, moving bed biofilm reactor (MBBR) configuration is determined to be effective for anammox enrichment (Gaul et al. 2005). Advantages of the MBBR in both technological applications and lab-scale experiments (Ling 2009; Rosenwinkel and Cornelius 2005; Schneider et al. 2009) include compactness, tolerance to inhibiting substances and avoiding biomass loss from the deammonification process. The MBBR type originated from Norway and was preliminary designed for operation in cold climate regions, where slow growing organisms were protected from wash-out (Jaroszynski et al. 2011).

The aims of this study were to start-up anammox process by enrichment of anammox biomass from reject water onto biofilm carriers. Also, to determine if the existence of nitrifying biomass on carriers shortens the anammox process start-up and prevents inhibition. The experiments performed with carriers without the initial biomass were set as reference. To cultivate anammox biofilm first and add nitritation step later makes it possible to achieve good quality anammox biofilm with less fluctuations occurring in the deammonification system start-up phase.

Materials and methods

Operation of MBBRs and batch tests

Two 20 l plexiglass reactors equipped with a water jacket and connected with a thermostat (Assistant

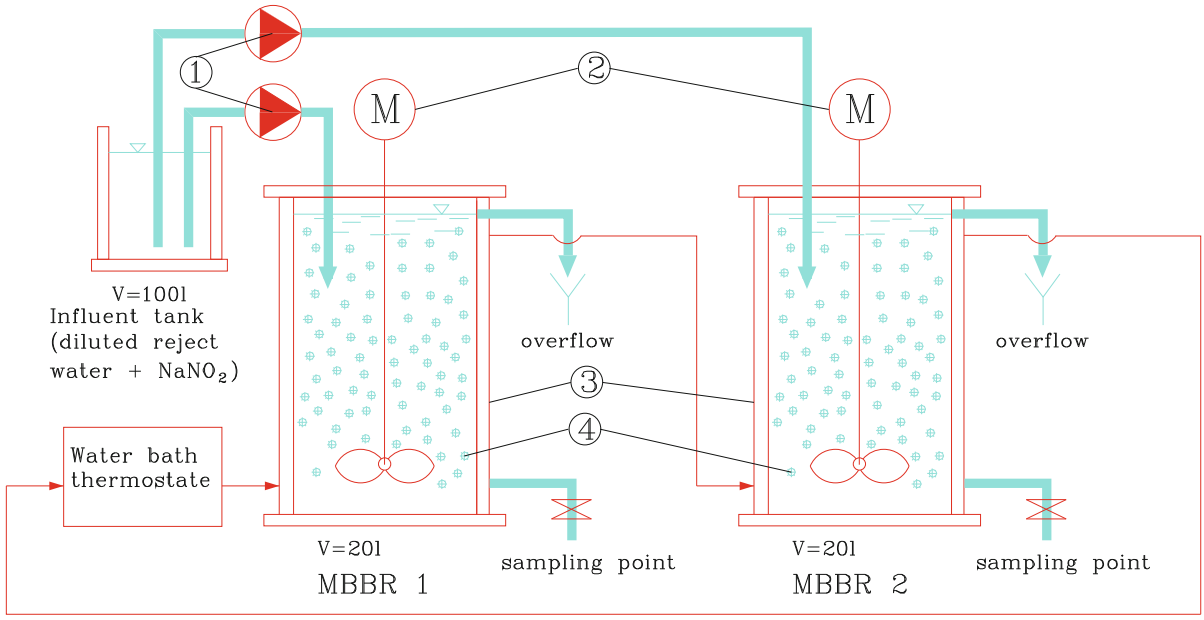

Fig. 1 Moving bed biofilm reactors configuration. *1* Influent pump. *2* Stirrer. *3* Water jacket. *4* Biofilm carriers

3180, Germany) were run at constant temperature (26.0 ± 0.5°C) (Fig. 1).

Influent was fed by peristaltic pumps (Seko P4, Italy) and no effluent recirculation was applied. In the start-up phase, lower mixing velocity of mechanical mixer (around 100 rpm) was applied in order to allow better attachment of microorganisms, later it was gradually increased to 200 rpm to avoid clogging up the carriers. $NaNO_2$ supplemented to the Tallinn wastewater treatment plant reject water containing NH_4^+ (with an average NO_2^-–N to NH_4^+–N molar ratio of 1.2:1) was used as a substrate. Reject water (parameters shown in Table 1) was diluted with tap water in ratios between 1:10 and 1:2 with a loading strategy depending on the parameters of reactor effluent. During the time course of operation of the reactors, the dilution factor of the influent was gradually decreased. NO_3^- concentration in the influent was mainly below 10 mg N/l.

Excess HCO_3^- concentration was removed from reject water by addition of 4 M HCl into the feeding medium, keeping HCO_3^- concentration in the influent below 400 mg HCO_3^-/l in the start-up phase and below 900 mg HCO_3^-/l in the high efficiency phase (checked by pH-metric titrations), except at the inhibition episode when HCO_3^- concentration increased to 1,500 mg HCO_3^-/l. HCO_3^- concentrations were controlled, because nitritation process (consuming HCO_3^-) was not applied.

Batch tests with 200 carriers containing anammox biomass were performed out of the MBBR reactors at temperature of 25 (±0.5°C) in 800 ml reactor liquid volume to determine anammox reaction during high efficiency period of MBBR1. An Assistant 3180 (Germany) water-bath thermostat maintained the stable temperature. Biofilm total suspended solids (TSS) concentration of 2,212 (±60) mg/l was maintained in the batch reaction. NH_4Cl–$NaNO_2$–$NaHCO_3$–water solution was used as a synthetic medium. 3 ml of acid microelement solution in addition to 3 ml of alkaline microelement solution were added into the batch substrate along with 40 ml of macroelement solution made according to Zhang et al. (2009). In one specific experiment NH_2OH was added in the form of $NH_2OH \times HCl$ to observe the formation of anammox-specific intermediate—hydrazine.

The ratio of NO_2^-–NH_4^+ at the beginning of each batch test was around 1.25:1 as an average ratio present in MBBR feeding medium.

Before the start of the reaction, headspaces of batch reactors were flushed with nitrogen gas or argon about 15 min to eliminate oxygen from dissolved phase. Then, batch reactors were sealed with butyl rubber stoppers. Sampling was performed with the aid of

Table 1 MBBR influent parameters (Zekker et al. 2011)

Parameter	Average concentration in Tallinn WWTP reject water (mg/l)
NH_4^+–N	680 ± 76
NO_2^-–N	1.21 ± 0.5
NO_3^-–N	0.2 ± 0.2
BOD_7	350 ± 90
HCO_3^-	3950 ± 100
pH	7.9 ± 0.3
COD	682 ± 210
PO_4^{3-}–P	10 ± 1
(S_{tot})	1.4 ± 0.4
TSS	750 ± 30
Ash weight	590 ± 28
VSS	160 ± 6
Mn	$0.18 \pm 4 \times 10^{-3}$
Fe	4.3 ± 10^{-2}
Mg	24 ± 0.3
Ca	30 ± 0.7
Zn	$5 \times 10^{-3} \pm 2 \times 10^{-4}$
B	0.45 ± 10^{-2}
Co	$9 \times 10^{-3} \pm 3 \times 10^{-4}$
Ni	$13 \times 10^{-3} \pm 6 \times 10^{-4}$

overpressure of N_2 or Ar created in the gas phase of reactors and the sample was removed through the tube placed into the liquid phase. The pH value was held consistently at 8.0–8.5. Concentrations of nitrogen species as also HCO_3^- concentrations were monitored after every 2 h.

Removal efficiencies of nitrogen compounds during the start-up periods of MBBR1 and MBBR2 were compared to determine the duration of the start-up period with and without the nitrifying biofilm matrix (supporting anammox microorganisms' attachment). Differently from other studies, which have used starting-up of bioreactors with sludge containing anammox microorganisms (Rosenwinkel and Cornelius 2005), in our case neither of the systems was typically (externally) inoculated with inoculum containing anammox microorganisms. Instead, anammox bacteria were enriched from reject water onto the carriers in two MBBRs: in the case of MBBR1 onto blank carriers (initially without a biofilm) and in the case of MBBR2 onto the carriers initially containing a nitrifying biofilm. Reject water contained anammox microorganisms (uncultured *Planctomycetales* bacterium clone *P4*) and sufficient amounts of microelements and other compounds needed for anammox process propagation (Table 1).

Biofilm carriers

Around 10,000 ring-shaped carrier elements made of polyethylene (Bioflow 9) were used as a microorganisms' support material in the MBBR1 and MBBR2 operation. The carriers placed into the MBBR2 containing nitrifying biomass were taken from a fish farm wastewater treatment facility, where they were subjected to a temperature of 15°C. The carriers occupied about 50% of the liquid volume of the reactors and their specific surface was 800 m^2/m^3 (carriers interior protected specific surface of 500 m^2/m^3).

Analytical methods

Samples from the influent and the effluent were collected simultaneously and analyzed right after that. NH_4^+–N, NO_2^-–N, NO_3^-–N (the sum of these species determined as TN); COD, BOD_7 and HCO_3^- were measured according to the standard methods (1985). TN loading and removal rates were determined as amounts (g) of TN loaded or removed, respectively per volume (m^3) or per surface area of biocarriers in the reactor (m^2) per time unit (day). The total suspended solid (TSS) concentration of the biofilm on the carriers was measured by weighing the carriers before and after biomass removal. pH was measured with a pH meter (Jenway, model 3520) connected with a pH electrode (Jenway). Humic substances were analyzed according to Ibrahim et al. (2008). The content of volatile fatty acids (VFA) was measured by a gas chromatograph Shimadzu GC-2014 using the Phenomenex Zebron ZB-WAXplus GUARDIAN column. Hydrazine concentration (measuring range 0–600 μg/l) was measured by Dr. Lange method (based on method described by Watt and Chrisp 1952): into 10 ml of sample 0.5 ml of *para*-dimethylaminobenzaldehyde dissolved in ethanol (Hydraver 2 reagent) was added and NO_2^- interference was eliminated by 0.5% sulfamic acid (George et al. 2008). After 12 min reaction time hydrazine concentration was measured at 458 nm.

Data and statistical analyses were performed by the MS Excel 2010 Analysis ToolPak. Homogeneity of group variances and the difference between group

means were checked using the *F*-test and the two-way *t* test, respectively. The level of significance was set at $\alpha < 0.05$.

Polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE) methodology

DNA extraction

DNA extraction from biomass was performed with the MoBio PowerSoil DNA extraction kit (MoBio Laboratories Inc.) according to the manufacturer's instructions.

PCR and DGGE primers, equipment

Fingerprinting of *Planctomycetes* microorganisms' communities was conducted via PCR and DGGE. The first PCR round for the amplification of *Planctomycetes* was performed with a wide-range primer set, Eub27f/Eub1492r (Lane 1991), and the second PCR round by using a *Planctomycetes*-specific primer Pla46f (Neef et al. 1998) with a GC-clamp (GC CGC CGC GCG GCG GGC GGG GCG GGG GC) coupled with the anammox-specific primer Amx368r (Sąnchez-Melsió et al. 2009). Nested PCR was carried out according to the thermocycling parameters described by Sąnchez-Melsió et al. (2009). To amplify 16S rDNA fragments from only anammox bacteria, PCR was conducted using primers Pla46f and Amx667r.

PCR was also performed to identify *Nitrospira* strains with the primer set NSR1113f/NSR1264r specific for *Nitrospira* 16S rDNA, using a PCR program described by Dionisi et al. (2002). The PCR products were purified with the JETquick Spin Column Kit (GENOMED GmbH) and then sequenced.

PCR–DGGE for detecting diversity of the most abundant microorganisms was conducted using the eubacterial primer set GC-BacV3f/907r as described previously (Muyzer et al. 1993, 1996; Koskinen et al. 2006).

The gene sequences were amplified in a Mastercycler Personal thermocycler (Eppendorf, Germany). The PCR reaction products were applied to agarose gel electrophoresis of a 1% agarose (SeaKem® GTG® Agarose, FMC Bioproducts, Rockland, Maine, USA) gel, which was stained with ethidium bromide and visualized under UV transillumination.

DGGE was performed using INGENY the PhorU System (INGENY, the Netherlands).

PCR products were loaded on a 30–65% denaturing gel and run for 17 h at 90 V at a constant temperature of 60°C. The gels were stained with an ethidium bromide solution in an 1× Tris-acetate–acetic acid–ethylenediaminetetraacetic acid (TAE) buffer to observe the bands by UV transillumination, subsequently the bands were excised for further reamplification and sequencing.

Sequencing

PCR for sequencing was performed with the BigDye® Terminator v3.1 Cycle Sequencing Kit (Life Technologies Corporation, USA). The acquired sequences were compared to the available database sequences via a Basic Local Alignment Search Tool (BLAST) search and the related sequences were obtained from the GenBank.

Phylogenetic analysis

In order to determine the phylogenetic position of the anammox 16S rRNA gene sequence acquired, it was compared with the available database sequences via a BLAST search, obtaining the related sequences from the GenBank. Further analysis was carried out with the MEGA software version 5.0 with the neighbour-joining method.

Fluorescence in situ hybridization (FISH)

FISH was performed to detect anammox bacteria. Biomass was harvested from the MBBRs, fixed in a 4% paraformaldehyde solution and FISH was performed according to Amann et al. (1990). The probe Amx820 with Cy3 label was used at 35% formamide to target the anammox genera "*Candidatus* Brocadia and Kuenenia" (Schmid et al. 2001). Images were acquired on a Carl Zeiss Axioskop 2 Plus epifluorescence microscope (Jena, Germany) equipped with differential interference contrast (DIC), and scales were added using ImageJ freeware.

Results and discussion

Enrichment of anammox bacteria and process performance in MBBRs

The anammox microorganisms were enriched from reject water onto the biofilm carriers of two different

MBBRs and the performance of the anammox process was divided into three periods with total durations of 450 and 407 days for the MBBR1 and for the MBBR2, respectively (Table 2). Nitrogen loading rate (NLR) was gradually increased from 14 to 1,200 g-N/m^3/day (0.04–3 g-N/m^2/day) with an influent TN concentration increase between 60 and 800 mg/l and with an increase of hydraulic retention time (HRT) from 0.75 to 2 days (Table 2).

The MBBR1 containing blank biofilm carriers at the start-up showed high average TN removal rates (500 g-N/m^3/day) (1.25 g-N/m^2/day) and efficiencies (80%) after a considerably long period of the adaptation of anammox microorganisms (Figs. 2a, 3a). During the high efficiency period there was only one major upset in TN removal efficiency when it decreased below 4% (Fig. 2a). It took about 7 days for the system to be recovered from inhibition and a full recovery from inhibition (reaching the same TN removal rate as before inhibition) occurred a few weeks later (described below). In the operation of MBBR2 there was one minor upset in TN removal efficiency when it decreased to 37%, followed by a rapid increase to 70% (Fig. 2b) after 4 days. This demonstrates the benefits of a nitrifying biofilm matrix on sustaining the anammox TN removal process and implies that it is possible to recover quickly from malfunction in this reactor. The average ratios of NO_2^-–N:NH_4^+–N concentrations of influents in both MBBRs as well as the ratios of the COD:TN concentrations were the same—around 1.2 and 0.1–0.8, respectively. The recalcitrant nature of organics present in the reactor ($COD:BOD_7 > 3$, no C_1–C_4 VFAs) indicated to promote and sustain the anammox process while keeping the activity of heterotrophic denitrifying bacteria low. Instead, the concentration of the humic substances detected (35 mg C/l as total organic carbon, TOC) was 2.5 times higher than the concentration of the non-humic substances (14 mg C/l as TOC) in reject water. It is

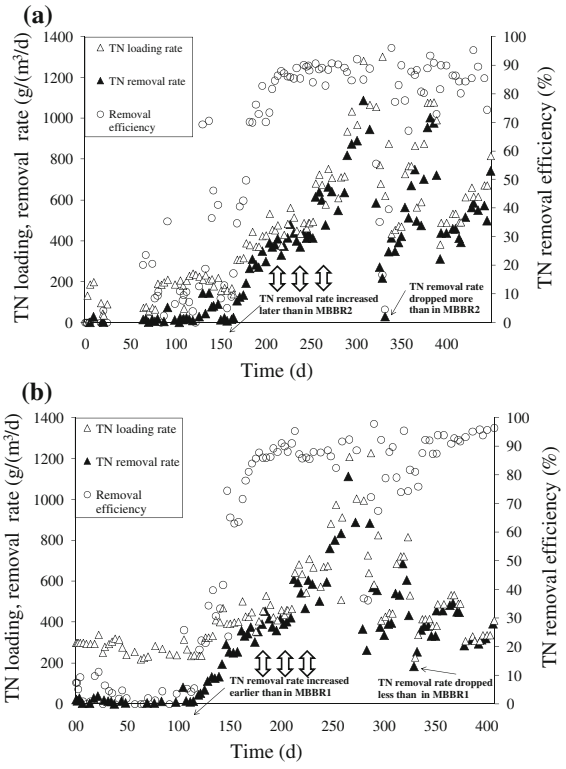

Fig. 2 **a**, **b** Total nitrogen loading rates (NLR *empty triangle*), total nitrogen removal rates (NRR *filled triangle*) and total nitrogen (TN) removal efficiencies (*empty circle*) in **a** MBBR1 and **b** in MBBR2 system

Table 2 Periods of MBBRs operation

Items	MBBR1 periods			MBBR2 periods		
	Start-up	Adaptation	High efficiency	Start-up	Adaptation	High efficiency
Days of operation	0–109	110–180	181–450	0–105	106–147	148–407
HRT (days)	0.75–1	0.6–0.75	0.75–2	0.5–0.75	0.75	0.75–2
Average TN removal rate (g-N/m^3/day)	13.8	65	510	14	107	460
Average TN removal efficiency (%)	10	23	80	25	24	85
Biofilm TSS (mg/carrier), standard deviation (±)	0	3.05 (±0.1)	5.96 (±0.2)–8.85 (±0.3)	1.6 (±0.3)–2.47 (±0.2)	4.42 (±0.2)	4.3 (±0.8)

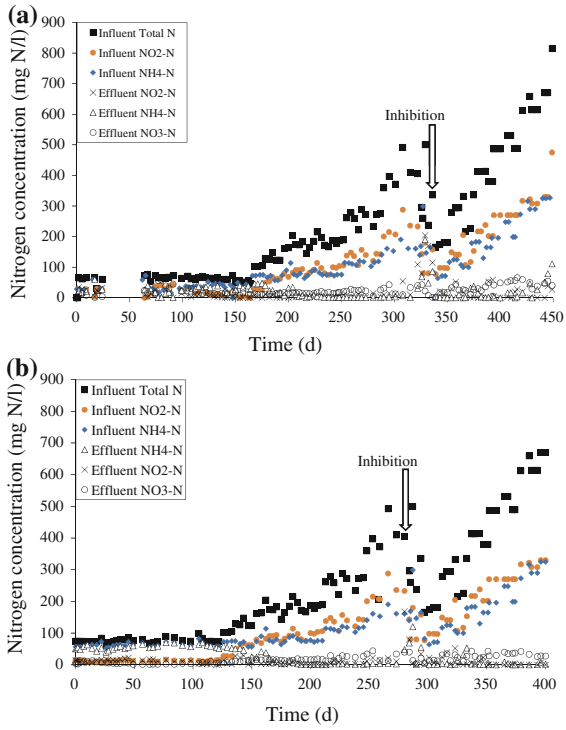

Fig. 3 Changes in the concentrations of nitrogen compounds in the influent and effluent in **a** MBBR1 system and **b** in MBBR2

unknown if anammox microorganisms could use humic substances as alternative electron acceptors in the absence of a terminal electron acceptor or a redox mediator as earlier determined in case of denitrifying bacteria (Aranda-Tamaura et al. 2007).

Start-up period of MBBR1 and MBBR2

The duration of the MBBRs' start-up period was determined as a period when autotrophic TN removal efficiencies exceeded 20% (showing stable values and a continuous increasing trend in TN removal efficiency). Influent and effluent NH_4^+–N concentrations during start-up of MBBR1 were in the range of 20–70 and 1–40 mg N/l, respectively. Influent and effluent NO_2^-–N concentrations during the start-up of MBBR1 were in the range of 10–40 and 5–30 mg N/l, respectively. Effluent NH_4^+–N and NO_2^-–N concentrations in MBBR2 were 40–70 and 5–20 mg N/l, respectively.

During the start-up NH_4^+–N was added in excess and HRTs were kept between 0.75 and 1 days and 0.5–0.75 days in MBBR1 and MBBR2, respectively. The start-up periods for both MBBRs were with similar duration, but ended up with different TN removal efficiencies, as shown in Table 2 and Fig. 2a, b. In MBBR2 on day 105 of the operation the TN removal efficiency increased abruptly from 2 (±1) to 25 (±5.6)% within around one week, but in MBBR1 for the same time the TN removal efficiency remained lower around 11 (±4.4)% with higher timely fluctuations occurring in the effluent concentrations.

In MBBR1 the average TN removal rate achieved was 13.8 (±8.5) g-N/m^3/day (0.03 g-N/m^2/day) and in MBBR2 it was quite similar—14 (±5.5) g-N/m^3/day (0.04 g-N/m^2/day). During the MBBR1 start-up period the highest TN removal rate with the HRT of 0.75 days was 17 (±7) g-N/m^3/day (0.04 g-N/m^2/day) that was achieved within 80 days of operation. In MBBR2 with the HRT of 0.5 days the rate was more than 20 (±11.3) g-N/m^3/day (0.05 g-N/m^2/day), which occurred already after the operation period of 3 weeks, showing benefits of the support of a nitrifying biofilm to trigger off the autotrophic TN removal process with less time.

NO_3^-–N concentration in the MBBR1 effluent increased from 2 to 45 mg N/l during the start-up. DO concentration in the systems (MBBR1, MBBR2) and in the feed tank was high enough to ensure the propagation of *Nitrospira* bacterial species. NO_3^-–N concentration of the effluent during the start-up period of MBBR2 (containing biofilm carriers with nitrifying biomass) was lower (2.4–12.5 mg N/l) than in MBBR1, possibly due to the denitrifying activity. *Nitrospira* bacteria are one of the most resistant NO_2^--oxidizing bacterial species in conditions of low DO concentration (Blackburne et al. 2007) (in our case the DO concentration inside the reactors was 0–0.2 mg O_2/l). As during the stationary anammox process phase *Nitrospira* bacterial species were also found in the biofilm bacterial consortium (described below), it demonstrated that no inhibition of the activity of these bacteria by long-term anoxic conditions occurred.

Benefits of nitrifying biofilm matrix over blank carriers in terms of achieving higher TN removal efficiencies with less time were recognized. Stoichiometrical and the DNA evidences confirming the presence of the anammox process are presented in paragraphs "Stoichiometry of anammox" and "PCR and DGGE observations."

Adaptation period of MBBR1 and MBBR2

In MBBR1 the average TN removal rate achieved was 65 g-N/m^3/day (0.16 g-N/m^2/day) and in MBBR2 it was 107 g-N/m^3/day (0.27 g-N/m^2/day), emphasizing that the adaptation time of anammox microorganisms could be shortened and higher TN removal efficiencies could be achieved if the carriers are initially covered with nitrifying biofilm. In order to increase the growth capacity of anammox microorganisms by nitrogen species supply, the NLR was increased step-wisely to keep the NO_2^- concentration in the effluent in the range of 1–10 mg N/l. With a stepwise increase in NLR, anammox microorganisms were well adapted to higher influent concentrations (around 300 mg N/l).

The chosen anammox biofilm growth strategy made it possible to grow an anammox biofilm tolerating increased influent loading rates without the occurrence of irreversible inhibition. By keeping NLR into MBBR1 180–230 g-N/m^3/day on days 133–147, TN removal efficiencies increased from 19 to 41% and TN removal rate increased from 43 to 89 g-N/m^3/day. The MBBR2 system responded to step-wisely increased influent nitrogen concentrations also positively as the influent's NLR was increased from 300 to 400 g-N/m^3/day during 133–147 day period, the TN removal rate increased from 130 to 290 g-N/m^3/day (from 0.3 to 0.7 g-N/m^2/day), with TN removal efficiency increasing from 40 to 74%.

Biomass concentration on carrier elements was also higher in MBBR2 as compared with MBBR1 (Table 2). Influent and effluent NH_4^+–N concentrations during adaptation phase of MBBR1 were in the range of 30–75 and 20–40 mg N/l, respectively. For MBBR2 effluent NH_4^+–N and NO_2^-–N concentrations were 15–70 and 1–30 mg N/l, respectively. At the end of the adaptation period satisfactory TN removal rates and efficiencies were observed. For MBBR1 the TN removal rate was 200 g-N/m^3/day (0.5 g-N/m^2/day) and TN removal efficiency 50%. For MBBR2 the TN removal rate was 290 g-N/^{3}/day (0.73 g-N/m^2/day) and TN removal efficiency 74%.

Different *Nitrospira* and anammox species were detected from MBBRs (see below) during the adaptation period. The coexistence of these bacteria was considered to be responsible for a disproportionately high effluent NO_3^- concentration in MBBRs. NO_3^-–N concentration in MBBR1 during days 110–150 gradually increased from 12 to 20 mg N/l and at the end of the adaptation period decreased back to 12 mg N/l, probably by the activity of denitrifying microorganisms (Fig. 3a). In MBBR2 the NO_3^-–N concentration increased from 5 to 15 mg N/l during this period. For the rest of the reactors' operation period the effluent's NO_3^-–N concentration did not show any significant increase in MBBR2, but the NO_3^-–N concentration kept increasing in MBBR1. It shows that in the system having a pre-existing biofilm matrix (MBBR2), under anoxic conditions denitrifying bacteria can also develop and improve the effluent quality in terms of lowering the concentration of NO_3^-–N. For thorough mass-balance analysis there are not enough data on COD, but its concentrations in the measured times in the effluent were around 50 mg/l, in the influent 86 mg/l. 38% of COD was removed, which could only be done by denitrifiers as also the concentration of NO_3^- in effluent was less than anammox stoichiometry would suggest. So, in day 182 of MBBR2 operation:

N balance Influent N: $NO_3^- + NO_2^- + NH_4^+ = 5.6 + 53.5 + 66.6 = 125.6$ mg N/l

Effluent N: $NO_3^- + NO_2^- + NH_4^+ = 12.6 + 2.22 + 22.8 = 37.6$ mg N/l, 70% removed.

Per one mole of NH_4^+–N 0.26 mol of NO_3^-–N should have been formed by anammox process, but in the experiment only 0.16 mol was formed. So, 38% less formation could refer to its utilization by denitrifiers.

Carbon balance Influent COD 86 mg/l. Effluent COD 50 mg/l. 36 mg/l removed.

As with anammox process no COD should be consumed (since low-molecular organic acids, a potential alternative substrate for the anammox bacteria, were absent in reject water) 36 mg/l removal should occur due to the activity of denitrifiers.

In MBBR1 and MBBR2 the average ratios of produced NO_3^-–N to consumed NH_4^+–N were 0.33 and 0.28, respectively. The ratios in general were close to the anammox stoichiometrical ratio of 0.26 (Strous et al. 1998), which fully indicated a high activity of anammox microorganisms in MBBRs. The colour of the biomass changing from light brown to the red colour characteristic of anammox microorganisms during this period was also an indicator of the effective adaptation of the anammox microorganisms.

High efficiency period of MBBR1 and MBBR2

MBBRs exhibited a relatively stable treatment performance with high average TN removal efficiencies between 80 and 85% and with a moderate average TN removal rate around 500 g-N/m^3/day (1.25 g-N/m^2/day). The highest TN removal rate achieved on day 308 in MBBR1 around 1,000 g-N/m^3/day (on average 500 g-N/m^3/day) at HRT of 0.75 days showed the system's ability to treat high TN loads effectively. Respective areal nitrogen removal rates were 2.5 g-N/m^2/day (at the maximum) and 1.25 g-N/m^2/day (on average). In MBBR2 fewer and lower-scale fluctuations appeared in terms of TN removal efficiencies than in MBBR1.

The MBBR1 process working at maximum capacity was observed when in addition to a 1/3 increase in the influent's NH_4^+–N concentration also the HCO_3^- concentration was increased twice, resulting in increased NO_2^-–N concentrations in the effluent. In detail, NO_2^-–N concentrations increased from 13 to 54 mg N/l in a week's time when the ratios of reject water (containing HCO_3^- concentration of 4,500 mg HCO_3^-/l) to tap water were increased from 1:4 to 1:2 in the influent. At the same time the NO_2^-–N and NH_4^+–N concentrations in the influent were increased from 200 to 288 mg N/l and from 200 to 300 mg N/l, in MBBR1 and MBBR2, respectively. High substrate concentrations in the feed with high NLR caused the system overloading. Effluent NH_4^+–N concentrations during inhibition were in the range of 50–200 mg N/l and effluent NO_2^-–N concentrations were in the range of 80–200 mg N/l, respectively. After the MBBR1 system having been working for 2 weeks with a high TN removal rate, on day 332 its TN removal efficiency and rate decreased promptly to 4% and 29 g-N/m^3/day (0.07 g-N/m^2/day), respectively due to the combined effects of high TN loading rate and high influent HCO_3^- concentrations. The occurring inhibition was one of the factors for a decreased TN removal rate in the later phase of the MBBRs' operation and caused the need for increasing HRT. The system recovered from inhibition within a week by using a higher influent NH_4^+–N:NO_2^-–N ratio (around 1.2) along with a lower HCO_3^- concentration of 750 mg HCO_3^-/l. As a result TN removal rate of 410 g-N/m^3/day (1.03 g-N/m^2/day) was achieved. During inhibition the free ammonia concentrations in the effluents of the reactors (5–10 mg N/l) still turned out to be below the inhibitory values of anammox microorganisms (13–15 mg N/l) (Plaza et al. 2011; Jaroszynski et al. 2011). This was also the case with free nitrous acid concentrations (below 0.1 mg N/l) due to the pH in the effluents being 7.5–8.5. On day 382, a TN removal rate of 1,000 g-N/m^3/day (2.5 g-N/m^2/day) was detected again, showing a full recovery of the process when the achieved NLR was as high as it was before the inhibition phase. Earlier, only few authors (Yang et al. 2010; Liao et al. 2008) have paid attention to the inhibiting effect of HCO_3^- on the anammox process, while others considered the concentrations of unionized NO_2^- (free nitrous acid) or NH_4^+ (free ammonia) concentrations as causes of inhibition (Jaroszynski et al. 2011). For precaution matters and preparation the series of batch tests the TN loading was held lower afterwards.

In the MBBR2 system the TN removal efficiency of 90% with a high TN removal rate of 420 g-N/m^3/day (1.05 g-N/m^2/day) was achieved after 200 days of operation, earlier than in MBBR1. The highest TN removal rate detected on day 266 was 1,110 g-N/m^3/day (2.78 g-N/m^2/day), higher than in MBBR1, which also showed high TN removal efficiencies (92%), but the average TN removal rate during the period was similar to that in MBBR1 (around 500 g-N/m^3/day) (1.25 g-N/m^2/day). In comparison, such a high TN removal rate by the anammox process has earlier been achieved within 295 days in a biofilm reactor treating reject water (Zhang et al. 2010). In MBBR2 the recovering from inhibition caused by effluent high NO_2^-–N and HCO_3^- concentrations took a shorter time period (about 4 days) and the inhibition itself was not that severe as in MBBR1—the TN removal rate and efficiency during the inhibition decreased less (Figs. 2a, b, 3a, b). The TN removal rate decreased from 890 g-N/m^3/day (2.23 g-N/m^2/day) to 260 g-N/m^3/day (0.65 g-N/m^2/day) and the TN removal efficiency from 89 to 36% in this case. TN removal rate of 880 g-N/m^3/day (2.23 g-N/m^2/day) was achieved again on day 287 after a short recovery period. Nitrifying biofilm matrix protected anammox microorganisms from the inhibiting amounts of NO_2^-–N and HCO_3^- and ensured the achievement of a high TN removal rate with less time than in MBBR1. In other studies the recovery from the inhibition caused by a HCO_3^- concentration (1,500 mg HCO_3^-/l) has also taken a short period of time (5 days) (Yang et al. 2010), consistent with the behavior observed in our

study. In contrast, recovering from NO_2^- inhibition has lasted up to 1 month (Ni et al. 2010). Appropriate inorganic carbon concentration in reject water could ensure a steady run of the anammox bioreactor, especially in systems where partial nitrification is not combined with the anammox process and high inorganic carbon concentrations could inhibit the anammox process (Yang et al. 2010).

If partial nitritation step would have been applied to the anammox MBBRs high HCO_3^- concentration in the effluent is not limiting the anammox process as ammonium oxidizing bacteria producing NO_2^- utilize HCO_3^- also.

TSS content on a biofilm carrier increased in MBBR1 from the start-up period till the end of the stationary phase (from days between 181 and 350) from 3.05 (±0.3) to 5.96 (±0.2) mg/per carrier bringing along a decline in the TN removal rate below 400 g-N/m^3/day (1 g-N/m^2/day).

In contrast, in MBBR2 the TSS content stayed constant throughout 330 days of the reactor operation and its efficiency was above 400 g-N/m^3/day (1 g-N/m^2/day) with HRT being 0.75 days. On day 368 a TN removal rate of 500 g-N/m^3/day (1.25 g-N/m^2/day) was achieved when the system was operated at the same HRT (2 days) as in MBBR1. In the period after 360 days free ammonia and free nitrous acid concentrations in the effluents of MBBR1 and MBBR2 were in range 0.01–0.1 and 0.01–0.08 mg N/l, respectively. Therefore, the concentrations of these compounds were not decreasing TN removal efficiency.

Stoichiometry of anammox

In the start-up phase of the MBBRs' process the ratios of the produced NO_3^-–N and consumed NO_2^-–N to the consumed NH_4^+–N determined were 1.39:1.75:1 in MBBR1 and 0.37:1.23:1 in MBBR2, respectively. In the stationary phase the ratios were 0.12:1.12:1 in MBBR1 and 0.10:1.19:1 in MBBR2, respectively. The ratios determined were different from the values characteristic to the anammox reaction (0.26:1.32:1) presented by Strous et al. (1999) and from the values achieved during the MBBRs' adaptation phase. Heterotrophic denitrifying bacteria may also have some role in higher NO_2^-–N consumption and lower NO_3^-–N production as they could get energy from the biodegradation of cellular organics or extracellular polymer substances released from the decayed bacteria. Anammox microorganisms can consume NO_3^- also by themselves to shift these ratios (Kartal et al. 2007a). Lower ratios (0.13:1.08:1) of the produced NO_3^-–N and the consumed NO_2^-–N to the consumed NH_4^+–N in the treatment of reject water were similarly detected by Zhang et al. (2010) with a stable achieved TN removal rate of 910 g-N/m^3/day (2.28 g-N/m^2/day).

The presence of recalcitrant organics ensured the anammox process avoiding overgrowth of denitrifying bacteria from anammox microorganisms. Possibly, TN removal efficiencies (including higher NO_3^- reduction) could also be improved through electron transfer processes mediated by humic substances (recalcitrant organics), which could also be able to be alternative electron acceptors.

Batch tests

Batch tests were carried out with MBBR1 biofilm carriers to determine anammox process occurring in biofilm. As seen from Fig. 4 rapid NO_2^- and NH_4^+ depletion with NO_3^- formation occurred in batch test. Utilization of NH_4^+ and production of NO_3^- are specific to anammox reaction. The ratio of produced NO_3^-:consumed NH_4^+ was 0.19:1. Both these factors imply to anammox reaction taking place in the biofilm system. Lower production of NO_3^- than characteristic to pure anammox culture implies to additional heterotrophic activity occurring in the biofilm. Formation of hydrazine from hydroxylamine (50 mg/l of hydroxylamine added) occurring in our batch (Fig. 4)

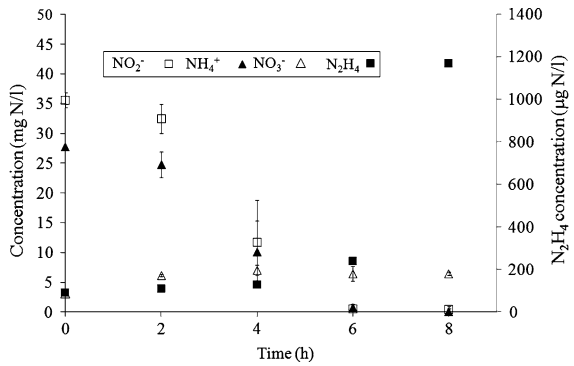

Fig. 4 Time dependent changes of NH_4^+ *filled triangle*, NO_2^- *filled square* and NO_3^- *empty triangle* and hydrazine *empty square* concentrations in time in batch experiment with MBBR1 biofilm carriers taken on reactor operation day 400 with added hydroxylamine (concentration of 50 mg/l)

is characteristic only to anammox microorganisms (van der Star et al. 2008).

FISH studies

FISH analyses performed after 5 months of operation showed the presence of anammox microorganisms in the biofilm of MBBRs belonging to "*Candidatus* Brocadia and Kuenenia" (Fig. 5a, b). The detected clusters of anammox bacteria were dense, and the anammox cells were abundantly present.

PCR and DGGE observations

PCR and DGGE analyses performed after 6 and 7 months of anammox process in MBBR1 and MBBR2, using anammox-specific primers Pla46f/Amx368r and Pla46f/Amx667r, resulted in the detection of sequences which were highly similar (99%) to DNA sequences belonging to the uncultured *Planctomycetales bacterium clone P4* (GenBank ID: DQ304521.2) (Fig. 6). Also highly similar (99%) sequences to uncultured *anaerobic ammonium-oxidizing bacterium clone W1* 16S ribosomal RNA gene, partial sequence (Genbank ID: HQ906639.1) were detected in MBBR2. The species of these microorganisms were detected before and after applying substrates at inhibiting concentrations.

PCR conducted with the primer set NSR1113f/NSR1264r indicated that Candidatus *Nitrospira defluvii* (GenBank ID: FP929003.1) (sequence similarity 99%) and *Nitrospira* sp. clone 53 (GenBank ID: HQ424565, (sequence similarity 95%) were detected in both the MBBR1 system and in the MBBR2 system

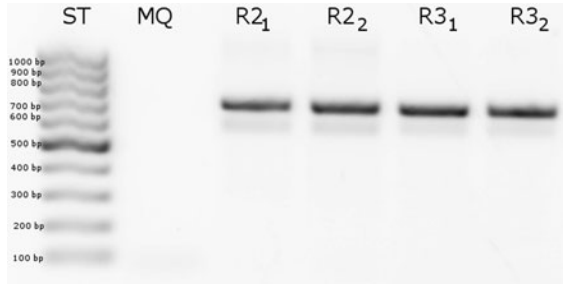

Fig. 6 PCR products amplified with the primer set Pla46f/Amx667r (~620 bp), visualized on agarose gel by UV transillumination. ST-GeneRuler 100 bp DNA Ladder (Fermentas), MQ-Sterile water used in PCR as negative control, R2$_1$-MBBR1 after 6 months of enrichment, R2$_2$-MBBR1 after 7 months of enrichment, R3$_1$-MBBR2 after 6 months of enrichment, R3$_2$-MBBR2 after 7 months of enrichment

and uncultured *Nitrospira* sp. *clone S1-62* (GenBank ID: HQ674926.1) (sequence similarity 95%) was found in the MBBR2 system.

PCR-DGGE conducted with the eubacterial primer set GC-BacV3f/907r also resulted in the detection of Candidatus *N. defluvii* in the MBBR1 and MBBR2 systems.

A phylogenetic neighbour-joining tree (Fig. 7) was constructed to show the phylogenetic relationships between the two anammox microorganisms detected in the systems and other anammox microorganisms including "*Brocadia fulgida*" and Candidatus "*Kuenenia stuttgartiensis*".

Gaul et al. (2005) have suggested that Brocadia strains, e.g. "*Brocadia anammoxidans*" strains are highly inhibited by elevated NO_2^- concentrations, and therefore they are not enriched in reactor systems of high

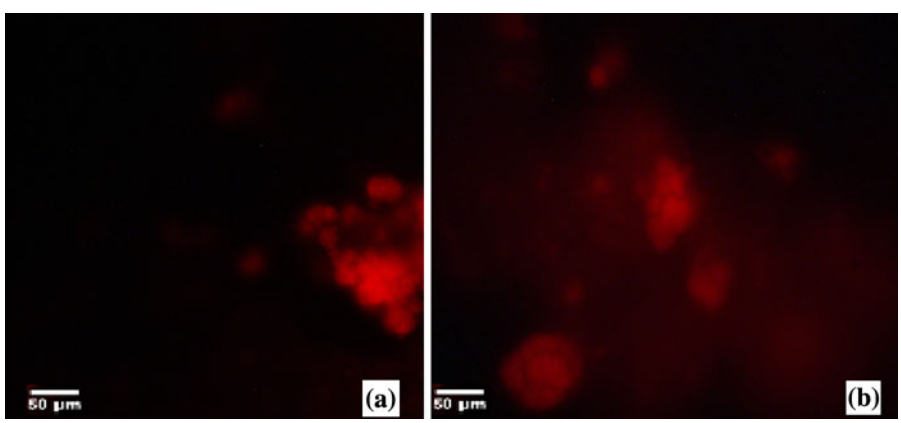

Fig. 5 Representative micrograph set of the MBBR anammox biofilm, with a FISH staining displaying anammox bacteria with Cy3-labeled Amx820 of **a** MBBR1 and **b** MBBR2

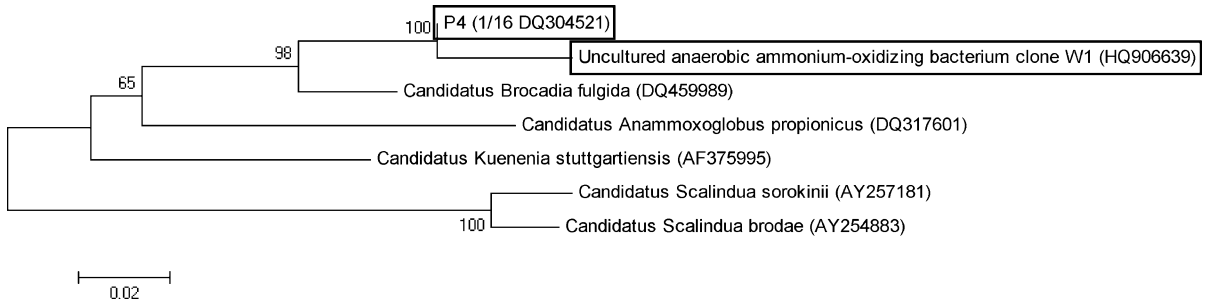

Fig. 7 Phylogenetic neighbour-joining tree, reflecting the relationships between some known anammox bacteria and the identified sequences (uncultured *Planctomycetales bacterium clone P4* and uncultured *anaerobic ammonium-oxidizing bacterium clone W1*), based on 16S rRNA genes amplified using *Planctomycetales*-specific primers. *Numbers* at the nodes are percentages of bootstrap values. Branch lengths correspond to sequence differences as indicated by the *scale bar*. The GenBank accession numbers are indicated

NO_2^- concentration, which temporarily occurred in our system. The reason is the lower affinity of "Brocadia" species for NO_2^- (Strous et al. 1999) as compared to Candidatus *K. stuttgartiensis* (0.2–3 μM) (van der Star et al. 2008). As opposed to this hypothesis, which had it that selective enrichment of Candidatus *K. stuttgartiensis* should take place, these microorganisms were not detected during the stationary phase in our reactors. Instead, the detection of anammox microorganisms (uncultured *Planctomycetales bacterium clone P4* and uncultured *anaerobic ammonium oxidizing bacterium clone W1*) related to *Candidatus B. fulgida* showed that this bacterium strain is tolerant to temporarily high TN loading rate, HCO_3^- and NO_2^--concentrations.

Other anammox species such as Candidatus "Scalindua" being detected under marine conditions (Schmid et al. 2003) and Candidatus "Anammoxoglobus" being enriched by adding propionate (Kartal et al. 2007b) were not found in our systems. The latter is possibly due to the fact that there were no VFAs present in our feeding medium.

On the basis of our results, anammox microorganisms in the biofilm reactors could be transferred from wastewater onto biofilm carriers containing nitrifying biomass and blank biofilm carriers resulting in a moderately high average TN removal rate around 510 g-N/m^3/day (1.28 g-N/m^2/day) and TN removal efficiency of between 80 and 85% in both systems.

Conclusions

(1) Anammox microorganisms were grown on biofilm of two MBBR systems in which similar average TN removal efficiency of 80–85% and the TN removal rate of 500 g-N/m^3/day (1.25 g-N/m^2/day) were achieved.
(2) A persistent and stable anammox process was achieved in MBBR systems with substrate TN concentration being gradually increased from 60 to 800 mg N/l.
(3) A better tolerance to high substrate loading and a short recovering period from the temporary interference of the activity of anammox microorganisms were detected in the system initially containing nitrifying biomass on biofilm carriers.
(4) Average stoichiometrical ratios of consumed NO_2^-:produced NO_3^-:consumed NH_4^+ determined during the stationary phase of the MBBRs' operating process were 1.12:0.12:1 and 1.19:0.10:1 in systems with and without the initially containing nitrifying biofilm matrix, respectively.
(5) Batch test confirmed the anammox reaction by production of hydrazine from hydroxylamine—characteristic only to anammox and by production of NO_3^-, although in lower amount than the anammox stoichiometry would predict (due to the activity of heterotrophic nitrifying bacteria). The ratio of produced NO_3^-:consumed NH_4^+ was 0.19:1.
(6) Uncultured *Planctomycetales bacterium clone P4,* Candidatus *N. defluvii* and uncultured *Nitrospira* sp. *clone 53* were detected from MBBR initially containing the blank carriers and from MBBR initially containing the nitrifying biomass, also, uncultured *ammonium-oxidizing bacterium clone W1* and *Nitrospira* sp. *clone S1-62* were detected.

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