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Segregation of Biomass in Cyclic Anaerobic/Aerobic Granular Sludge Allows the Enrichment of Anaerobic Ammonium Oxidizing Bacteria at Low Temperatures

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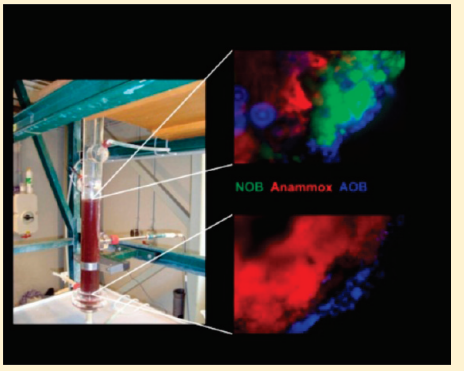
Supporting Information

ABSTRACT: A cyclic anaerobic/aerobic bubble column reactor was run for 420 days to study the competition for nitrite between nitrite oxidizing bacteria (NOB) and anaerobic ammonium oxidizing bacteria (Anammox) at low temperatures. An anaerobic feeding period with nitrite and ammonium in the influent followed by an aerated period was applied resulting in a biomass specific conversion rate of 0.18 ± 0.02 [$gN_2 - N \cdot gVSS^{-1} \cdot day^{-1}$] when the dissolved oxygen concentration was maintained at 1.0 $mgO_2 \cdot L^{-1}$. An increase in white granules was observed in the reactor which were mainly located at the top of the settled sludge bed, whereas red granules were located at the bottom. FISH, activity tests, and qPCR techniques revealed that red biomass was dominated by Anammox bacteria and white granules by NOB. Granules from the top of the sludge bed were smaller and therefore had a higher aerobic volume fraction, a lower density, and consequently a slower settling rate. Sludge was manually removed from the top of the settled sludge bed to selectively remove NOB which resulted in an increased overall biomass specific N-conversion rate of 0.32 ± 0.02 [$gN_2 - N \cdot gVSS^{-1} \cdot day^{-1}$]. Biomass segregation in granular sludge reactors gives an extra opportunity to select for specific microbial groups by applying a different SRT for different microbial groups.



INTRODUCTION

The process of Anammox is a shortcut in the nitrogen cycle whereby ammonium is oxidized with nitrite to nitrogen gas.[1] For the application of Anammox for ammonium removal from wastewater, it is required to produce nitrite by ammonium oxidizing bacteria (AOB) and combine it with the anaerobic oxidation of ammonium at the expense of the nitrite produced. Since its discovery by Mulder in 1992[2] many Anammox based treatment systems have successfully been implemented for cost-efficient full-scale nitrogen removal from anaerobic sludge digestion rejection water.[3−5] To establish good nitrogen removal Anammox and AOB need to be enriched in the reactor system, while nitrite oxidizing bacteria (NOB) need to be outcompeted. Different strategies have been developed to control this competition, and a suite of processes is applied at full scale for nitrogen removal from wastewater with relative high ammonium content and higher temperatures (>20 °C).[6]

In a single reactor for high activity ammonium removal over nitrite (Sharon) system the preferential production of nitrite instead of nitrate from ammonium is accomplished by exploiting the higher growth rate of AOB compared to NOB at higher operational temperatures. This allows for selection of AOB and wash-out of NOB simply by reducing the solid retention time to approximately one day.[7] In the subsequent Anammox reactor AOB and NOB cannot grow due to absence of oxygen. In processes which combine nitritation and Anammox in one reactor, also the higher growth rate of AOB (as compared to NOB) contributes to preventing nitrate formation by NOB. Current research studying the effect of low temperatures on Anammox activity is showing that Anammox can cope with lower temperatures.[8−10] However, for application of a one-stage Nitritation/Anammox process at lower temperatures, like those encountered in sewage, it is necessary to gain more insight in the microbial competition between AOB, NOB, and Anammox. In a one stage system the competition between Anammox and NOB for nitrite and between AOB and NOB for oxygen is the major issue in selecting the desired population. Low oxygen concentrations have been proposed to favor AOB in preference to NOB for their electron acceptor oxygen.[11] In oxygen limited one-stage Anammox systems, all 3 groups of bacteria do not grow in suspension but are agglomerated in one compact granule. The minimum sludge retention time (SRT) required is therefore defined by the slow growing Anammox bacteria, making it impossible to select against NOB based on growth rate.

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Recently we demonstrated that in aerobic granular sludge vertical segregation of granules occurs based on small differences in settling velocity of the granules.[12] Granules with a higher density and diameter accumulate at the bottom of the sludge blanket. Since biomass in the bottom and the top of the sludge bed are exposed to different substrate concentrations during feeding, different microbial communities may develop as a function of height in the sludge bed. Selective sludge withdrawal from either top or bottom of the sludge bed can therefore be used as a possibility to control the microbial community structure in a granular sludge reactor. Interestingly, mathematical models have recently indicated that microbial populations in granules can be influenced by particle size distribution. For a nitritation/anammox granular sludge process it was demonstrated that NOBs are favored to grow in smaller granules supposedly because of the higher fractional aerobic volume for nitrification.[13] Bigger granules have a smaller aerobic volume fraction which is in advantage of Anammox.[14] Anammox can accumulate to larger amounts in these large granules and therefore maintain a low nitrite concentration inside the granule leading to an outcompetition of NOB. If selective particle density/size based sludge control is applicable an extra operational variable would be created in a one-stage complete anaerobic nitrogen removal over nitrite (CANON) system potentially allowing selective removal of NOB from a system at low temperatures. The objective of this work was to investigate the potential to use segregation of granules in a granular sludge bed to obtain a process dominated by nitritation/anammox in the absence of nitrite oxidation.

EXPERIMENTAL SECTION

Cycle Operation and Measurements. Anammox enriched granular sludge from Rotterdam Dokhaven was used as seed sludge for a laboratory fed batch granular sludge reactor operated with anaerobic aerobic cycles. The operation during one cycle is schematically drawn in Figure 1. It consisted of 60 min anaerobic feeding period of 1.5 L media from the bottom of the reactor in a plug flow regime followed by a 112 min of aeration, 3 min settling period, and 5 min effluent withdrawal. During the effluent period half of the reactor volume (1.5 L) was discharged and half remained in the system. System performance for N-removal during one cycle was evaluated by sampling every 10−20 min to measure ammonium, nitrite, and nitrate by means of flow injection analysis (Quick Chem8500, Lachat instruments) (Figure 2). Opposed to normal reactor operation substrate was added all at once during a cycle measurement. Mixing was performed with dinitrogen gas during the first 60 min to keep anaerobic conditions. Temperature was not controlled and varied between 17 and 22 °C. In the aeration period the pH was controlled similar to other studies at 7.0 ± 0.2 using sodium hydroxide and hydrochloric acid.[15] The dissolved oxygen (DO) concentration was controlled at a specific value between 0.2 and 1.0 $mgO_2 \cdot L^{-1}$. During long-term reactor operation the oxygen set-point was stepwise elevated starting with an initial DO of 0.2 $mgO_2 \cdot L^{-1}$ for the first 90 days (phase I). From day 90 on the oxygen concentration was set to 0.5 $mgO_2 \cdot L^{-1}$ until day 250 (phase II) and from there on 1.0 $mgO_2 \cdot L^{-1}$ until day 400 (phases III and IV). The DO was set by recirculating the off-gas and blending with either dinitrogen gas (anaerobic period) or fresh air (aerobic period). In this way the DO concentration could be regulated, while the superficial airflow was kept constant at 2 L/min.[16] The autotrophic feed medium (1.5 L) consisted of

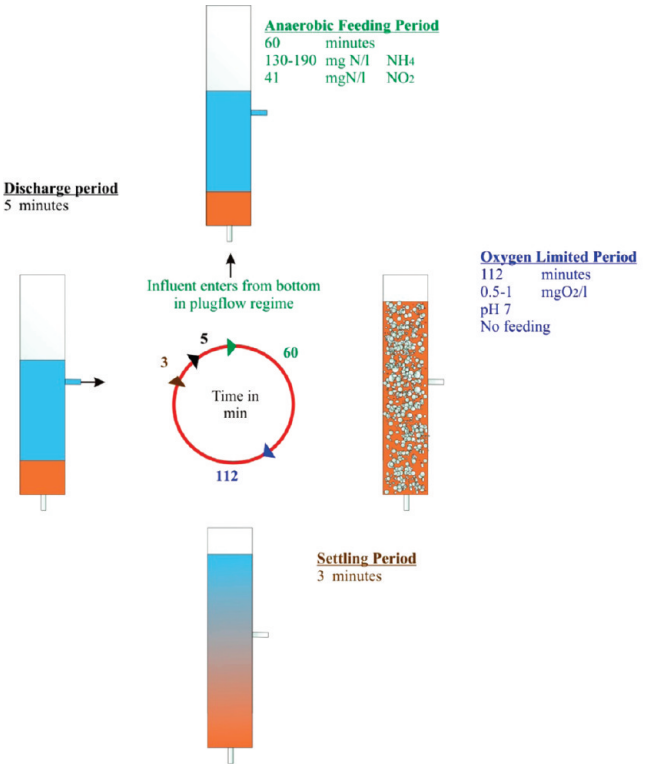

Figure 1. Cycle operation in the laboratory SBR reactor consisting of 1) an anaerobic feeding period of 1.5 L medium from the bottom of the reactor in a plug flow regime, 2) an aeration period, 3) a settling period, and 4) a discharge period.

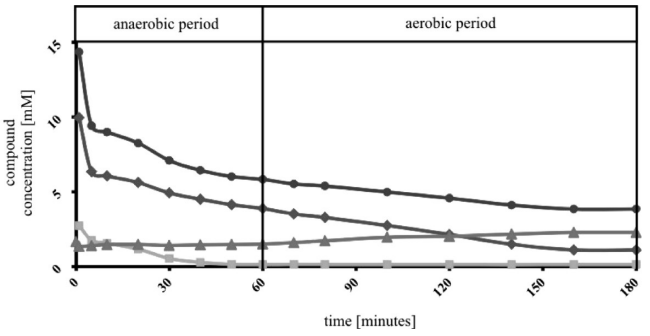

Figure 2. Concentration changes during one cycle in phase III of the aerobic granular sludge reactor: nitrate (▲), nitrite (■), ammonium (♦), and total nitrogen (●). Aeration starts after 60 min and is controlled at a dissolved oxygen concentration of 1.0 mgO_2/L. Note that first point is calculated and difference to second sampling point is largely due to adsorption of ammonium to the granular sludge.

0.2 mM $MgSO_4 \cdot 7H_2O$, 0.2 mM KCl, 1.5 mM $NaHCO_3$, 4 mM $NaNO_2$ (41 mgN/L), 0.2 mM K_2HPO_4, and 0.1 mM KH_2PO_4 (9.1 mg P/L). During phases I and II ammonium concentration was 9.1 mM NH_4Cl (129 mg N/L) and was elevated to 13.6 mM NH_4Cl (190 mg N/L) after changing DO from 0.5 to 1 $mgO_2 \cdot L^{-1}$ in phases III and IV. 'Vishniac and Santer' solution was used to provide trace elements.[17] During phases I, II, and III the SRT was controlled by the sludge which washed-out spontaneously with the effluent withdrawal (1.5 L). In phase IV, sludge was manually withdrawn from the upper part of the sludge bed, and SRT was controlled on average at 40 days and hydraulic retention

time was 6 h. Measurements of volatile suspended solids (VSS) as well as calculation of SRT followed the protocol from earlier research.[12] Nitrogen removal efficiency and rates are based on the difference in total soluble nitrogen compounds (i.e. as measured in the bulk) in the feed and effluent of the reactor. The aerobic NOB activity was evaluated by defining the ratio of NO_3 produced to NH_4 converted during aerobic period, excluding the produced nitrate and consumed ammonium by Anammox from an- and aerobic period $f_{NO_3/NH_4} = (NO_{3(NOB)} - NO_{3(AMX)})/(NH_{4(AOB)}^{+} - NH_{4(AMX)}^{+})$ in N-mol/N-mol.

Granule Characterization. Biomass samples were taken from the bottom and the top of the sludge bed over time for microscopic analysis in order to assess morphology and the microbiological community structure. Specific biomass density was measured with a pycnometer,[12] and size distribution measurements were conducted by means of an image-analyzer. Microscopic images from white and red granules of sliced and regular granules were taken with a light microscope (Axioplan 2, Zeiss). Slicing was accomplished after fixation in 4% paraformaldehyde. Granules were embedded in a tissue freezing medium (Leica Microsystems) hardened by freezing (−20 °C) and cut in the frozen state with a microtome-cryostat (Leica CM1900-Cryostat) into 25 μm thin slices. Dried slices were kept on a microscopic glass slide, and fluorescence in situ hybridization (FISH) was performed on them for determination of Anammox (Cy3), AOB (Cy5), and NOB (Fluos) microbial populations in the same manner as recorded previously.[12] Probe sequences are listed in Table 4, see the Supporting Information.

qPCR. qPCR was conducted on white and red granules using primers targeting NOB and AOB as well as Anammox primer sets. Primers were checked in the database Arb as well as with the Ribosomal Database Project (rdp). All samples were measured in triplicates. DNA extraction was conducted with an UltraClean Microbial DNA Isolation Kit. First a normal PCR was prepared followed by a purification step with a QIAqiuck PCR Purification Kit. The PCR product was used for a qPCR procedure with a variable primer concentration and 25 μM iCycler mix (Table 5, see the Supporting Information). All primers were optimized with a gradient qPCR. The resulting conditions and primer concentration as well as the DNA used as a standard for the qPCR are listed in Table 5, see the Supporting Information. A picogreen protocol was used to determine the amount of DNA template in order to normalize all C_T values to 5 ng DNA. ΔC_T was calculated by the following equation: $\Delta C_T = C_{T(ref)} - C_{T(target)}$.[18] For determination of the ratios of one target organism (e.g., AOB) to the total reference community (here Eub) the following equation was applied: $ratio_{target/ref} = 2^{\Delta C_T}$.

Biological Oxygen Demand. Maximum aerobic NH_4^+ and NO_2^- oxidizing capacity was analyzed for top and bottom biomass under oxygen saturated conditions. Twenty g of granules was transferred into a 50 mL vessel containing water. In order to measure the activity of AOB, NH_4^+ (50 mg/L) was injected and oxygen consumption was monitored over time. For the measurement of the NOB, 80 μM allythiourea (ATU) was used to inhibit AOB.[19] Subsequently NO_2^- was injected reaching an end concentration of 13 $mgNO_2^- \cdot L^{-1}$. Volatile suspended solids (VSS) were determined, and the ammonium and nitrite oxidizing capacity was quantified with a linear regression fit of accumulative oxygen uptake rate (OUR).

Calculation of the Oxygen Penetration Depth. The oxygen penetration depth was calculated according to the following equation: $\delta_{pf} = ((2D \cdot C_{si})/(q_s^{max} \cdot c_{xf}))^{1/2}$ [μm].[20] With a biomass concentration in the biofilm (c_{xf}) of $3.38 \cdot 10^3$ [$mol \cdot m^{-3}$] and $q_{O_2}^{max}$ from a nitrifying culture $9.72 \cdot 10-5$[$molO_2/Cmol_x$], a diffusion coefficient for oxygen of $1.97 \cdot 10^{-9}$ [m^2/s] and an average granular diameter of 0.45 mm for small and 1.2 mm for big granules. Based on the oxygen penetration depth the relative volume available for nitrification was calculated using the following equation $V_{pf} = V_{aerob}/V_{total}$ [$\mu m^3/\mu m^3$] · 100[%].

Table 1. Dissolved Oxygen Concentration, Application of SRT Control, Volumetric Total Nitrogen Removal Conversion Rate, and Maximum Biomass Specific Conversion Rate of Total Nitrogen Removed in the Different Experimental Phases (on Influent Effluent Basis)

phase	DO mg/L in aerated period	SRT control	volumetric nitrogen conversion rate [$gN_2 - N \cdot m^{-3} \cdot day^{-1}$]	max. biomass specific nitrogen removal rate [$gN_2 - N \cdot gVsSS^{-1} \cdot day^{-1}$]
I	0.2	no	905 ± 35	0.06 ± 0.01
II	0.5	no	913 ± 58	0.08 ± 0.01
III	1.0	no	1940 ± 96	0.18 ± 0.02
IV	1.0	yes	1990 ± 39	0.32 ± 0.02

Microelectrode Measurements. pH profiles were measured with a 25 μm thick micro electrode obtained from Unisense (Arhus, Denmark). Medium concentrations were chosen to be the same as during reactor operation as listed in the section cycle operations. The data acquisition system consisted of a picoamperometer (Unisens, Denmark, model PA2000), an A/D converter (Pico Technology Ltd., UK; model ADC-101) and a computer to retain data and monitor microsensor motion. Motion control consisted of a motorized two-dimensional stage (Phytroninc., USA, model MT-65) and a motorized micromanipulator (Unisense, Denmark, model MM3M), controlling the position of the microelectrode in three axis. For the small reactor setup conditions were chosen to be similar to the reactor conditions. pH gradients of red granules were tested for Anammox activity. Media contained ammonium and nitrite and was sparged with N_2 for anaerobic conditions. Activity of white granules was tested for pH profiles. Media for the aerobic period contained ammonium only, and the dissolved oxygen concentration was 100%.

RESULTS

Reactor Performance during One Cycle of Operation. A typical reactor performance during one cycle of operation in phase III is depicted in Figure 2. One cycle lasted 3 h starting with 60 min of anaerobic feeding during which all incoming ammonium and nitrite were fed from the bottom of the reactor in a plug flow regime. During the anaerobic feeding period in a plug-flow regime from the bottom all nitrite was metabolized by Anammox to nitrogen gas and a small portion to NO_3^- as its anabolic byproduct.[1] During the aerobic period ammonium was converted by AOB to nitrite and depending on competition factors further metabolized by either Anammox to nitrogen gas or by NOB to nitrate.

Long-Term Operation. The reactor was operated during four phases over a time period of 420 days as summarized in Table 1. The volumetric and biomass specific nitrogen conversion rates are shown in Figure 3. The initial biomass specific nitrogen removal rate was 0.06 ± 0.01 [$gN_2 - N \cdot gVSS^{-1} \cdot day^{-1}$] when

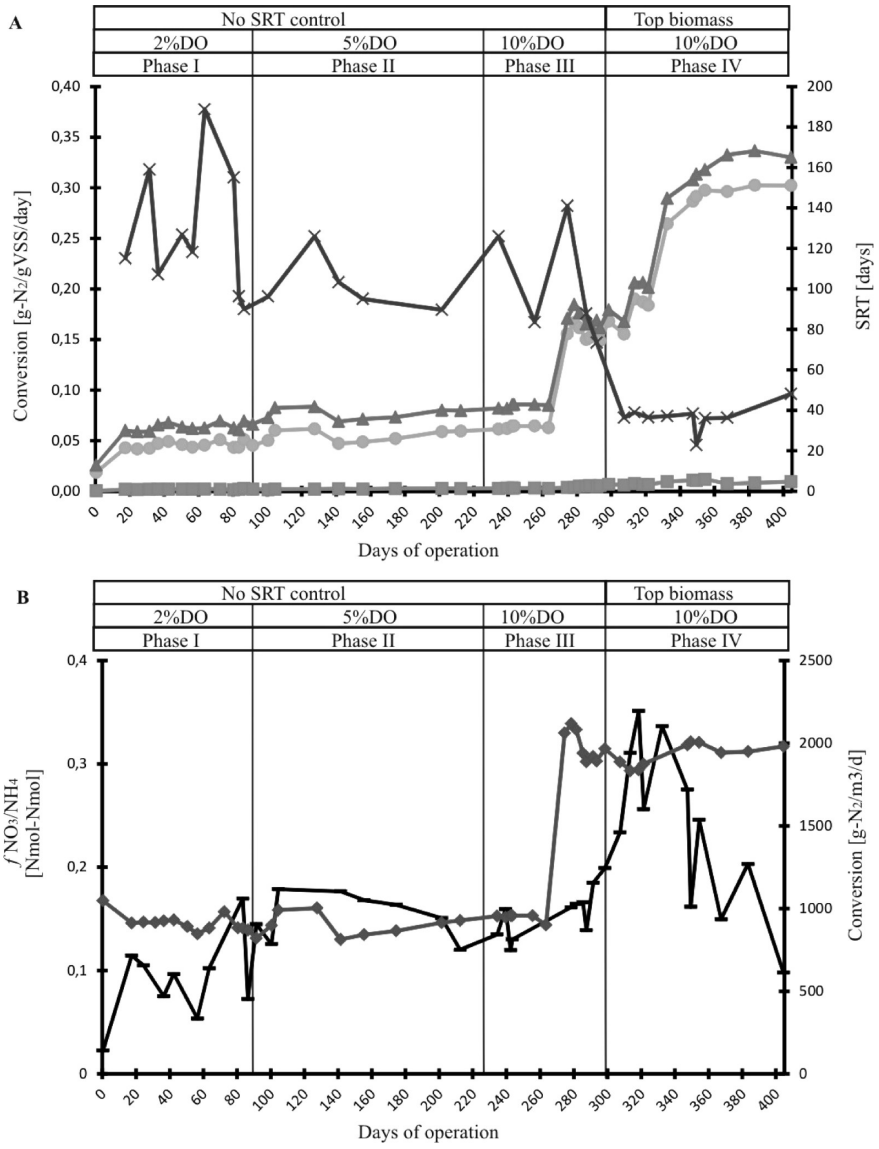

Figure 3. General behavior of the experimental reactor system. Biomass specific nitrogen conversion rates in $[gN_2 - N \cdot gVSS^{-1} \cdot day^{-1}]$ of NH_4^+ (●), total nitrogen (▲), NO_3^- (■), and SRT (×) over time. The dissolved oxygen during the aerated period was set at different set points for each experimental phase. The f_{NO_3/NH_4}-values (-) as well as the volumetric N-conversion rates $[gN_2 - N \cdot m^{-3} \cdot day^{-1}]$ (♦) are shown in part B.

the reactor was run at a DO of 0.2 $mgO_2 \cdot L^{-1}$ to slowly adapt the biomass to aerated conditions (Figure 3 phase I). The Anammox granules used as inoculum were obtained from a full-scale anaerobic Anammox reactor operated at 35 °C (versus 20 °C in the laboratory reactor). In phase II the oxygen was increased to 0.5 $mgO_2 \cdot L^{-1}$ to enhance the nitrification in the aerated period. During this period an average biomass specific removal rate of 0.08 ± 0.01 $[gN_2 - N \cdot gVSS^{-1} \cdot day^{-1}]$ was measured (Figure 3 phase II). In order to further enhance the nitrogen removal capacity in phase III, the oxygen concentration was raised to 1.0 $mgO_2 \cdot L^{-1}$ resulting in an average biomass specific conversion rate of 0.18 ± 0.01 $[gN_2 - N \cdot gVSS^{-1} \cdot day^{-1}]$ (Figure 3 phase III). During this phase there was an increase in white granules observed in the upper part of the settled sludge bed which were dominated by NOB as demonstrated by FISH and qPCR. At the same time the nitrate production in the aerated phase increased gradually (Figure 3b). During phases I, II, and III the SRT was not actively controlled and therefore the resultant from the wash-out of sludge during the effluent withdrawal period. In order to selectively remove white NOB-dominated granules from the system in the fourth experimental phase biomass was withdrawn from the upper part of sludge bed. The amount of biomass withdrawn was such that an SRT of 40 days was established. Within 2 weeks the biomass specific nitrogen removal efficiency increased to 0.32 ± 0.02 $[gN_2 - N \cdot gVSS^{-1} \cdot day^{-1}]$ (Figure 3 phase IV), while the volumetric conversion rate remained the same as it was the case in phase III (Table 1).

The trend of f_{NO_3/NH_4} in the different phases is shown in Figure 3b. The f_{NO_3/NH_4}-value equals 0 if all nitrite produced is consumed by Anammox bacteria and 1 if full oxidation of ammonium to nitrate occurs. The values for f_{NO_3/NH_4} increased with increasing dissolved oxygen concentration reaching the largest value of 0.32 at 1.0 $mgO_2 \cdot L^{-1}$ (phase III). After removal

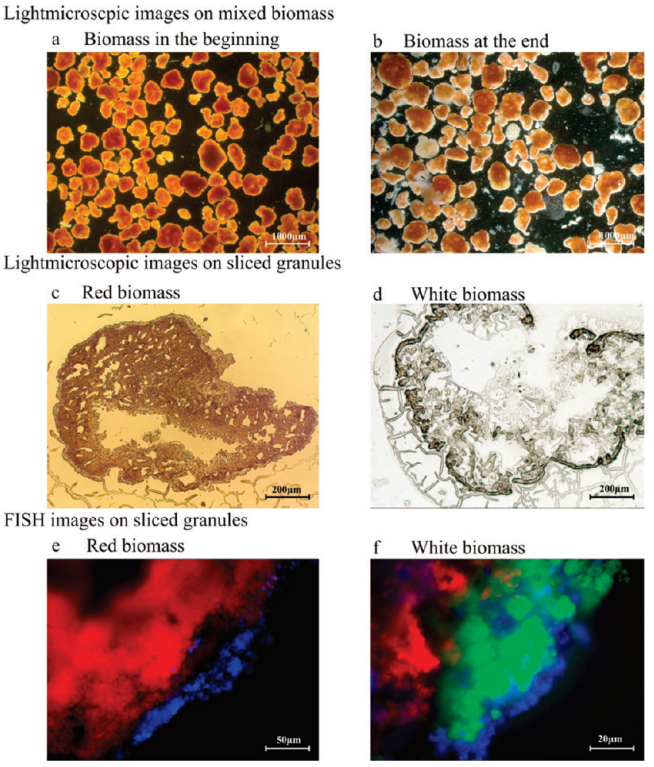

Figure 4. Microscopic images of granules a) at the beginning of the experiment and b) at the end. As well as c) sliced big red granules and d) sliced small white granules e) FISH image of a sliced red granule and f) FISH image of a sliced white granule. FISH was conducted on sliced granules and hybridization was accomplished with Cy3-red (Anammox), Cy5-blue (AOB), and Fluos-green (NOB)-labeled probes.

of the top biomass in phase IV, while maintaining the same DO concentration, the value for f_{NO_3/NH_4} decreased again to its approximate initial value of 0.1.

Microscopic Analyses of Granular Sludge Samples. During the reactor operation the red granules originating from the full scale Anammox reactor system (Figure 4a) developed over time into red and a few white granules (4b). Structure and shape differed for red and white granules. Bigger white granules were mostly hollow in the middle (4c), and smaller white granules had a porous structure (image not shown). Red Anammox dominated granules were densely populated regardless of the diameter (4c). In both granules FISH revealed that AOB were the dominant organisms on the outer layers. In the red granules NOB were barely present (4e). However, in white granules a mix of NOB, AOB, and Anammox was determined (4f). Overall there was only a limited number of eubacteria present not reacting with the FISH-probes for AOB, NOB, and Anammox used.

Density and Size Distribution Measurements of Top and Bottom Granules. The top layer of the settled sludge bed was dominated by white granules, and the middle and bottom sections of the sludge bed were dominated by red granules (visual observation). The ash content of red bottom granules was higher than for white top granules. This was also reflected in a higher density of the bottom granular sludge. The measured values were 1008 ± 2.3 [$g \cdot L^{-1}$] for bottom granules versus 1003 ± 1.5 [$g \cdot L^{-1}$] for top granules. The diameter of bottom granules was also larger (Table 2). The physical parameters obtained were used to estimate the settling velocity applying Stokes law as

Table 2. Physical, Kinetic, and Microbial Properties of Bottom and Top Granules during the Period with Sludge Wasting from the Top of the Sludge Bed (Stage IV)[a]

parameter	top	bottom
ash content [%]	7.4 ± 1.5	11.6 ± 2.7
density [$g \cdot L^{-1}$]	1003.0 ± 1.5	1008.0 ± 2.3
average diameter[1] [mm]	0.78 ± 0.30	0.93 ± 0.27
settling velocity calculated [$m \cdot h^{-1}$]	5.7	16.5
q_{NOB} [$mgO_2/gVSS \cdot h$]	10.5	1.2
q_{AOB} [$mgO_2/gVSS \cdot h$]	20.1	27.7
	white	red
$ratio_{AOB/Eub}$	3.5	0.7
$ratio_{AMX/Eub}$	0.6	3.5
$ratio_{NOB/Eub}$	0.1	0.003

[a] The estimated ratio of Anammox, NOB, and AOB versus bacterial population as estimated by EUB probe from qPCR data.

Table 3. Oxygen Penetration Depth and Aerobic Volume Fraction Available for Nitrification at 0.5 and 1 mg $O_2 \cdot L^{-1}$ in Big and Small Granules

	small granules 0.45 [mm]	big granules 1.2 [mm]
Dissolved O_2:	0.5 $mgO_2 \cdot L^{-1}$	
$\delta_{pf(O_2)}$ [μm]	13.3	13.1
V_{pf} [%]	16.7	6.4
Dissolved O_2:	1.0 $mgO_2 \cdot L^{-1}$	
$\delta_{pf(O_2)}$ [μm]	18.9	18.8
V_{pf} [%]	23.2	9.1

described previously.[12] The estimated settling rate of top granules of 5.7 [$m \cdot h^{-1}$] was just enough to allow them to settle before effluent was removed from the top half of the reactor.

Microbial Characterization of Granules. Biological oxygen consumption measurements were used to evaluate the activity of AOB and NOB in the granular sludge. These measurements revealed a somewhat higher biomass specific nitrite oxidizing capacity for top granules compared to bottom granules. Aerobic ammonium oxidizing capacities were similar for bottom and top biomass however differed considerably in nitrite oxidizing capacity with a 10-fold higher oxygen consumption for top biomass (Table 2). For the experiment white granules were not separated from red granules, but 20 g of granules was taken from top and bottom biomass, respectively. For qPCR measurement white and red granules were separately analyzed. From the derived Ct values ratios were calculated for AOB, NOB, and Anammox as a fraction of the general bacterial population (here referred to as reference). The resulting ratios were used to compare e.g. the difference resulting from the ratio between NOB and Anammox from a white and red granule. Ratios were not used to describe community composition within one sample because of the bias that Anammox is not detected by general bacterial primers. In a white granule the ratio for Anammox was 0.6-fold lower and for NOB 33-fold higher than observed in a red granule (Table 2). In big and small granules the estimated oxygen penetration depth increased from approximately 13 to 19 μm when dissolved oxygen concentration was increased from 0.5 to 1 $mgO_2 \cdot L^{-1}$. The volume fraction of a granule available for nitrification $V_{(O_2)}$ [%] for small granules was ca. 3-fold higher as it was for big granules (Table 3).

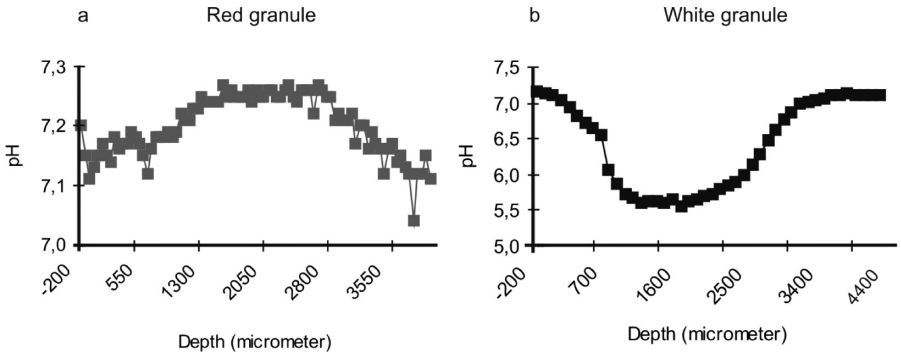

Figure 5. pH profile over a granule a) a red granule and b) a white granule. Media conditions were chosen to be the same as during reactor operation.

pH Microelectrode Measurements. pH measurements were conducted on white and red granules. Since it was not possible to fit small white granules in the microelectrode setup, a bigger white granule was picked to see the difference in pH profiles. In white granules the pH dropped under aerated conditions from 7 to 5.5 as expected for nitrifying granules. When red granules were anaerobically incubated with nitrite and ammonium a pH increase of 0.2 pH units was measured (Figure 5).

DISCUSSION

Earlier research has shown that particle size- and density-induced segregation of granules occurs as a function of height over a settled sludge bed in the aerobic granular sludge process.[12] Larger and denser granules accumulate due to their higher settling rate at the bottom of the sludge bed. In a plug flow reactor fed from the bottom larger granules get more substrate and grow faster as compared to smaller granules at the top of the sludge bed. Granules that are exposed to higher substrate concentrations grow more which may enhance the segregation of biomass. In this study we succeeded to favor the development of bigger Anammox-dominated granules by an anoxic feeding period with nitrite and ammonium in the feed and therewith a segregation of biomass. An extra factor contributing to the segregation of the granular sludge bed is the observations that Anammox dominated granules tend to have an elevated internal pH, whereas nitrification dominated particles have a decreased pH (Figure 5). In the anabolic pathway of anaerobic ammonium oxidation carbonate is reduced and assimilated into biomass. This is a proton consuming process which causes an increase of the pH in the bulk.[21] At higher pH chemical precipitation is provoked[22,23] which might explain the higher observed ash percentage in Anammox dominated granules, leading to a larger specific density and thereby a higher settling velocity of these granules.

At limiting oxygen concentrations AOB already get a preferential advantage over NOB. However, in the competition between AOB, NOB, and Anammox bacteria not only the oxygen concentration in the bulk liquid but also the particle size seems to be a determining factor.[24] It has been proposed based on mathematical modeling that Anammox will grow in bigger granules, while NOB grow in smaller.[13,24] This was also experimentally observed for a CANON reactor.[14,25] Since small and large granules are subjected to the same bulk oxygen concentrations, the oxygen penetration depths are similar. The aerobic volume fraction for larger granules is therefore smaller (Table 3), and Anammox can likely more efficiently outcompete the NOB for nitrite.

FISH images demonstrated that larger granules were dominated by AOB and Anammox, whereas smaller granules contained also NOB (Figure 4). qPCR revealed the same trend with more Anammox in the large granules and more AOB and NOB in the small granules. Activity measurements showed similar ammonium oxidizing capacity for top and bottom biomass but a higher nitrite oxidizing capacity for top biomass, which had a large fraction of smaller white granules (Table 2). The N-removal capacity was measured over time, and the removal rate increased from 905 ± 35 to 1940 ± 96 $[gN_2 - N \cdot m^{-3} \cdot day^{-1}]$ when dissolved oxygen concentration was elevated from 0.2 $mgO_2 \cdot L^{-1}$ to eventually 1.0 $mgO_2 \cdot L^{-1}$ (Table 1). Without selective sludge wasting from the top of the bed white-NOB dominated granules accumulated at the top of the sludge bed. When the solid retention was manually maintained by removing granular sludge from the top of the bed a considerable increase in biomass specific removal rate from 0.18 ± 0.02 to 0.32 ± 0.02 $[gN_2 - N \cdot gVSS^{-1} \cdot day^{-1}]$ (Figure 3 phases III–IV, Table 1) was observed. The observed volumetric conversion was in the order of magnitude with reported values of other Anammox based treatment systems ran at higher temperatures.[26,27] Moreover, the ratio for aerobic NO_3^- produced/NH_4^+ consumed positively correlated with the overall results. The ratio was low when the dissolved oxygen concentration was low (0.3–0.5 $mgO_2 \cdot L^{-1}$) and increased with increasing oxygen concentration (1 $mgO_2 \cdot L^{-1}$) indicating an ingrowth of NOB. When top biomass was extracted to selectively remove NOB, the ratio dropped to its approximate initial ratio of 0.1 for pure anammox sludge, in spite of a higher oxygen concentration (1 $mgO_2 \cdot L^{-1}$). This further indicates the selective removal of NOB.

Like in our previous work related to competition between phosphate and glycogen accumulating bacteria, biomass segregation in aerobic granular sludge reactors was shown to enable removal of an unwanted microbial population by a combination of minimizing SRT for that population (here the NOB) and feeding preferentially the desired population (here Anammox).[12] This study has shown that it was possible with selective sludge control to counter select against NOB in preference to AOB and Anammox bacteria in a granular sludge reactor at ambient temperatures. It was reported that at high temperature in a continuously operated granular sludge (CANON) systems, selective washed-out of smaller particles lead to higher nitrogen conversion rates,[25,28] suggesting that smaller aggregates were enriched in NOB. The exact mechanism why NOB are outcompeted in larger granules needs further investigation, but it is likely that even at lower temperatures NOB would be preferentially accumulating in smaller granules due to their affinity to

oxygen. Feeding of nitrite and ammonium could be used to promote the growth of bigger Anammox dominated granules and hence enhance segregation during a CANON stratup process at mesophilic temperatures. Modeling work showed that thicker biofilms (i.e., larger granules) are needed to sustain Anammox conversion at lower temperature.[29] A selective wash-out of smaller NOB dominated granules gives the opportunity to introduce Anammox conversions in main stream wastewater treatment plants. This will greatly increase the sustainability of the process with respect to energy.[30,31]

APPENDIX

$\delta_{pf} = ((2D \cdot C_{si})/(q_s^{max} \cdot c_{xf}))^{1/2}$ [μm]
δ_{pf} = oxygen penetration depth [m]
D = oxygen diffusion coefficient [m^2/s]
C_{si} = oxygen concentration in the biofilm interphase [mol/m^3]
$q_{O_2}^{max}$ = maximal oxygen specific uptake rate [1/s]
c_{xf} = biomass in the bilfilm [mol/m^3]
$V_{pf} = V_{aerob}/V_{total}$ [$\mu m^3/\mu m^3$] $\cdot$ 100[%]
$r_{anaerob} = \delta_{pf} - d_{granule}$
$V_{tot} = (3/4)\pi r_{granule}^3$
$V_{anaerob} = (3/4)\pi r_{anaerob}^3$
$V_{aerob} = V_{tot} - V_{anaerob}$
V = volume [m^3]
d = diameter [m]
r = radius [m]

ASSOCIATED CONTENT

Supporting Information. Tables 4 and 5. This material is available free of charge via the Internet at http://pubs.acs.org.

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