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Nitrate reduction by organotrophic Anammox bacteria in a nitritation/anammox granular sludge and a moving bed biofilm reactor

Mari K.H. Winkler [a,1], Jingjing Yang [b,1], Robbert Kleerebezem [a], Elzbieta Plaza [b], Jozef Trela [b], Bengt Hultman [b], Mark C.M. van Loosdrecht [a,*]

[a] Delft University of Technology, Department of Biotechnology, Julianalaan 65, 2628 BC Delft, The Netherlands
[b] Royal Institute of Technology (KTH), Department of Land and Water Resources Engineering, Teknikringen 76, SE-100 44 Stockholm, Sweden

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ABSTRACT

The effects of volatile fatty acids (VFAs) on nitrogen removal and microbial community structure in nitritation/anammox process were compared within a granular sludge reactor and a moving bed biofilm reactor. Nitrate productions in both systems were lower by 40–68% in comparison with expected nitrate production. Expected sludge production on VFAs was estimated to be 67–77% higher if heterotrophs were the main acetate degraders suggesting that Anammox bacteria used its organotrophic capability and successfully competed with general heterotrophs for organic carbon, which led to a reduced sludge production. FISH measurements showed a population consisting of mainly Anammox and AOB in both reactors and oxygen uptake rate (OUR) tests also confirmed that flocculent biomass consisted of a minor proportion of heterotrophs with a large proportion of AOBs. The dominant Anammox bacterium was Candidatus "*Brocadia fulgida*" with a minor fraction of Candidatus "*Anammoxoglobus propionicus*", both known to be capable of oxidizing VFAs.

1. Introduction

Partial nitritation/anammox process is commonly used to remove nitrogen from ammonium rich wastewater. During this process ammonium is firstly oxidized to nitrite by ammonium oxidizers (AOB) (1) after which the remaining ammonium and nitrite are converted into dinitrogen gas by Anammox bacteria (2).

$$NH_4^+ + 1.5O_2 + 2HCO_3^- \rightarrow NH_4^+ + NO_2^- + 2CO_2 + 3H_2O \quad (1)$$

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 0.066CH_2O_{0.5}N_{0.15} + 2.03H_2O \quad (2)$$

Anaerobic ammonium oxidizing bacteria (Anammox) are capable of oxidizing ammonium with nitrite as electron acceptor. During this reaction a nitrate yield on ammonium of 0.26 gNO_3-N/gNH_4-N is expected due to the use of nitrite as electron donor for carbon dioxide reduction to biomass components. This leads to incomplete nitrogen removal in Anammox based nitrogen removal processes (Strous et al., 1999). Until recently it was considered that Anammox bacteria were obligate autotrophs not capable of converting organic carbon substrates. However, lately it was found that Anammox bacteria have the capacity to oxidize volatile fatty acids with nitrate as electron acceptor, while forming ammonium with nitrite as intermediate (Guven et al., 2005; Kartal et al., 2008). Anammox bacteria do not incorporate the fatty acids into biomass, but completely oxidize it into CO_2 thereby maintaining a low biomass yield (Kartal et al., 2007a). This organotrophic potential of Anammox bacteria has advantages for wastewater treatment. The conversion of fatty acids by Anammox bacteria prevents a high sludge production and allows the removal of nitrate yielding in lower total nitrogen effluent concentration (Winkler et al., 2012). Heterotrophic denitrifying bacteria oxidize fatty acids with either nitrate or nitrite as electron acceptor with a biomass yield on organic compounds is ca. 0.5 gVSS/gCOD. The produced biomass is an unwanted and costly by-product in wastewater treatment. Investigations showed that the most critical point in the competition between organotrophic Anammox and heterotrophs for acetate is the C/N ratio in the influent. If this ratio increases to values larger than 1 gCOD/gN the slow growing Anammox bacteria seem to lose the competition against general heterotrophs (Chamchoi et al., 2008; Guven et al., 2005; Pathak et al., 2007).

Simultaneous partial nitrification, Anammox and denitrification (SNAD) processes treating wastewater with an approximate ratio of VFA/N of 0.5 gCOD/gNH_4-N were developed in different reactor configurations, including UASB (Lan et al., 2011), non-woven rotating biologic contactor (NRBC) (Chen et al., 2009) and a sequencing batch reactor (Xu et al., 2010). All these studies were conducted at temperatures of 30–36 °C under constantly aerated conditions and partly

* Corresponding author. Tel.: +31 152782628; fax: +31 152782355.
E-mail address: M.C.M.vanLoosdrecht@tudelft.nl (M.C.M. van Loosdrecht).
[1] The first two authors equally contributed to this research.

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with nitrate and nitrite in the influent. However real wastewater usually does not contain nitrate or nitrite and is not high in temperature. Moreover, the presence of acetate and oxygen (strong electron donor and acceptor) at the same time is favorable for general heterotrophic bacteria hence outcompeting Anammox for the VFA. A better strategy would be to anoxically feed acetate and to promote the oxidation of VFAs by nitrate which is produced within the process (Winkler et al., 2012). The temperature of the wastewater will also have large impact on the competition of bacteria performing nitrogen removal. Pure cultures of *Nitrosomonas* sp. and *Nitrobacter* sp. have an optimum temperature of 35 °C and 38 °C, respectively (Grunditz and Dalhammar, 2000). At temperatures above 25 °C the specific growth rate of AOB is higher than for the NOB. Therefore it is possible to effectively washout the NOB by applying a short solid retention time (SRT) which is lower than the minimum retention time required for NOB (Hellinga et al., 1998; Jetten et al., 1999). Below 15 °C the maximum growth rate of AOB is lower than that of NOB making a control based on growth rate impossible (Hellinga et al., 1998). Previous work has demonstrated that bacterial competition is influenced in granular sludge by particle size. NOB tend to grow in smaller particles due to a larger aerobic volume fraction whereas Anammox bacteria dominate in bigger granules due to smaller aerobic volume fraction (Volcke et al., 2010; Winkler et al., 2011b). The selective sludge removal of smaller granules has shown to minimize the growth of NOB in CANON type systems (Winkler et al., 2011b). Therefore, we here also studied the microbial differences of flocculent sludge and the biofilm to see if selective sludge removal could be used to minimize heterotrophic growth. In this study, we focused on the potential of Anammox bacteria to simultaneously convert ammonium and nitrite to nitrogen gas as well as to oxidize fatty acids with nitrate as electron acceptor, under low dissolved oxygen (1.5–3 mg O_2/l) concentrations and ambient temperature (18–25 °C) conditions. For this we operated an aerobic granular sludge (AGS) and a moving bed biofilm reactor (MBBR) system under similar conditions. Both reactors received wastewater with a COD/N ratio of approximately 0.5 gCOD/gNH_4-N and were operated under aerobic/anoxic conditions with a ratio of 3. We then compared both systems for their nitrate effluent concentrations, sludge production and the microbial community composition of biofilm (biomass fixed on the MBBR carriers and in granular form) and flocs (biomass not fixed on the MBBR carriers or granules and suspended in the reactor).

2. Methods

2.1. Operation of aerobic granular sludge

An aerobic bubble column reactor was run for 180 days with Anammox dominated granular sludge from previous research (Winkler et al., 2012). The influent contained 190 mgNH_4-N/l (NH_4Cl)) and 100 mgCOD/l (C_2H_3OONa). The medium further consisted of 0.2 mM $MgSO_4 \cdot 7H_2O$, 0.2 mM KCl, 2 mM $NaHCO_3$, 0.2 mM K_2HPO_4, and 0.1 mM KH_2PO_4 .'Visniak and Santer' solution was used to provide trace elements (Visniak and Santer, 1957). The pH value was kept constant at 7.2 ± 0.2 and dissolved oxygen (DO) concentration was controlled at 1.5 mg O_2/l. The DO was set by recirculating the off-gas and blending with fresh air. In this way, DO could be regulated while the superficial airflow was kept constant at 2 l/min (Mosquera-Corral et al., 2005). The reactor was operated in a sequencing fed batch mode. During the mixed anoxic feeding period (60 min) effluent with nitrate from the aerobic period was returned to the influent containing acetate and ammonium. Next an aerated period for partial nitrification was introduced lasting 172 min, followed by a settling period (3 min), and an effluent withdrawal period (5 min). During the effluent period half of the reactor volume (1.5 l) was discharged and half (1.5 l) remained in the system. During influent addition, the medium was mixed with the remaining 1.5 l which contained nitrate from previous cycle in order to allow acetate oxidation and nitrate reduction by Anammox bacteria. Fig. 1A shows the cycle operation (1) mixed anaerobic feeding period with recycled nitrate from previous aerobic period (2) aerobic period (3) settling period (4) discharge period. Ammonium, nitrite and nitrate were measured by means of flow injection analysis (Quick Chem8500, Lachat instruments). Acetate was measured by using a High Performance Liquid Chromatography (HPLC). Biomass production was monitored over time by catching effluent from one cycle and determining dry weight and ash content.

2.2. Operation moving bed biofilm reactor

A nitritation/anammox process was investigated in a moving bed biofilm reactor (MBBR) at Hammarby Sjöstadsverk research station run over a time frame of 480 days (Stockholm, Sweden). The reactor had a volume of 200 l and 40% of the volume was filled with Kaldnes biofilm carriers. The effective specific surface area of the carriers was 500 m^2/m^3. The MBBR was continuously fed with a loading rate of 739 ± 55 gNH_4-N/m^3/day as well as 406 ± 42 gCOD/m^3/day with reject water from a sludge digester. The reactor was operated at 25 °C. Conductivity, redox and pH were measured online. The air was supplied intermittently with 45 min aerated phase and 15 min non-aerated phase in each hour (Fig. 1B). DO was controlled by a PID controller and kept at 3 mg/l during aerated phase. The pH value inside the reactor was not controlled and it varied between 7 and 8. In MBBR, there was mechanical mixing installed and air was supplied from the bottom of the reactor, so it could almost be considered as a homogenous condition inside reactor. NH_4-N, NO_3-N and COD in the influent and effluent were determined spectrophotometrically with Hach Lange tests kits. The concentration of solids, total suspended solids (TSS) and volatile suspended solids (VSS) were measured according to Standard Methods. VSS in influent was rather small and was neglected in this study. The composition of VFA in reject water was analyzed by using HPLC analysis (Yang et al., 2011).

2.3. OUR measurements

In order to determine the activities of different oxygen consumers, which in this study were heterotrophic bacteria, AOB and NOB, we performed oxygen uptake rate (OUR) tests at 25 °C on biofilm carriers and flocculent biomass. The OUR test was based on the measurement of the oxidation rate of NH_4^+-N, organic matter content and NO_2^--N being produced during the test. Selective inhibition was achieved by subsequently injecting $NaClO_3$ for the inhibition of NOB and ATU (allylthiourea) for the inhibition of AOB. The methodology of the experiments was adapted from Surmacz-Gorska et al. (1996). The buffer solution contained 100 mg/l NH_4^+-N and COD of 100 mg O_2/l, respectively. Activity of heterotrophic bacteria, AOB and NOB were calculated according to the maximum slope of oxygen consumption rate along the time.

2.4. Microscopical characterization of biomass

Biomass samples were taken from flocculated sludge as well as from the granules and biocarriers over time for microscopic analysis. Samples were fixed in 4% paraformaldehyde and incubated for 120 min at room temperature. After fixation, samples were centrifuged for 2 min at 16,000 rpm, washed twice in 1× Phosphate buffer saline (PBS), and re-suspended in volume of 1:1 ethanol/PBS buffer for storage at −20 °C. For hybridization, the fixed samples were dried on a hybridization slide with 6 wells preventing mixing of probe in adjacent wells and dehydrated by incubating

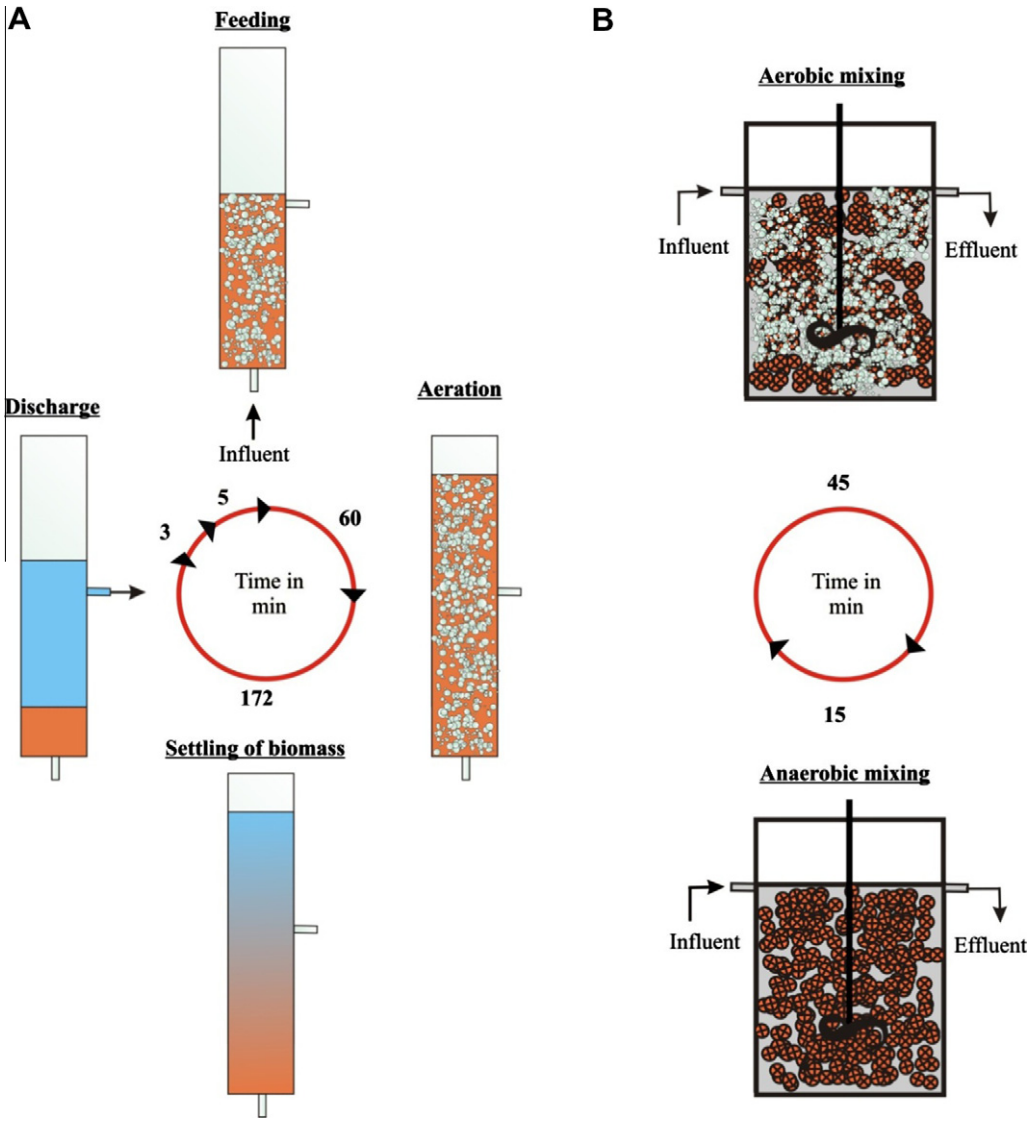

Fig. 1. (A) shows the cycle operation in experimental set-up of (1) mixed anaerobic feeding fed batch period with recycled nitrate from previous aerobic period and ammonium and COD in the influent (2) aerobic period (3) settling period (4) discharge period (B) shows the cycle operation in MBBR with (1) 45 min aerated phase and DO was 3 mg/l and (2) 15 min non-aerated phase and DO was 0 mg/l.

the microscope slides in 50%, 80% and 100% ethanol for 3 min in each solution. After dehydration, the hybridization solution (10 ml) and 25 mg of oligonucleotide probe tagged with a fluorescent label (Fluos, Cy5 or Cy3) was added to each well, and the samples were incubated for 2 h in a humid chamber at 46 °C. The hybridization buffer consisted of a mixture of 360 ml of 5 M NaCl, 40 ml of 1 M Tris (pH 8), 10 ml of a 10% (w/v) sodium dodecylsulfate buffer (SDS), 700 ml of formamide, and 900 ml of MilliQ water. After hybridization, the microscope slides were washed at 48 °C for 15 min by immersing them into 50 ml of washing solution consisting of 800 ml of 5 M NaCl, 500 ml of 0.5 M EDTA, 1000 ml of 1 M Tris (pH 8), and 50 ml of 10% SDS (w/v). The samples were dried and prepared with 2 ml antifade fluorescent mounting oil and analyzed with an epifluorescence microscope (Axioplan 2, Zeiss). Oligonucleotide probes were tagged with three different fluorescent dyes (Cy3, Cy5, Fluos). Probe sequences are listed in Table 1. For the determination of Anammox bacteria, a mixture of probes targeting Candidatus "*Brocadia fulgida*" and Candidatus "*Anammoxoglobus propionicus*" was used. Light microscopic images were taken with a light microscope (Leica).

Table 1
Oligonucleotide probes and primers target microorganisms, and references used in this study.

Probes	Sequence (from '5 to '3)	Specificity	Reference
Bfu613	GGATGCCGTTCTTCCGTTAAGCGG	Candidatus *Brocadia fulgida*	Kartal et al. (2008)
Apr820	AAACCCCTCTACCGAGTGCCC	Candidatus "*Anammoxoglobus propionicus*"	Kartal et al. (2007b)
EUB 338	GCTGCCTCCCGTAGGAGT	*Most bacteria*	Amann et al. (1990)
EUB 338 III	GCTGCCACCCGTAGGTGT	*Verrucomicrobiales*	Daims et al. (1999)
NSO190	CGATCCCCTGCTTTTCTCC	*All AOB*	Mobarry et al. (1996)
NSO1225	CGCCATTGTATTACGTGTGA	*All AOB*	Mobarry et al. (1996)

Anammox probes were tagged with the fluorescent dye Fluos (green) AOB with Cy3 (red) and EUB with Cy5 (blue). For analysis probes of one target group were mixed.

Table 2
Biomass yields obtained from literature for Anammox, AOB and heterotrophic bacteria and calculated the corresponding biomass concentration and relative community composition according to consumed substrate in reactor during one day of operation in phase II. (a) Based on assumption that all acetate was converted by Anammox and (b) based on assumption that all acetate was converted by heterotrophic bacteria.

	$Y_{X/VFA}$	Y_{X/NH_4^+}	gx/m^3/day		Community composition [%]				Reference for yields
			MBBR	AGS	MBBR		AGS		
Anammox[a]	–	0.066	21	30	32[a]	16[b]	32[a]	7[b]	van der Star et al. (2007)
AOB	–	0.14	45	63	68[a]	34[b]	68[a]	15[b]	Blackburne et al. (2007)
Heterotroph[b]	0.4	–	67	336	–	50[b]	–	78[b]	Beun et al. (2001)

Table 3
Comparison of operational data of the MBBR and AGS.

Parameters	Abbreviations	Unit	MBBR	AGS	Note
Operational parameters					
Temperature	*T*	[°C]	25 ± 0.2	18 ± 3	
Ratio aerobic anaerobic	r_ae/an	[–]	3.0	2.9	Ratio between aerobic and anaerobic duration in each operation cycle
Volume	*V*	[l]	200	3	
Biomass parameters					
Biofilm/granular biomass	x	[gVSS/l]	5.6 ± 0.03	7.3 ± 0.3	In order to do the comparison, concentration of biofilm in MBBR converted from [g VSS/m^2] to [gVSS/l] (measured values)
Flocculent sludge measured	x_f	[gVSS/m^3/d]	44 ± 3	100 ± 48	
Heterotrophic biomass expected	x_het	[gVSS/m^3/d]	67	336	Calculated values (Based on table 2)
Autotrophic biomass expected	x_aut	[gVSS/m^3/d]	67	93	
Less biomass produced than expected	x_exp	[%]	67	77	Calculation: 1-x_f/(x_het + x_aut)
COD parameters					
Load COD	L_COD	[gCOD/m^3/d]	120 ± 6	600[a]	Measured values
Volumetric removal capacity	R_COD	[gCOD/m^3/d]	120 ± 6	600[a]	
Biomass specific removal capacity	q_COD	[gCOD/VSS/d]	0.02 ± 0.001	0.08[a]	
N parameters					
Ammonium load	L_N	[gN/m^3/d]	739 ± 7	1140[a]	Measured values
Volumetric nitrogen removal capacity	R_N	[gN/m^3/d]	647 ± 6	956 ± 34	
Biomass specific removal capacity	q_N	[gN/VSS/d]	0.11 ± 0.002	0.12 ± 0.005	
NO_3 production	R_NO3 m	[gNO_3/m^3/d]	52 ± 4.3	40 ± 5	
NO_3 production expected (autorophic)	R_NO3exp	[gNO_3/m^3/d]	84	124	Calculated values(Based on anammox reaction stoichiometry)
Less NO_3 produced than expected	NO3 exp	[%]	38	68	Calculation: 1-R_NO_3m /(R_NO_3exp)

[a] Synthetic media assured constant loading rates for ammonium and COD. COD was always completely removed.

2.5. Biomass yields

The estimated community composition was based on produced biomass per consumed acetate ($Y_{X/HAC}$) and ammonium (Y_{X/NH_4^+}), respectively (Table 2). It was assumed that all acetate which was fed into the reactor would be metabolized by either Anammox (2.a) or heterotrophic bacteria (2.b). In case of Anammox being the only active bacteria half of the consumed ammonium was supposed to be used for partial nitrification (AOB) and the other half by anaerobic ammonium oxidation. Since Anammox does not incorporate acetate in biomass no growth on acetate for Anammox was assumed. For the conversion from COD to VSS a factor of 1.4 was used (Scherer et al., 1983). The expected reduction for biomass production was based on the comparison between the flocculent sludge concentration measured and the theoretic calculation of produced sludge assuming a degradation of VFAs by heterotrophs only. The reduction for biomass production [in%] was calculated according to following equation: $x_{exp} = 1 - x_f/(x_{het} + x_{aut}) \times 100\%$ (Table 3). The reduction in effluent nitrate concentrations was obtained by comparing the measured nitrate concentration in the effluent with the expected produced nitrate according to known Anammox stoichiometry according to following equation $NO_{3\,exp} = 1 - (R_{NO_3 m}/R_{NO_3\,exp})$. The expected volumetric nitrate production rate $R_{NO_3\,exp} = (R_N/2) \cdot Y_{NH_4/NO_3}$ the experimentally obtained yield of nitrate on ammonium of 0.26 (Strous et al., 1999) was used and symbols are given in Table 3.

3. Results and discussion

3.1. Operational parameters

Table 3 presents operational, biomass, nitrogen and COD parameters from both reactors. Both reactors were run under

comparable operational conditions at ambient temperatures (18–25 °C), with an aerobic/anaerobic time ratio of 3 and received wastewater with a COD/N ratio less than 0.5 gCOD/gNH_4-N. Despite the fact that the reactor volume of the MBBR was 67 times larger than the volume of AGS, the biomass concentration of both reactors was very comparable (5.6–7.3 gVSS/l). The values for biomass specific N removal capacity were also very similar with values of 0.11–0.12 gN/VSS/day. Presence of COD in AGS influent was due to the addition of acetate and for the MBBR, the composition of volatile fatty matter in reject water was tested on a regular basis for COD and once for general VFA composition by HPLC. Results from HPLC revealed the presence of acetic acid in concentration of 139 mg/L. Analysis was also conducted for succinate, propionic acid, butyric acid, methanol and valeric acid all named VFAs were detected but measured concentrations were below the calibration threshold of the HPLC machine.

The flocculent biomass production was monitored over time and was compared to what could be expected if all VFA would have been used by normal heterotrophs. Calculations were done assuming that all acetate was consumed by general heterotrophs. Compared to measured sludge production these calculations revealed that calculated sludge production was 67–77% higher than the measured sludge production (Table 3). Moreover, measured nitrate effluent concentrations were in both reactors 40–68% lower than what could be expected to be produced by autotrophic ammonium oxidation (Table 3). The flocculent sludge showed a higher aerobic activity than the biofilm biomass. Calculations based on the OUR tests showed that the activity of AOB in both flocculent biomass and biofilm was higher than the activity of heterotrophic bacteria (Table 4). NOB showed the lowest activity.

3.2. Microscopic observation

FISH analysis revealed a domination of Candidatus "*B. fulgida*" in the biofilms (carriers and granules) of both reactors. In the MBBR also a small fraction of Candidatus "*Anammoxoglobus propionicus*" was found. For all further studies a mix of both primers sets was used to determine Anammox bacteria. Except from nitrifiers and anammox bacteria, there was only a limited number of eubacteria in the biofilm (carriers and granular) showing that Anammox could maintain itself in both reactors despite of the presence of VFAs. The flocs were also measured for their microbial community composition and results showed a domination of AOB bacteria in both systems whereas in the MBBR the heterotrophic fraction was slightly higher than for the granular sludge system.

3.3. Biomass yields

In order to investigate the conversion of acetate in the reactor we evaluated the biomass production and composition on calculated biomass production based on two conversion routes. If only Anammox and AOB would dominate the system and all acetate and ammonium would be consumed by Anammox and AOB a low sludge production is expected and hence a strongly Anammox (32%) and AOB (68%) dominated system. If all acetate was assumed to be oxidized by heterotrophs and all ammonium assumed to be converted by partial nitrification in combination with anaerobic ammonium oxidation the sludge production was estimated to be 429 gVSS/m^3/d for the AGS and 134 gVSS/m^3/d for the MBBR. If all volatile fatty acids were fully converted by heterotrophs it could be expected that the community of both systems was strongly dominated by heterotrophic bacteria (for MBBR 50% and SBR 78%), with only a minor fraction of Anammox (for MBBR 16% and SBR 7%) and AOB (for MBBR 34% and SBR 15%) (table 2).

Table 4
Activities of AOB, NOB and Heterotrophic bacteria in the biofilm obtained from oxygen uptake rate (OUR) results from MBBR.

g O_2 g VSS^{-1} d^{-1}	AOB	NOB	Heterotrophic bacteria
Biomass on biofilm carriers	0.17 ± 0.014	0.042 ± 0.008	0.093 ± 0.012
Flocculent sludge	0.39 ± 0.01	0.11 ± 0.01	0.21 ± 0.01

3.4. Discussion

Our studies showed that Anammox bacteria could outcompete heterotrophic bacteria at ambient temperatures in two different Anammox based treatment systems. Acetate oxidation was combined with anaerobic ammonium oxidation while keeping sludge production and nitrate effluent concentrations low. The influent of both systems contained ammonium and COD only. The nitrite needed for the oxidation of ammonium and the nitrate needed to oxidize acetate were produced during the aeration period. Until now there are only studies which are reporting the usage of organotrophic capacity of Anammox bacteria with nitrite in the influent (Chen et al., 2009; Lan et al., 2011). In fact, in our study the concentration of nitrite was close to zero all the time even under aerobic condition in both systems and the presence of nitrate was mainly due to the Anammox reaction. Nitrogen removal rates in our research were similar to those reported in systems based on one-reactor nitrition-Anammox processes operated without significant amount of organic matter in the influent (Abma et al., 2010; Siegrist et al., 2008; Wett, 2007). Both systems were operated under aerobic/anoxic conditions which gave possibilities to allow the organorophic oxidation of VFA with nitrate by Anammox bacteria and limit aerobic oxidation of VFA by normal heterotrophs. In this study, chemical analysis results showed that excess nitrate production was reduced by 40–68% as compared to expectations based on anammox stoichiometry (Strous et al., 1999). The denitrification could be due to either heterotrophic bacteria or organotrophic Anammox bacteria. The flocculent biomass production inside both reactors was measured to be 67–77% less than what could be expected based on the assumption that all organic compound were consumed by heterotrophic bacteria (Table 3). Since FISH analysis also showed that heterotrophic bacteria accounted for only a minor fraction of the whole bacterial population within the flocculent sludge and biofilm we conclude that in both reactors Anammox used its organotrophic capability and oxidized an considerable amount of the VFAs with nitrate as electron acceptor, which is in line with earlier studies which have shown that certain Anammox bacteria can fully oxidize organic matter to CO_2 (Kartal et al., 2007a).

OUR tests conducted on flocculent sludge as well as on biofilm carriers showed in both cases that the AOB showed a 2–3 times higher respiration activity than the heterotrophic bacteria. NOB activity was minimal within flocculent and biofilm biomass (Table 4). The results are comparable with the data obtained from a deammonification reactor which also showed that activity of AOB was 2.5 times higher than the activity of heterotrophic bacteria (Gut et al., 2005). Our results hence show that AOB were the dominating group among the oxygen consumers and henceforth successfully outcompete the heterotrophic bacteria. It is generally believed that it is hard to avoid the presence of heterotrophic bacteria in one stage partial nitritation/Anammox process when organic matter will be present. Other studies have also shown a minimal activity of heterotrophic bacteria in nitritation/anammox processes containing organic matter, by keeping the influent COD/N ratio below 0.5 (Guven et al., 2005; Winkler et al., 2012).

Calculations showed that if all acetate was converted by general heterotrophs Anammox bacteria would only account for a minor fraction of the whole population. However, FISH images revealed that a much larger fraction of Anammox than the estimated 6–17% was present in both systems investigated in this study (Table 2). FISH analysis showed that in both systems the microbial composition of flocculent sludge was dominated by AOB. NOBs were barely detected by FISH analysis which is in line with these results obtained from OUR tests. Earlier granular sludge based Anammox studies showed an increased growth of nitrifiers in smaller/flocculent biomass and also that slow growing organisms (here Anammox) are expected to grow in the inner part of a biofilm (Volcke et al., 2010; Winkler et al., 2011b). Mathematical modeling has shown that faster growing organisms (here AOB and Eubacteria) are pushed towards the rim where they are detached from the biofilm (Okabe et al., 1996; Picioreanu et al., 2004; Wanner and Reichert, 1996). Since flocs and barely any granules or bio-carriers will be washed out most of the measured sludge production accounted hence from flocculent sludge dominated by AOBs and a minor fraction of Eub and no anammox bacteria.

Segregation of biomass and selective sludge removal of a certain microbial population in aerobic granular sludge has been shown in earlier studies (Winkler et al., 2011a,b). At higher COD/N ratios than 0.5 gCOD/gN it might be an extra option to counter select against heterotrophic bacteria by selectively removing flocculent biomass hence enriching Anammox bacteria and washing out general heterotrophs. Earlier studies, conducted in a nitrifying–denitrifying Biofilm Airlift Suspension reactor, have already shown that growth of nitrifiers in the biofilm and growth of heterotrophs in suspension could be controlled by manipulating the hydraulic retention time (Van Benthum et al., 1997). Here we showed an option to oxidize volatile fatty acids by Anammox bacteria while obtaining a stable nitrogen removal process at lower temperatures and low COD/N ratios. We showed that Anammox successfully outcompeted general heterotrophs for acetate in laboratory as well as pilot plant tests. These observations show that also for an influent with a low COD/N ratio the nitritation Anammox process can be used, resulting in a lower sludge production and nitrate effluent concentration. This enlarges the operational flexibility of anammox processes and strengthens its relevance for future wastewater treatment process development.

4. Conclusions

Anammox bacteria can outcompete heterotrophic denitrifying bacteria when the ratio of C/N in influent was less than 0.5 gCOD/gN under ambient temperatures in nitritation/Anammox AGS and MBBR. The measured sludge production and nitrate concentration in effluent from both reactors were much less than the expected calculated values. The explanation was that Anammox bacteria organotrophically used organic matter supplied in the influent leading to a low sludge production and low concentration of nitrate in effluent. The dominating bacteria in both reactors were Anammox bacteria and AOB, oxygen uptake rate tests and FISH analysis confirmed these conclusions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version,
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