  Water Science & Technology—WST | 59.3 | 2009

Erratum

Water Science and Technology **58**(5) 1113–1120

Granular biomass capable of partial nitritation and anammox

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Publisher's note. We regret that an outdated version of this article was used in production; the correct final version, which incorporates several amendments and different authorship, is printed below.

Granular biomass capable of partial nitritation and anammox

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ABSTRACT

A novel and efficient way of removing nitrogen from wastewaters poor in biodegradable organic carbon is the combination of partial nitritation and anoxic ammonium oxidation (anammox), as in the one-stage oxygen-limited autotrophic nitrification/denitrification (OLAND) process. Since anammox bacteria grow very slowly, maximum biomass retention in the reactor is required. In this study, granular and rapidly settling biomass was obtained in an oxygen-limited lab-scale sequencing batch reactor (SBR). Operated with 1 h cycles, OLAND granules were formed in 1.5 months and the nitrogen removal rate increased from 50 to 450 mg N L^{-1} d^{-1} in 2 months. The average diameter and settling velocity of the granules were 1.8 mm and 55 m h^{-1}, respectively. Fluorescent *in-situ* hybridization (FISH) analyses on the granules demonstrated the presence of both aerobic ammonium oxidizers and anammox bacteria, and their activities were well equilibrated to perform the OLAND reaction. The presented results show the feasibility of rapidly settling granular biomass for one-stage partial nitritation and anammox.

Key words | anammox, CANON, granulation, nitritation, OLAND, SBR

S. E. Vlaeminck
L. F. F. Cloetens
H. De Clippeleir
M. Carballa
W. Verstraete
Laboratory of Microbial Ecology and Technology (LabMET),
Ghent University,
Coupure Links 653,
Ghent 9000,
Belgium
E-mail: *Siegfried.Vlaeminck@UGent.be*; *LievenC@Gmail.com*; *Haydee.DeClippeleir@UGent.be*; *Marta.Carballa@UGent.be*; *Willy.Verstraete@UGent.be*

INTRODUCTION

In the last years, the technology of one-stage nitrogen removal via partial nitritation and anoxic ammonium oxidation (anammox) has developed rapidly, as demonstrated by the construction of several pilot and full scale reactors tabulated by Cema *et al.* (2006) and van der Star *et al.* (2007). One of these processes is oxygen-limited autotrophic nitrification/denitrification (OLAND), with aerobic ammonium-oxidizing bacteria (AOB) oxidizing ammonium to nitrite, and anammox bacteria oxidizing the residual ammonium with nitrite into dinitrogen gas and some nitrate (Pynaert *et al.* 2003). The overall process stoichiometry is the following (Vlaeminck *et al.* 2007*a*):

$$NH_4^+ + 0.85\,O_2 \rightarrow 0.44\,N_2 + 0.11\,NO_3^- + 1.08\,H^+ + 1.44\,H_2O \quad (1)$$

One of the bottlenecks in all anammox based applications is the slow growth of these bacteria (Strous & Jetten 2004). To prevent biomass washout, the choice of a good biomass retention mechanism is an important aspect in the design of OLAND-type reactors. One way of avoiding biomass loss is a biofilm-based setup, such as a rotating contactor (Pynaert *et al.* 2003), a moving bed reactor (Cema *et al.* 2006), or a fixed bed reactor (Furukawa *et al.* 2006). In suspended growth systems, measures have to be taken to prevent biomass loss. In a chemostat, a biomass settling device in the outflow tube can be used (Third *et al.* 2005), whereas a three-phase separator can ensure biomass retention in an airlift reactor (Sliekers *et al.* 2003). In a sequencing batch reactor (SBR), high biomass retention is obtained by applying a low minimum biomass settling velocity, which is the ratio between the settling time and the vertical distance from the water surface to the effluent discharge point. Reported minimum biomass settling velocities for OLAND-type SBRs are about 0.3 m h^{-1} (Third *et al.* 2001; Sliekers *et al.* 2002; Wett 2006).

In suspended growth systems, biomass aggregate morphology has an influence on the settling properties. However, the morphology of aggregated AOB and anammox bacteria is vague or hardly discussed in literature. In most studies, mainly floccular biomass was formed (Third *et al.* 2001; Sliekers *et al.* 2002; Sliekers *et al.* 2003; Third *et al.* 2005) and, to the best of our knowledge, granular biomass in OLAND-type systems was only mentioned by Nielsen *et al.* (2005) and Innerebner *et al.* (2007). Given the good settling capacities of granules, and thus the easy biomass retention in the reactor, the goal of this study was to obtain granules in an OLAND SBR, and to study their physical, physiological and microbiological characteristics.

METHODS

Lab-scale reactor set-up

The OLAND SBR consisted of a cylindrical vessel with an internal diameter of 14 cm and a working volume of 1.87 L. The reactor was inoculated with OLAND biomass harvested from a lab-scale rotating contactor described by Pynaert *et al.* (2003), at an initial biomass concentration of 1.6 g VSS L^{-1}. Tap water-based influent consisted of $(NH_4)_2SO_4$ at an initial concentration of 0.1 g N L^{-1}, 0.308 g KH_2PO_4 L^{-1}, and 2 mL L^{-1} of a trace elements solution (Kuai & Verstraete 1998) amended with final concentrations of 0.050 mg Ni L^{-1}, 0.050 mg Se L^{-1} and 0.002 mg B L^{-1}. To provide buffering capacity, 1 mole of $NaHCO_3$ was added per mole of nitrogen. If necessary, the latter ratio was increased temporarily to ensure that the reactor pH did not drop below 7.4. The reactor temperature was controlled at 33 ± 1°C. The reactor dissolved oxygen (DO) concentration was controlled, either automatically (Consort R305 controller with DO probe SZ10T), or manually by daily adjustment of aeration intervals and air flow rate. Reactor mixing was done with a magnetic stirrer.

The reactor was operated with a 1 h cycle (Figure 1). Each cycle, 0.37 L (less than 10% deviation) of synthetic medium was fed into the reactor. Both during the feeding and the reaction phase, the reactor was mixed and aerated. Subsequently, the biomass was allowed to settle for two minutes, giving rise to a minimum biomass settling velocity of 0.73 m h^{-1}. Finally, the effluent pump removed the supernatant. The reactor was operated with a fixed residual volume of 1.50 L, which resulted in a volumetric exchange ratio of 20% per cycle and a hydraulic residence time of 5.0 h.

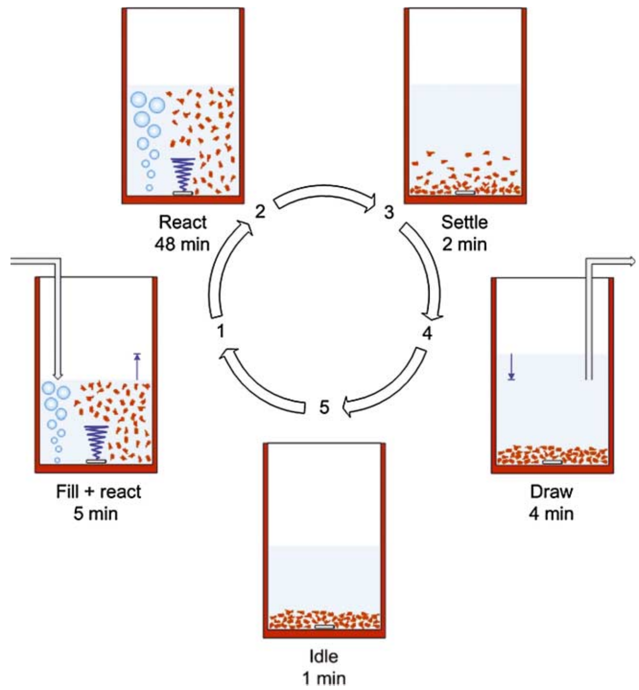

Figure 1 | Schematic representation of the OLAND SBR cycle.

Aerobic and anoxic batch activity tests

After 1.5 months of reactor operation, two different forms of biomass aggregates could be distinguished visually, i.e. flocs and granules. All granular biomass was retained by a 1.0 mm pore size sieve, while the residual fraction of the biomass, retained by a 0.1 mm sieve, consisted of floccular biomass. Prior to the batch activity tests, biomass was centrifuged (5 min at 5,000 × g) and washed twice with a phosphate buffer (100 mg P L^{-1}; pH 8) to remove residual dissolved reactor compounds. Aerobic and anoxic ammonium conversion tests were described more in detail by Vlaeminck *et al.* (2007*b*). In short, biomass was incubated in a shaking Erlenmeyer with ammonium as substrate for the aerobic activity tests. For the anoxic tests, biomass incubation occurred in a gas-tight anoxic serum flask with ammonium and nitrite as substrates. During the incubation, liquid samples were taken over time for ammonium, nitrite and nitrate analysis. Since little SBR biomass was available, no replicas were included. However, the inoculum was tested in triplicate and the ammonium oxidation rates of the replicas differed less than 10% from the average oxidation rate, indicating the reproducibility of the batch tests.

Fluorescent *in-situ* hybridization (FISH)

Both flocs and granules from the OLAND SBR were fixed in a 4% paraformaldehyde solution. FISH was performed according to Amann *et al* (1990). The probes used in this study were Nso1225 for β-proteobacterial AOB (Mobarry *et al.* 1996), Amx820 for the anammox bacteria "*Candidatus* Brocadia" and "*Candidatus* Kuenenia" (Schmid *et al.* 2000), NIT3 and its competitor for *Nitrobacter* (Wagner *et al.* 1996) and Ntspa662 and its competitor for *Nitrospira* (Daims *et al.* 2001). Image acquisition was done on a Zeiss Axioskop 2 Plus epifluorescence microscope.

Granule diameter

ImageJ software was used to calculate the area of 50 biomass granules on images obtained with a stereomicroscope. The average diameter was determined as a circle-equivalent diameter.

Granule settling velocity

The settling velocity of 25 granules was determined by measuring the time needed for each granule to travel 1.25 m through a transparent vertical column (Ø 10 cm) filled with tap water (20°C).

Analytical methods

Ammonium was determined colorimetrically with Nessler reagent according to *Standard Methods* (Greenberg *et al.* 1992). Both nitrite and nitrate were determined using a Metrohm 761 Compact Ion Chromatograph equipped with a conductivity detector. The total and volatile suspended solids (TSS and VSS) content of the biomass were determined according to *Standard Methods* (Greenberg *et al.* 1992). pH was determined potentiometrically with a portable Consort C532 pH meter and DO concentration and water temperature were measured with a portable Endress-Hauser COM381 DO meter.

RESULTS AND DISCUSSION

OLAND SBR performance

The reactor pH was always between 7.4 and 7.8. According to changes in the aeration of the reactor, the SBR performance was subdivided into seven operational periods (Figure 2).

At the initial setpoint of 0.85 mg O_2 L^{-1} (period I), increasingly more ammonium was converted into nitrite and nitrate (Figure 2), but the nitrogen removal rate remained fairly constant. To overcome possible oxygen inhibition of the anammox bacteria (Strous *et al.* 1997), the DO setpoint was lowered to 0.4 mg O_2 L^{-1} in order to limit the oxygen penetration depth in the biomass aggregates. As a result, anammox activity increased in period II, as follows from the sharp increase of the nitrogen removal rate.

In period III, a lower oxygen supply, derived from rapid drifting of the probe, limited the increase in removal rate. Since this could not be overcome by daily calibration and maintenance, the automatic DO control was replaced by a manual control mechanism in period IV.

From this moment on, the initial oxygen sensitivity of the anammox bacteria seemed to have disappeared, since higher DO levels (1.1 mg O_2 L^{-1}) were not inhibitory for the nitrogen removal, and thus the anammox reaction. Moreover, the higher DO levels (periods IV and VI) resulted in increasing nitrogen removal rates.

In periods V and VII, the oxygen supply was decreased (lower setpoint/anoxic phase) to lower the nitrite effluent concentrations in order to prevent nitrite inhibition of anammox bacteria (Strous *et al.* 1999). However, this measure did not reduce the nitrite levels, probably due to the development of floccular biomass with a high nitrite producing activity, which is discussed more in detail below.

The presented reactor removed up to 0.45 g N L^{-1} d^{-1}, and a second parallel SBR even reached values of 1 g N L^{-1} d^{-1} (data not shown). The theoretical maximum removal

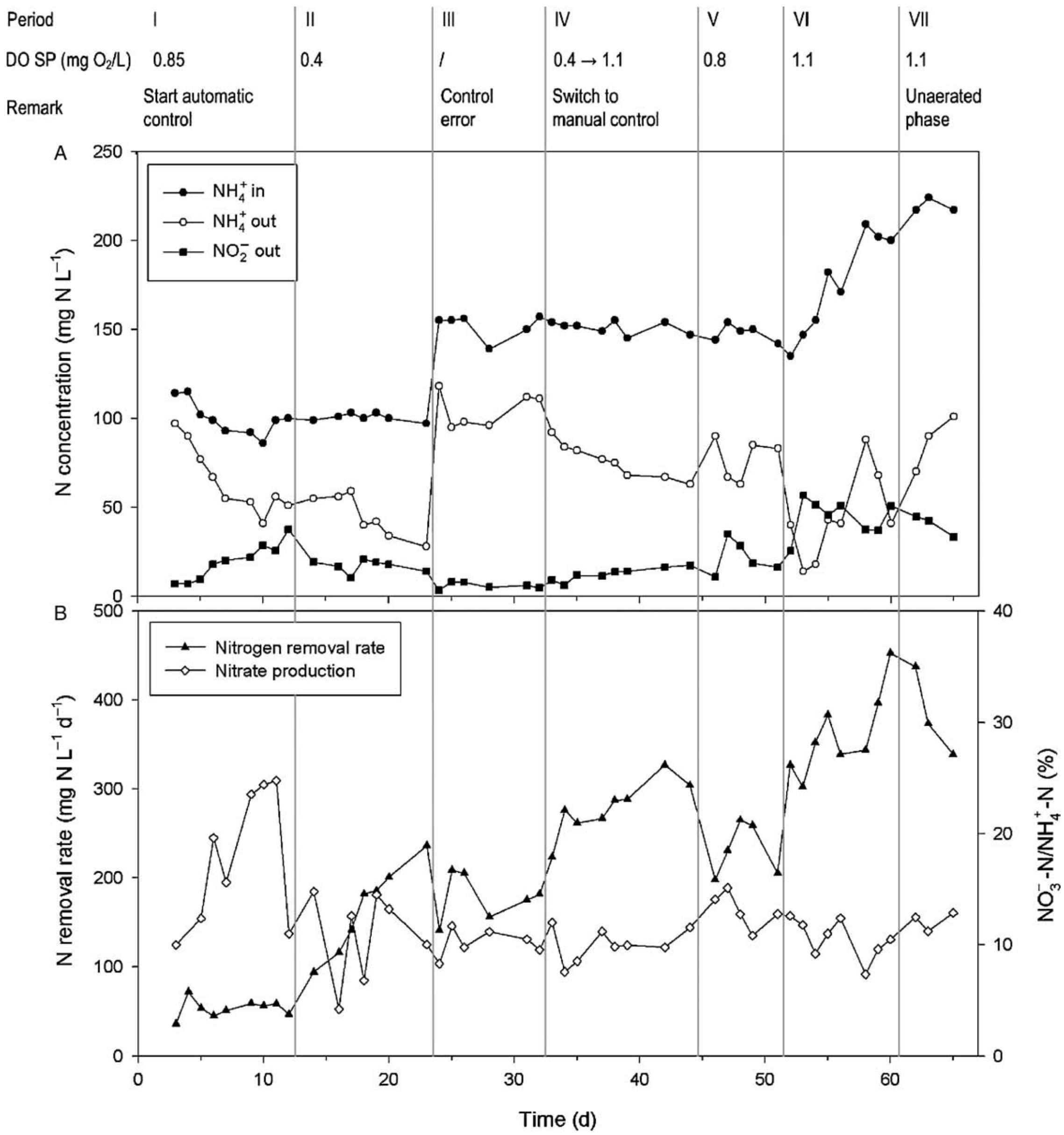

Figure 2 | Performance of the OLAND SBR. DO setpoints (SP) and remarks on the DO control mechanism are indicated per operational period. In period III, probe drifting resulted in a lower DO concentration compared to period II. In period VII, eight minutes without aeration were introduced at the end of the reaction phase. (A) Ammonium concentration in influent and effluent, and nitrite concentration in effluent. (B) Nitrogen removal rate and nitrate production expressed as a percentage of the ammonium removal.

for suspended one-stage systems is $8\,g\,N\,L^{-1}\,d^{-1}$, based on oxygen transfer calculations (van der Star *et al.* 2007). However, the highest removal rate reported in practice is $1.5\,g\;N\;L^{-1}\;d^{-1}$ (Sliekers *et al.* 2003). Thus, the achieved removal rates obtained in this study were competitive for a one-stage nitritation/anammox system. In comparison, higher removal rates are usually obtained in two-stage systems (e.g. $9\,g\,N\,L^{-1}\,d^{-1}$; van der Star *et al.* 2007), but the main disadvantage of the two-stage process is the high complexity to control two subsequent reactors. For instance, an incidental breakdown of the on-line nitrite analyzer rapidly results in inhibitory nitrite levels, leading to failure of the anammox reactor (van der Star *et al.* 2007).

When fitting the increase of the removal rate over the full operational period to an exponential curve, a doubling time of 21 days could be calculated ($R^2 = 79\%$). This value

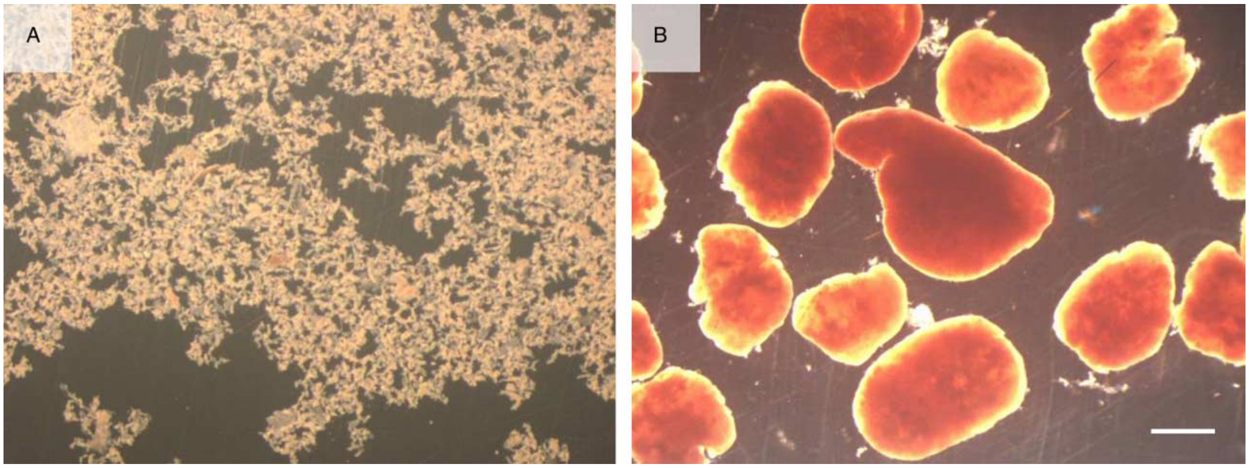

Figure 3 | Morphological differences in the OLAND SBR biomass. (A) Floccular biomass (*B*) Granular biomass. Scale bar = 1.0 mm.

lies within the reported range of anammox doubling times of 14 to 21 days (Strous & Jetten 2004). Since the increase in the removal rate was limited by the oxygen supply in some periods and by biomass wash-out, it is likely that the real doubling time of the OLAND biomass was less than 21 days.

Biomass characterization

The OLAND inoculum aggregates derived from a 5 mm thick biofilm with no distinct or uniform morphology. During SBR operation, the morphology of the biomass gradually changed and from period V on, two different forms could be distinguished (Figure 3).

One form of biomass was rather floccular, slowly settling and brownish, with an aggregate size between 0.1 and 1.0 mm (Figure 3A). These flocs made up 43% of the reactor's VSS content retained on a 0.1 mm sieve. The larger VSS fraction consisted of granular biomass, which was more reddish (Figure 3B). The granules had a diameter of 1.8 ± 0.3 mm and a settling velocity of 55 ± 10 m h^{-1}.

Floccular and granular biomass were harvested on day 65 and examined for their aerobic and anoxic ammonium-oxidizing activity (Figure 4). Compared to the inoculum, floccular biomass was specialized in aerobic ammonium oxidation (426 mg N g^{-1} VSS d^{-1}), while granular biomass was specialized in anoxic ammonium oxidation (133 mg N g^{-1} VSS d^{-1}). Similarly but without discussing the biomass morphology (flocs/granules), Nielsen *et al.* (2005) reported that OLAND-type aggregates smaller than 500 μm were specialized in aerobic ammonium oxidation, and those larger than 500 μm were specialized in anoxic ammonium oxidation.

The aerobic activity of the floccular biomass was more than ten times higher than the anoxic activity. It is therefore likely that the undesired nitrite accumulation in the reactor has been caused by the floccular biomass. Interestingly, the aerobic and anoxic activities of granular biomass were in the same order of magnitude, suggesting a well equilibrated composition of the granular biomass to perform the OLAND reaction.

FISH analyses revealed the presence of both β-proteobacterial AOB and anammox bacteria *("Candidatus*

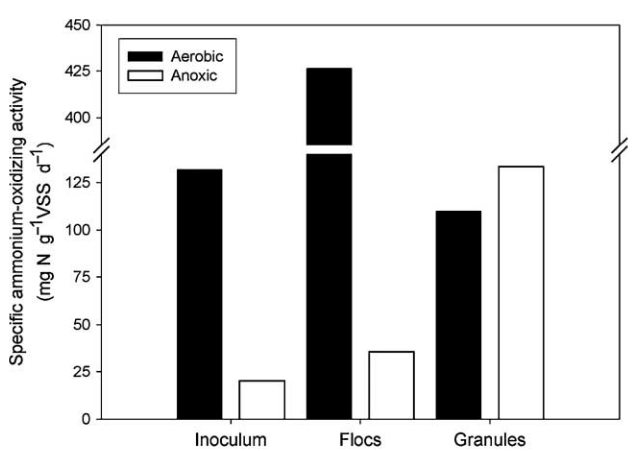

Figure 4 | Specific aerobic and anoxic ammonium-oxidizing activity of the SBR inoculum, flocs and granules on day 65 (*n* = 1). Note the break in the ordinate.

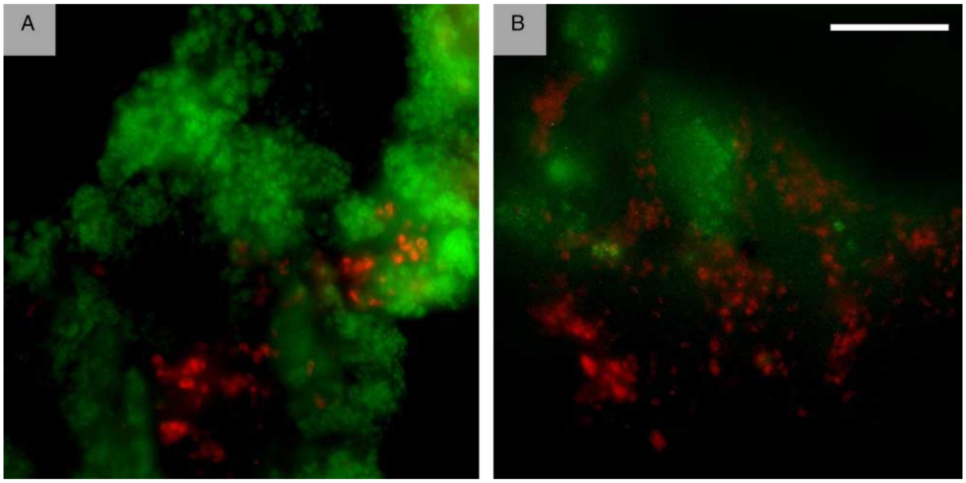

Figure 5 | FISH on OLAND SBR biomass. AOB (probe Nso1225 labelled with FITC) appear green, and anammox bacteria (probe Amx820 labelled with Cy3) appear red. (A) Floccular biomass (B) Granular biomass. Scale bar = 20 μm. Subscribers to the online version of *Water Science and Technology* can access the colour version of this figure from http://www.iwaponline.com/wst.

Brocadia" and "*Candidatus* Kuenenia") in the SBR flocs and granules (Figure 5). As expected from the results of the activity batch tests, anammox bacteria were more abundant in the granules compared to AOB, and vice versa in the flocs. Likewise, Innerebner *et al.* (2007) detected anammox bacteria in their SBR granules, but in contrast to our results, their floccular biomass fraction did not contain anammox bacteria.

The development of nitrite oxidizing bacteria (NOB) in the SBR was negligible at the applied operational conditions. No nitrate was produced in the aerobic batch tests (data not shown), and neither *Nitrobacter* nor *Nitrospira* could be detected with FISH. The observed nitrate production in the reactor per ammonium removed was 11 ± 2% from period III on (Figure 2), corresponding well with the expected nitrate production from anammox (equation 1). It is likely that both the DO concentration and the free ammonia level played a role in suppressing NOB growth. On one hand, the chosen DO setpoints ($0.4 - 1.1$ mg O_2 L^{-1}) were in the range allowing AOB to outcompete NOB, given the lower oxygen affinity constant of AOB (Laanbroek & Gerards 1993). On the other hand, the calculated free ammonia concentrations ($0.3 - 6.0$ mg N L^{-1}) exceeded the reported NOB inhibition level of $0.1 - 1.0$ mg N L^{-1} (Anthonisen *et al.* 1976). Free nitrous acid was not a factor in achieving partial nitrification, since the calculated concentrations (<0.004 mg N L^{-1}) were far below the NOB inhibition level of 0.2 mg N L^{-1} (Anthonisen *et al.* 1976).

Overall, OLAND granules had good settling properties and nitrogen conversion capacities. Another advantage of granules is that they can shield the anammox bacteria from oxygen exposure. Initially, low DO concentrations (0.4 mg O_2 L^{-1}) were required to achieve an increase in the anammox activity in the reactor. However, with the development of granules from period IV on, higher DO concentrations (1.1 mg O_2 L^{-1}) were not inhibitory for anammox activity. Based on the measured specific aerobic ammonium oxidation activity and assuming spherical granules of 1.8 mm diameter and zero-order substrate uptake (Perez *et al.* 2005), an maximum oxygen penetration depth of 120 μm was calculated in the OLAND granules. As such, the presence of oxygen-consuming AOB in the outer granule layer created a large central anoxic core in the granule (65%, v/v), which is ideally suitable for the oxygen-sensitive anammox bacteria.

Liu *et al.* (2005) state that a high minimum biomass settling velocity, allowing only rapidly settling granules to stay in the reactor, is the major selection pressure responsible for SBR granulation. In most SBR granulation studies with activated or nitrifying sludge, minimum biomass settling velocities are at least 4.5 m h^{-1} (Beun *et al.* 1999; Kim & Seo 2006; Kishida *et al.* 2006). In this study however, we applied a much lower minimum settling velocity of 0.7 m h^{-1}, selecting for biomass aggregate sizes above 190 μm, as calculated from Stokes' sedimentation law. It seems therefore that a high selection pressure was not prerequisite for the formation of OLAND granules. On the other hand, higher selection might have prevented the nitrite accumulation in the reactor, by washing out the predominantly nitrifying flocs.

The key factor to achieve OLAND-type granulation is yet unknown. Likewise to the observations of Innerebner *et al.* (2007), the formation of the granules occurred when the system showed a certain removal rate and thus anammox activity. Research on factors leading to OLAND granulation is ongoing and will be most useful to obtain granules in practice.

CONCLUSIONS

- With 1 h SBR cycles, granular OLAND biomass was successfully developed after 1.5 months. After 2 months, the nitrogen removal increased from about 50 to 450 mg N L^{-1} d^{-1}, which is a competitive rate for OLAND-type reactors.
- Aerobic batch tests and FISH confirmed that NOB did not develop in the reactor.
- Granular OLAND biomass had an average diameter of 1.8 mm and an average settling velocity of 55 m h^{-1}.
- The aerobic and anoxic ammonium-oxidizing activities of the granules were well equilibrated to perform the OLAND reaction without excessive nitrite production. In addition, the presence of both AOB and anammox bacteria was demonstrated by FISH.

ACKNOWLEDGEMENTS

This research was funded by a PhD grant (Aspirant) for Siegfried E. Vlaeminck from the Fund of Scientific Research-Flanders (Fonds voor Wetenschappelijk Onderzoek (FWO) Vlaanderen), and by a postdoctoral grant for Marta Carballa from the Spanish Ministry of Education and Science (EX2006-0963). The authors gratefully thank Jeroen Bursens for assistance with the reactor design, Winnok De Vos for assistance with the microscopy and image processing, and Kim Windey, Peter De Schryver, Tuba Hande Ergüder and Lieven Wittebolle for the inspiring discussions.

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