

Successful hydraulic strategies to start up OLAND sequencing batch reactors at lab scale

Thomas Schaubroeck,^{1,2} Samik Bagchi,²
Haydée De Clippeleir,² Marta Carballa,³
Willy Verstraete^{2*} and Siegfried E. Vlaeminck²

¹Research Group ENVOC, Ghent University, Coupure Links 653, 9000 Gent, Belgium.

²Laboratory of Microbial Ecology and Technology (LabMET), Ghent University, Coupure Links 653, 9000 Gent, Belgium.

³Department of Chemical Engineering, School of Engineering, University of Santiago de Compostela, Rúa Lope Gómez de Marzoa, s/n, 15782 Santiago de Compostela, Spain.

Summary

Oxygen-limited autotrophic nitrification/denitrification (OLAND) is a one-stage combination of partial nitrification and anammox, which can have a challenging process start-up. In this study, start-up strategies were tested for sequencing batch reactors (SBR), varying hydraulic parameters, i.e. volumetric exchange ratio (VER) and feeding regime, and salinity. Two sequential tests with two parallel SBR were performed, and stable removal rates $> 0.4 \text{ g N l}^{-1} \text{ day}^{-1}$ with minimal nitrite and nitrate accumulation were considered a successful start-up. SBR A and B were operated at 50% VER with 3 g NaCl l^{-1} in the influent, and the influent was fed over 8% and 82% of the cycle time respectively. SBR B started up in 24 days, but SBR A achieved no start-up in 39 days. SBR C and D were fed over 65% of the cycle time at 25% VER, and salt was added only to the influent of SBR D (5 g NaCl l^{-1}). Start-up of both SBR C and D was successful in 9 and 32 days respectively. Reactor D developed a higher proportion of small aggregates (0.10–0.25 mm), with a high nitrification to anammox rate ratio, likely the cause of the observed nitrite accumulation. The latter was overcome by temporarily including an anoxic period at the end of the reaction phase. All systems achieved granulation and similar biomass-specific nitrogen removal rates ($141\text{--}220 \text{ mg N g}^{-1} \text{ VSS day}^{-1}$). FISH revealed a close juxtapositioning of aerobic and anoxic

ammonium-oxidizing bacteria (AerAOB and AnAOB), also in small aggregates. DGGE showed that AerAOB communities had a lower evenness than *Planctomycetes* communities. A higher richness of the latter seemed to be correlated with better reactor performance. Overall, the fast start-up of SBR B, C and D suggests that stable hydraulic conditions are beneficial for OLAND while increased salinity at the tested levels is not needed for good reactor performance.

Introduction

The discovery of anoxic ammonium-oxidizing bacteria (AnAOB) some 15 years ago has led to the development of several partial nitrification/anammox processes for biological nitrogen removal, including the one-stage oxygen-limited autotrophic nitrification/denitrification (OLAND) process (Kuai and Verstraete, 1998; Pynaert *et al.*, 2003; Vlaeminck *et al.*, 2009a). OLAND consumes 100% less organic carbon, produces about 90% less sludge and consumes almost 60% less oxygen compared with nitrification/denitrification (Mulder, 2003), resulting in overall cost savings of 30–40% for the treatment of wastewaters with a low biodegradable carbon (C) to nitrogen (N) ratio, such as for instance sewage sludge digestates (Wett, 2006; Joss *et al.*, 2009; Jeanningros *et al.*, 2010), but also pretreated sewage (Verstraete and Vlaeminck, 2011; De Clippeleir *et al.*, 2011a) and other types of wastewater. In the OLAND process two reactions occur, the first reaction (partial nitrification) consists of the aerobic oxidation of about half of the ammonium to nitrite, performed by aerobic ammonium-oxidizing bacteria (AerAOB). The second reaction (anammox) is performed by AnAOB, a group of *Planctomycetes*, and comprises the anoxic oxidation of the residual ammonium with nitrite to mainly dinitrogen gas and some nitrate. Depending on the wastewater characteristics and reactor operation, additional nitrogen conversions can take place, including the aerobic nitrite oxidation to nitrate (nitrification) by nitrite oxidizing bacteria (NOB) and the anoxic reduction of nitrate or nitrite with organic carbon to nitrogen gas (heterotrophic denitrification). In absence of nitrification, denitrification or excess nitrite production, OLAND converts 89% of the oxidized ammonium to nitrogen gas and 11% to nitrate (Barnes and Bliss, 1983; Strous *et al.*, 1998). Distinct advantages of a one-stage (OLAND)

Received 31 August, 2011; accepted 7 December, 2011. *For correspondence. E-mail willy.verstraete@ugent.be; Tel. (+32) 9 2645976; Fax (+32) 9 2646248.

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versus two-stage process configuration include, for instance, lower investment costs, less complex process control and a lower risk of nitrite inhibition on AnAOB.

Sequencing batch reactors (SBR) are a popular OLAND technology (Wett, 2006; Joss *et al.*, 2009; Jeanningros *et al.*, 2010), because of their operational flexibility allowing to easily vary feeding regime, cycle duration, aerobic/anoxic reaction time, minimum settling velocity (MSV) for the aggregates and the volumetric exchange ratio (VER) per cycle (Siegrist *et al.*, 2008; De Clippeleir *et al.*, 2009). Given the high doubling time of 7–14 days for AnAOB (Strous *et al.*, 1998), as opposed to about 0.5–1.0 days for AerAOB (Wiesmann, 1994), start-up times can be very high if no active inoculum is available and if biomass retention is not sufficiently high. A fast start-up can be achieved more efficiently in case of large aggregates or granules settling at 35–55 m h⁻¹ (De Clippeleir *et al.*, 2009; Vlaeminck *et al.*, 2009b). Yet, OLAND granulation mechanisms are not clearly understood so far (Vlaeminck *et al.*, 2010). Table 1 shows that granulation mostly did not involve the typical high selective settling pressure (high MSV) and high shear (high superficial air flow rates), as known for aerobic granulation (Adav *et al.*, 2008). Moreover, Table 1 shows that the ideal hydraulic conditions for OLAND activity are not clear, with large variations for applied VER and feeding regime (pulse-fed, intermittently fed or continuously fed). For a pulse-fed SBR operated in cycles of 1 h, De Clippeleir and colleagues (2009) showed that no start-up could be achieved at a high VER (40%), and that a low VER (25%) was crucial. Hypothesis 1 of this study was therefore that an OLAND microbial community is enhanced by a hydraulically stable environment. Indeed, the latter leads to a chemically stable environment, and possibly avoids too much dilution of stimulatory molecules (De Clippeleir *et al.*, 2011b). Additionally, a higher hydraulic stability could decrease fluctuations in shear forces, which may affect biomass aggregate structure. According to hypothesis 1, SBR with a shorter operational cycle would need a more spread-out feeding pattern and a lower VER.

The tolerance of a salt-adapted OLAND community to 30 g NaCl l⁻¹ (about 55.5 mS cm⁻¹) was first reported by Windey and colleagues (2005). Yet, sudden exposure of non-adapted biomass to such high salt concentrations led to almost complete inhibition of the anammox activity. Dapena-Mora and colleagues (2007) showed that addition of 2.9, 5.9, 8.8 and 11.7 g NaCl l⁻¹ to a non-adapted AnAOB community resulted in about 120%, 110%, 95%, 85% of the anammox activity relative to the activity without salt respectively. In a follow-up study, it was shown that the specific anammox activity of salt-adapted biomass increased for salt concentrations up to

15 g NaCl l⁻¹ (Dapena-Mora *et al.*, 2010). In a similar salinity/conductivity range, successful anammox performance has been reported for the treatment of several real effluents: sludge reject water containing 2.0–6.2 and 1.1–2.7 mS cm⁻¹ (~ 0.4–1.1 g NaCl l⁻¹) after treatment (Szatkowska *et al.*, 2007; Siegrist *et al.*, 2008; Joss *et al.*, 2009; Jeanningros *et al.*, 2010; Zubrowska-Sudol *et al.*, 2011), black water digestate containing 7.9 mS cm⁻¹ (Vlaeminck *et al.*, 2009a), digestate from fish canning industry containing 8–10 g NaCl l⁻¹ (Dapena-Mora *et al.*, 2006), landfill leachate containing 11–65 mS cm⁻¹, and up to 28 mS cm⁻¹ (~ 13 g NaCl l⁻¹) after treatment (Siegrist *et al.*, 1998; Rusalleda, 2012). Hypothesis 2 of this study was therefore that some salinity (3–5 g NaCl l⁻¹) would be beneficial for OLAND biomass growth and activity. Increased salinities or conductivities also indirectly level out the osmotic pressure variations in a SBR cycle, as such providing more stable physicochemical conditions.

In this study, four start-up strategies were compared in two sequential tests with two parallel scale SBR with a short operational cycle (1 h) and MSV of 0.7 m h⁻¹. In each test, the two reactors only differed in one key parameter. In test 1, the effect of the 'hydraulic stability' was tested, i.e. feeding over the first 8% of the operation cycle (pulse; SBR A) versus feeding over 82% of the cycle (semi-continuous; SBR B). For both reactors, influent salinity was 3 g NaCl l⁻¹ and VER was 50%. In test 2, the effect of influent salinity was tested, i.e. no salt was added to the influent of SBR C whereas 5 g NaCl l⁻¹ was added to SBR D. For both reactors, the optimal hydraulic strategy of test 1 was applied, i.e. semi-continuous feeding, and a low VER of 25% was applied. The operational cycles of the four reactors are schematized in Fig. 1. In order to score a 'successful' start-up, some performance criteria were specifically defined for this study: (i) reasonably high volumetric removal rates (average above 400 mg N l⁻¹ day⁻¹, as achieved in most studies in Table 1) over a period of at least 7 days with no outliers below 300 mg N l⁻¹ day⁻¹, and (ii) balanced nitrogen removal, i.e. low nitrite accumulation (< 10 mg NO₂⁻ N l⁻¹, minimizing anammox inhibition; Wett *et al.*, 2007) with no or negligible nitrification (NO₃⁻ produced per NH₄⁺ converted to N₂ < 15%). High nitrogen removal efficiency was not included as a start-up criterion since this can in principle easily be achieved by fine-tuning the nitrogen loading rate once the abovementioned criteria are met. Besides reactor performance, size-specific biomass characteristics were examined. Specific aerobic and anoxic ammonium oxidation rates, AerAOB–AnAOB juxtaposition (fluorescent *in situ* hybridization, FISH), and AerAOB, *Planctomycetes* and bacterial community structure (denaturing gradient gel electrophoresis, DGGE) were determined.

Table 1. Overview of the typical parameters of OLAND-type SBR systems.

Reactor scale	Reference	Granulation	MSV (m h ⁻¹)	Cycle duration (h)	Feeding (% of cycle)	VER (%)	HRT (h)	H/D (-)	Superficial air flow rate ^a (m ³ m ⁻² h ⁻¹)	C _{NH₄⁺} (g N l ⁻¹)	B _v (g N l ⁻¹ day ⁻¹)	Biomass concentration (g VSS l ⁻¹)	DO level (mg O ₂ l ⁻¹)
Lab	Vlaeminck <i>et al.</i> (2009b)	Yes	0.7	1	8	20	5	0.9	NA	0.10–0.20	0.5–0.9	1.6	0.4–1.1
	De Clippeleir <i>et al.</i> (2009)	Yes	0.7	1	8	25	4	0.9	2.6	0.10–0.30	0.65–1.5	2.3	0.3–0.7
	Li <i>et al.</i> (2011)	Yes	0.08	48 ^e	1	71	72 ^c	7	NA	0.07	0.022 ^c	5.2	0.3–0.8
	Winkler <i>et al.</i> (2011) ^d	Yes	9	3	33 ^e	50	6	14	39	0.36 ^f	1.96	6.2–10.8	1.0
	SBR A-D (this study) ^b	Yes	0.7	1	8–82	25–50	2–4	0.9	0.16–0.65	0.10–0.25	0.20–1.03	1.4–4.7	0.3–0.9
Full	Weissenbacher <i>et al.</i> (2010)	Yes	0.38	6	75	10	72	0.3 ^g	6.1	1.8	0.54	3.1	0.3
Lab	Third <i>et al.</i> (2001)	No	0.42	12	96	50	6	1.5	0.03	0.17	0.336	1	0.2
	T. Vanslambrouck (unpublished)	No	0.12	1	50	12.5	7.6	1.3	0.53	0.20	0.6	2	0.1–0.3
Pilot	Jeanningros <i>et al.</i> (2010)	No	1.4	8	NA	20	41	4	0.83–0.96	0.40–0.80	0.74	NA	0.3–0.8
Full	Joss <i>et al.</i> (2009; 2011) ⁱ	No	3	8–9 ^h	18–15	24–25	41–35	0.4 ^g	5.9–6.9	0.65	0.45	3.4–3.8	0.5–0.4

a. Assuming that the air flow rate was equally spread over the horizontal reactor section.

b. The applied ranges for all tested systems are given; details per reactor system are presented in Table 2.

c. Values at the end of the start-up period, in which the HRT and hence cycle duration were gradually decreased, increasing B_v.

d. Values from final two operational periods.

e. Anoxic feeding period.

f. Besides ammonium, the influent also contained 80 mg NO₂⁻-N l⁻¹.

g. Rectangular reactors: average of length and width was taken as diameter.

h. Cycle duration dependent on activity.

i. In case of two values: data for reactors Nord and Süd respectively. MSV, minimum settling velocity; VER, volumetric exchange ratio; HRT, hydraulic retention time; H/D, reactor height/diameter; C_{NH₄⁺}, ammonium influent concentration; B_v, volumetric loading rate; DO, dissolved oxygen; NA, not available.

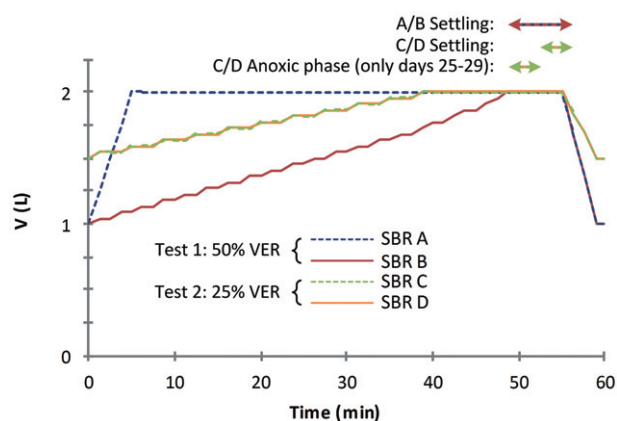


Fig. 1. Schematic representation of the operational cycles of the tested sequencing batch reactors (SBR). VER, volumetric exchange ratio.

Results

Performance

Effect of feeding regime (test 1). In the first parallel test, a fast pulse-feeding regime (reactor A) was compared with a slow semi-continuous feeding regime (reactor B) (Fig. 2, Table 2).

The nitrogen removal rate of reactor A lingered around $200 \text{ mg N l}^{-1} \text{ day}^{-1}$, and the nitrate balance was highly variable. At day 24, the air flow rates of reactors A and B were lowered from 40 to 30 l h^{-1} , to keep the effluent nitrite levels in reactor A $< 10 \text{ mg N l}^{-1}$, but reactor A did not develop sufficient nitrogen removal, and the pulse-feeding strategy was considered not to be successful over the test period of 39 days.

For reactor B, the removal rate increased rapidly at first, yet decreased after 10 days (minimum of $84 \text{ mg N l}^{-1} \text{ day}^{-1}$ at day 19) and the nitrate balance began to fluctuate more. This was presumably caused by the downfall in the loading rate, with a minimum of $597 \text{ mg N l}^{-1} \text{ day}^{-1}$ at day 17 as a consequence of an accidental drop in the influent ammonium concentration. SBR B recovered and from days 24 until 39: the removal rate remained high with an average of $0.43 (\pm 0.077) \text{ g N l}^{-1} \text{ day}^{-1}$ and a stable and low nitrate balance (10.4%). The decreased air flow rate from day 24 on was not considered to be causal for the start-up of B, since the increasing trend in nitrogen removal rate was already set in before this (Fig. 2).

Effect of salt (test 2). In the second parallel test, the effect of salt in the influent was tested by adding 5 g NaCl l^{-1} to SBR D and not to SBR C (Fig. 2, Table 2). This series of tests was started at a low air flow rate (10 l h^{-1}), which was gradually increased over time to keep the dissolved oxygen (DO) overshoot low at the beginning of the experiment (see also *Experimental procedures*).

SBR C started up quickly: from day 9 on, a nitrogen removal rate of $0.50 \pm 0.16 \text{ g N l}^{-1} \text{ day}^{-1}$ was achieved. Nitrite in the effluent did not exceed 7 mg N l^{-1} and the nitrate balance was always below 12%. There were some technical problems with online DO measurements, hence the higher standard deviation for DO levels in this reactor (Table 2).

The removal rate of reactor D rose steadily and rapidly with on day 22 a removal rate of $811 \text{ mg N l}^{-1} \text{ day}^{-1}$. The nitrate balance was always c. 11%, indicating negligible nitrification. However, nitrite levels gradually increased, coincident with the increase in air flow rate. As a consequence of the high nitrification activity and concurrent fast DO uptake, the DO level did not reach its setpoint at the given air flow rate, and was lower compared with reactor C (Table 2). On day 23, a concentration of $67 \text{ mg NO}_2^- \text{ N l}^{-1}$ in the effluent was measured. To counteract this nitrite build-up, an anoxic phase of 5 min with mixing without aeration was implemented from day 25 until 29 in both reactors. Nitrite concentrations rose up to 132 mg N l^{-1} on days 25 and 26, concurrent with decreased nitrogen removal rates, presumably caused by some AnAOB inhibition, but then nitrite levels quickly decreased. To further suppress nitrification rates, the air flow rate of reactor D was lowered to 20 l h^{-1} on day 28 for this reactor only (Fig. 2), not to jeopardize the good performance of reactor C. From day 28 on, we thus deviated from the concept of a strict parallel test. The day thereafter (day 29), nitrite in the effluent dropped below $10 \text{ mg NO}_2^- \text{ N l}^{-1}$ and the reactor had recovered, so the anoxic phase was eliminated. Next, on day 30, the ammonium influent of SBR D was increased from 200 to 250 mg N l^{-1} to provide sufficient substrate. After 32 days, SBR D was considered to have started up, reaching up to day 45 a high nitrogen removal rate of $1.03 \pm 0.11 \text{ g N l}^{-1} \text{ day}^{-1}$.

Biomass characterization

Size distribution. During tests 1 and 2, granules were observed in reactors A/B and C/D from days 19 and 7 on respectively. Both red and brown granules were present (Figs S1 and S2), in accordance with De Clippeleir and colleagues (2009). At the end of the tests, biomass was divided into size fractions: $0.10\text{--}0.50 \text{ mm}$ and $> 0.50 \text{ mm}$ for reactors A/B and $0.10\text{--}0.25 \text{ mm}$, $0.25\text{--}0.50 \text{ mm}$ and $> 0.50 \text{ mm}$ for reactors C/D. The size distribution of the biomass aggregates is shown in Table 2. In reactor A, which did not start up, the particles $> 0.50 \text{ mm}$ were more represented than the fraction $0.10\text{--}0.50 \text{ mm}$, in contrast to the minority of larger particles in reactor B. In reactor C, the vast majority of the biomass (86%) was $> 0.50 \text{ mm}$, compared with only 35% of this fraction in reactor D.

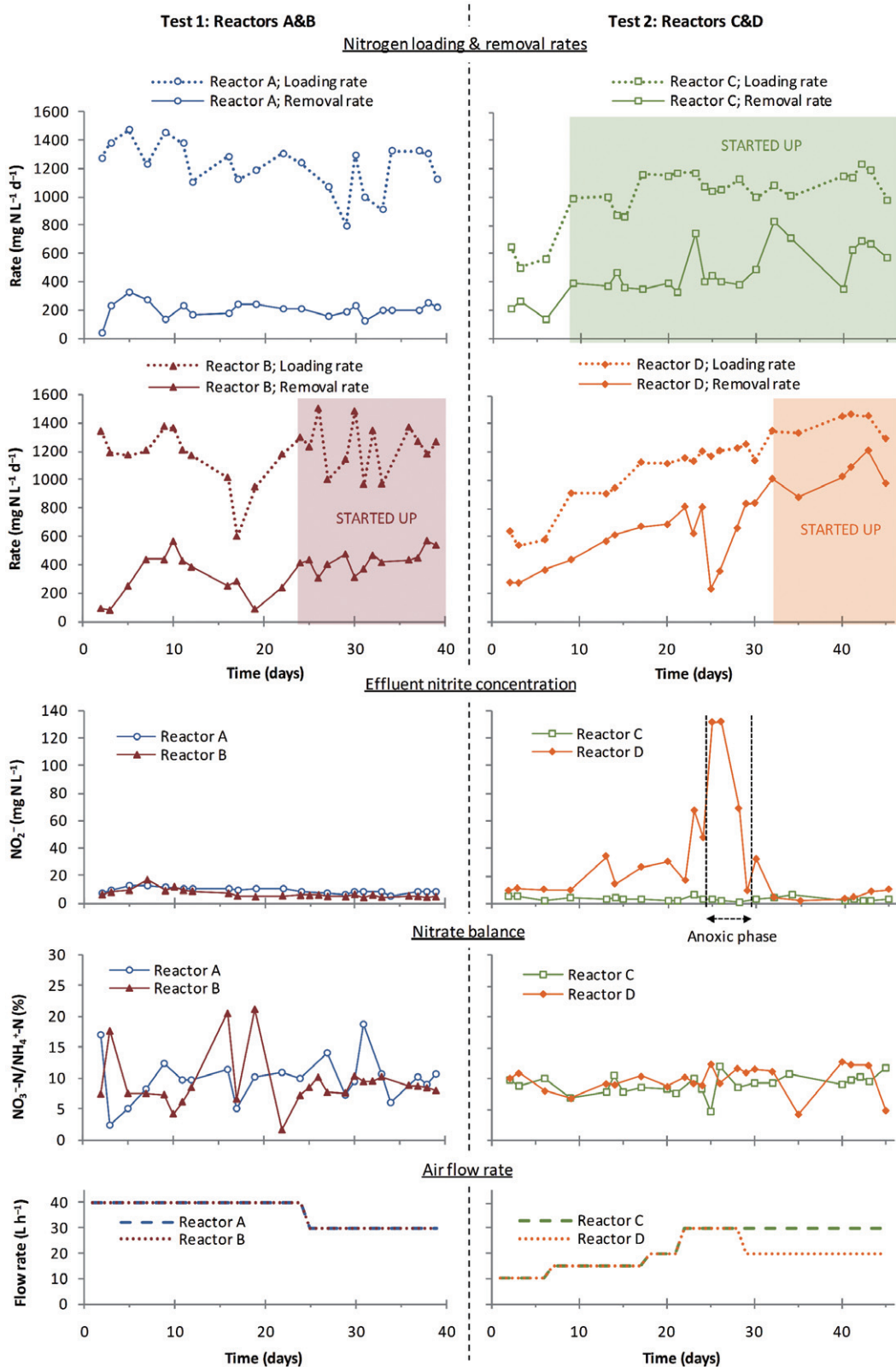


Fig. 2. Performance of reactors A and B (test 1, left) and reactors C and D (test 2, right). The period with an anoxic phase in reactors C and D is shown in the graph of the effluent nitrite concentration.

Table 2. Set-up and performance characteristics of the reactors of the two parallel SBR tests.

Reactor tests	Test 1				Test 2			
	Reactor A	Reactor B	Reactor C	Reactor D	Reactor A	Reactor B	Reactor C	Reactor D
Hydraulic parameters								
VER, %	50	50	25	25	50	50	25	25
HRT, h	2	2	4	4	2	2	4	4
Feeding regime (% of cycle duration)	Pulse (8%)	Semi-continuous (82%)	Semi-continuous (65%)	Semi-continuous (65%)	Semi-continuous (82%)	Semi-continuous (82%)	Semi-continuous (65%)	Semi-continuous (65%)
Influent								
Salt, g NaCl l ⁻¹	3	3	0	5	3	3	0	5
Initial NH ₄ ⁺ level, mg N l ⁻¹	100	100	100–150–200 ^b	100–150–250 ^b	100	100	100–150–200 ^b	100–150–250 ^b
Biomass retention								
Minimum settling velocity, m h ⁻¹	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7
Aeration								
Air flow, l h ⁻¹	30–40	30–40	10–30	10–30	/	/	10–30	10–30
Anoxic phase (% of reaction phase)	/	/	Day 25–29 (10%)	Day 25–29 (10%)	/	/	Day 25–29 (10%)	Day 25–29 (10%)
average DO, mg O ₂ l ⁻¹	0.80 (±0.14)	0.72 (±0.10)	0.76 (±0.27)	0.43 (±0.14)	0.72 (±0.10)	0.72 (±0.10)	0.76 (±0.27)	0.43 (±0.14)
Performance ^c								
Nitrogen removal rate, g N l ⁻¹ day ⁻¹	0.20 (±0.06)	0.43 (±0.08)	0.50 (±0.16)	1.03 (±0.11)	0.20 (±0.06)	0.43 (±0.08)	0.50 (±0.16)	1.03 (±0.11)
Nitrogen removal efficiency, %	17 (±5)	36 (±8)	47 (±14)	74 (±6)	17 (±5)	36 (±8)	47 (±14)	74 (±6)
Start-up time, days	(> 39)	24	9	32	(> 39)	24	9	32
Other								
pH	7.4 (±0.1)	7.5 (±0.1)	7.7 (±0.2)	7.5 (±0.2)	7.4 (±0.1)	7.5 (±0.1)	7.7 (±0.2)	7.5 (±0.2)
Effluent FA, ^a mg NH ₃ -N l ⁻¹	1.7 (±0.6)	1.5 (±0.8)	3.6 (±2.4)	0.7 (±0.7)	1.7 (±0.6)	1.5 (±0.8)	3.6 (±2.4)	0.7 (±0.7)
Biomass characterization								
Total, g VSS l ⁻¹	1.43	3.05	3.31	4.67	1.43	3.05	3.31	4.67
Diameter, mm	0.10–0.50	0.10–0.50	0.25–0.50	0.25–0.50	0.10–0.50	0.10–0.50	0.25–0.50	0.25–0.50
Distribution, % of total	44	56	86	> 0.50	44	56	86	> 0.50
Aerobic activity, mg NH ₄ ⁺ -N g ⁻¹ VSS day ⁻¹	ND	ND	605 (±4)	731 (±84)	ND	ND	605 (±4)	731 (±84)
Anoxic activity, mg NH ₄ ⁺ -N g ⁻¹ VSS day ⁻¹	ND	ND	442 (±104)	376 (±36)	ND	ND	442 (±104)	376 (±36)
NARR	ND	ND	0.96	0.41	ND	ND	0.96	0.41

a. Calculated from the measured pH, temperature and effluent ammonium concentration (Anthonisen *et al.*, 1976).

b. Concentrations were changed at day 7, day 15 and day 30 (reactor D only).

c. For A, B and D, data reflected the period after start-up, but for reactor C data reflect the complete test period.

For some parameters, average values (± standard deviation) are given.

VER, volumetric exchange ratio; HRT, hydraulic retention time; DO, dissolved oxygen; FA, free ammonia; ND, not determined; NARR, nitrite accumulation rate ratio. For each test, the key difference parameters for the two parallel SBR are printed in bold.

Biomass-specific activity rates. The final biomass concentrations of reactors A–D followed the same trend as the obtained nitrogen removal rates (Table 2), i.e. the higher the nitrogen removal rate obtained, the more biomass had developed. As such, dividing the volumetric nitrogen removal rates by the biomass concentrations yields similar specific activities of 142, 141, 150 and 220 mg N g⁻¹ VSS day⁻¹ for reactors A, B, C and D respectively.

For reactors C and D, separate aerobic and anoxic batch activity tests were performed for the three size classes, and none of the biomass showed nitrification activity, in agreement with the very low nitrate balance in these reactors (Fig. 2). For both reactors, specific nitrification rates decreased with increasing size, and anammox rates increased from 0.10–0.25 mm to 0.25–0.50 mm fraction, but not to the fraction of > 0.50 mm (Table 2). Aerobic and anoxic activity rates of the SBR D biomass were lower compared with those of SBR C, and the anoxic rates of SBR D were below the expected 220 mg N g⁻¹ VSS day⁻¹, presumably due to the absence of salt in the batch tests. The aerobic activity of the inoculum was 170 ± 11 mg NH₄⁺-N g⁻¹ VSS day⁻¹ and the anoxic activity 104 ± 10 mg NH₄⁺-N g⁻¹ VSS day⁻¹, so all specific activity had increased in the reactors, except the anammox activity in the smallest aggregates (0.10–0.25 mm).

The nitrite accumulation rate ratio (NARR) was calculated for the different size fractions (Vlaeminck *et al.*, 2010), defined as the nitrite production rate measured in the aerobic batch test fed with ammonium (nitrification minus nitrification) divided by the nitrite consumption rate measured in the anoxic batch test fed with ammonium and nitrite (anammox). NARR values were 11–14 for the smallest fraction (0.10–0.25 mm) of reactors C and D, indicating that these acted as net nitrite sources. The larger fraction 0.25–0.50 mm in C and 0.25–0.50 and > 0.50 mm in D displayed quite well-balanced aerobic and anoxic activities with a NARR of almost 1.0–1.6. In contrast to D, the largest C fraction (> 0.50 mm) had a NARR of 0.4, acting a net nitrite sinks.

AerAOB–AnAOB juxtaposition (FISH). FISH was performed to qualitatively examine the presence, abundance and juxtaposition of AerAOB (*Nitrosomonas* spp.) and AnAOB (*Candidatus Brocadia* and *Candidatus Kueneenia*). Differences in aggregate structure between the reactors A–D were minor compared with those between the different size fraction (Fig. S3), and therefore only reactors C and D are discussed here. In agreement with the batch activity tests, AnAOB were also detected in the smallest aggregates (Fig. S3, C1 and D1), and the abundance of AerAOB decreased with increasing aggregate size, whereas the abundance of AnAOB increased.

AerAOB and AnAOB were always closely juxtapositioned. The AerAOB microcolonies were quite scattered and not very dense. In contrast, AnAOB appeared mostly in dense and spherical clusters, or pleomorphic zones in the larger particles (Fig. S3, C3), except for the abundantly present small flocs in salt-fed reactor D, where AnAOB were quite dispersed (Fig. S3, D1).

Structure of the communities (DGGE). DGGE was performed to compare the structure of AerAOB, *Planctomycetes* and bacterial communities between the reactor systems and different size fractions (Fig. 3). In general, reactors from the same test (A/B versus C/D) were more similar, and the AerAOB and *Planctomycetes* communities of different sizes classes in one system were more similar than the communities of different systems. Furthermore, the similarities between AerAOB communities were very high (> 90%), in contrast to the similarities between *Planctomycetes* communities (60–90%). This was likely caused by the fact that 1–2 AerAOB bands were quite dominant, whereas the abundance of the different *Planctomycetes* was more equally spread. This is also reflected in the values for the community organization, which were consistently lower for *Planctomycetes* compared with AerAOB (Fig. 3), showing a higher evenness for *Planctomycetes* communities. Also, *Planctomycetes* similarities were higher between reactors A and B (90%) than those between reactors C and D (75%), although the reactors of each parallel test were inoculated with the same biomass. In terms of richness, reactors C and D had about twice as much species in their *Planctomycetes* community than reactors A and B.

Discussion

Effect of hydraulic regime

As proposed in the first hypothesis, a low VER of 25% and/or a semi-continuous feeding regime (reactor B, C and D) resulted in good biomass growth and hence a fast start-up (< 1 month) with good reactor performance (balanced nitrogen removal rates > 0.4 g N l⁻¹ day⁻¹) at a short operational cycle (1 h). Both the semi-continuous feeding regime and the low VER resulted in relatively stable chemical conditions for the biomass. The only reactor which could not be started up (reactor A) did not have a semi-continuous feeding regime nor a low VER.

The biomass content of the different systems showed a clear trend with the achieved removal rates, and reactor A had the lowest biomass levels (1.43 g VSS l⁻¹; Table 2). The relative increases in biomass content over the test periods were –0.6, 1.1, 1.0 and 2.4 g VSS l⁻¹ for systems A, B, C and D, respectively, or –15, 27, 22 and 53 mg VSS l⁻¹ day⁻¹, in a linear approximation. The

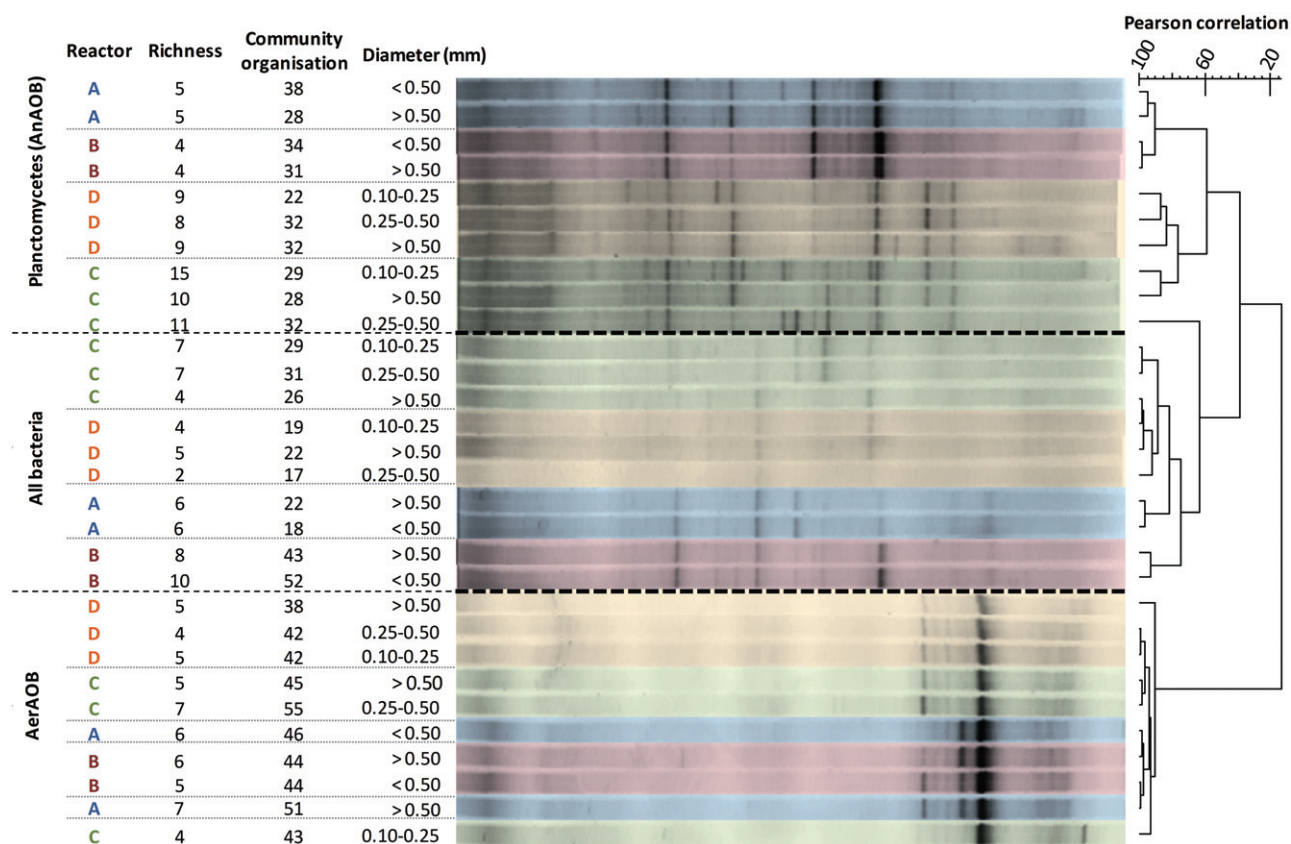


Fig. 3. DGGE gels of the different size classes of reactors A and B (test 1) and reactors C and D (test 2) for β -proteobacterial aerobic ammonium-oxidizing bacteria (AerAOB), all bacteria and Planctomycetes, harbouring anoxic ammonium-oxidizing bacteria (AnAOB). The number of bands represents the richness and the community organization represents the evenness (Read *et al.*, 2011).

decreased biomass content in SBR A indicated that there was some washout, and that true biomass growth was likely higher than these estimated values. Since the biomass of all systems removed nitrogen at similar rates ($141\text{--}220\text{ mg N g}^{-1}\text{ VSS day}^{-1}$), the applied hydraulic strategies leading to more stable chemical conditions resulted mainly in a higher biomass growth and development in systems B and C, and especially in D. For the latter, salt at 5 g NaCl l^{-1} could have played an additional role, as is discussed in the next section.

Since all reactors had the same low MSV (0.7 m h^{-1}), this parameter was not decisive to achieve a good SBR start-up for OLAND. De Clippeleir and colleagues (2009) also concluded that of the parameters critical MSV and low VER, the latter was the driving force for start-up. Still, a low MSV increases biomass retention in the system, and can therefore be requisite for a good start-up.

The FISH results could not be clearly linked to the successful start-up of a reactor. DGGE showed that the communities' similarities were higher between two reactors of the same test. There are two possible reasons for this. Firstly, the inoculum communities for A/B and C/D

likely differed, although the inocula were derived from the same rotating biological contactor. Such biofilm usually displays a large heterogeneity in community structure (Vlaeminck *et al.*, 2009a), additionally depending on biofilm thickness. Further, the period between inoculum sampling for A/B and C/D was 2 months, in which the inoculum community might have evolved. Secondly, the different hydraulic conditions in A/B (VER 25%) versus C/D (VER 50%) might have influenced the community. Such a community change is possible over the tested time-course, as exemplified by the divergence of the slowest growing community, i.e. the *Planctomycetes*, between C and D. As such, the richer *Planctomycetes* communities in reactors C/D compared with A/B might be related to the better performance of these reactors.

Appreciably high nitrogen removal rates were obtained in SBR B–D. Furthermore, the nitrogen conversions were well balanced. No clear signs of nitrification were observed in any of the SBR. Low DO ($< 1\text{ mg O}_2\text{ l}^{-1}$) and moderately high free ammonia ($0.7\text{--}3.6\text{ mg N l}^{-1}$) levels successfully inhibited NOB activity over the test period. Only reactor D suffered from nitrite build-up, which could effectively be

cured by temporarily including an anoxic period at the end of the reaction phase. The latter was also demonstrated by De Clippeleir and colleagues (2009).

Based on the fact that reactor C started up considerably faster than reactor B, we propose the combination of low VER, in accordance with the study of De Clippeleir and colleagues (2009), and semi-continuous feeding as the best strategy.

Effect of salinity

The effect of salt on the performance was difficult to unravel. A salt concentration of 3 g NaCl l⁻¹ did not have a negative effect on reactor performance, as shown by the start-up of reactor B. A concentration of 5 g NaCl l⁻¹ in the influent could have had a positive effect on reactor performance as reactor D obtained the highest removal rates (> 1 g N l⁻¹ day⁻¹) and biomass growth. However, reactor D was the only one troubling with a nitrite build-up, likely due to the high NARR value of the abundantly present small aggregates (0.10–0.25 mm).

In fact, the higher salinity is believed to have played a role in the abundant formation of small aggregates in reactor D. The salt concentration (5 g NaCl l⁻¹) clearly influenced the biomass structure and activity as indicated by several facts. Firstly, reactor D was the only one to develop a large fraction of small aggregates (0.10–0.25 mm), with a high NARR value and hence nitrite accumulation potential, demonstrated by the nitrite build-up of reactor D. Secondly, the FISH results showed a quite dispersed AnAOB appearance in the small flocs. Together with the previous fact, this might indicate an influence of elevated salinity on the production of extracellular polymeric substances (EPS), but such mechanism remains to be studied. Thirdly, the anoxic batch activity rates of reactor D yielded lower rates than expected from the reactor performance, likely due to the absence of salt in the batch test, indicating a salt-adapted community. The latter adaptation is also suggested from the fourth fact, i.e. the relatively low similarities in *Planctomycetes* community between reactors C and D, which was supposedly a salt effect.

The aeration strategy for reactors C/D was to start at a lower air flow rate at the beginning of the operation of reactors C/D, to initially 'protect' AnAOB from high DO levels from a higher overshoot at higher air flow rates and to keep the shear stress initially lower. Subsequently, the air flow rate was gradually increased. For reactor C, this strategy did not result in the accumulation of nitrite. Yet, in reactor D, the rise in nitrite in the effluent was coincident with the increase in air flow rate (Fig. 2). Through a link with EPS, the increased salinity might have rendered particles more vulnerable to shear and disintegration in reactor D.

Overall, an increased salinity or conductivity through the addition of salt to the influent was not necessary to obtain a fast start-up, since reactor C started up in only 9 days in the absence of salt.

Effect of aggregate size

Each of the reactors developed a different aggregate size distribution, with smaller particles (< 0.50 mm) dominating in systems B and D, and larger particles in systems A and C. Batch activity tests of the biomass of reactors C and D showed that nitrification rates decreased with increasing size, whereas anammox rates increased except for the largest size fraction (Table 2). Similarly, qualitative FISH indicated decreasing AerAOB abundance with size, and increasing AnAOB abundance. These findings and those on the aggregate structure are in accordance with Vlaeminck and colleagues (2010).

Reactor C had few aggregates in the fraction of 0.25–0.50 mm and reactor D had few larger than 0.50 mm. Nonetheless, stable and balanced nitrogen removal by OLAND could be obtained. This indicates that the smaller particles with high NARR values (net nitrite sources) worked efficiently together with the larger particles with lower NARR values (net nitrite sinks). Also within one aggregate, the close juxtaposition of AerAOB and AnAOB as seen by FISH indicates an efficient transfer of nitrite. Although AnAOB are supposedly more vulnerable to oxygen inhibition in small aggregates since they are not protected by a thick layer of oxygen consuming AerAOB, reactor D was not suffering from lower anammox rate. Overall, biomass existing solely out of aggregates with an optimal size for OLAND is thus not a prerequisite for good reactor performance of an OLAND reactor, as concluded by Vlaeminck and colleagues (2010) in their work.

Experimental procedures

OLAND SBR

The lab-scale OLAND SBR consisted of a cylindrical vessel with an internal diameter of 14 cm (working volume of 2 l). Most operational details are presented in Table 2. All reactors were inoculated with OLAND biofilm harvested from the rotating biological contactor described by Pynaert and colleagues (2003), at an initial biomass concentration of 2 g VSS l⁻¹ (A and B), 2.3 g VSS l⁻¹ (C and D). The reactors were fed with synthetic wastewater containing an initial ammonium concentration of 100 mg NH₄SO₄-N l⁻¹, 10 mg KH₂PO₄-P l⁻¹ and 2 ml l⁻¹ of a trace elements solution (Kuai and Verstraete, 1998). To provide both buffering capacity and inorganic carbon, 1.5 mol of bicarbonate was added per mole of nitrogen and adjusted temporarily to ensure that the reactor pH did not drop below 7.4. In addition, the influent ammonium concentration was increased with 50 mg N l⁻¹ for both reactors of a parallel test whenever the effluent concentration of

one reactor was below c. 25 mg N l⁻¹, except the increase from 200 to 250 mg N l⁻¹ for reactor D, which was not executed in reactor C. Reactors were mixed with magnetic stirrers at 50–180 rpm and the temperature was 30–34°C (temperature controlled room). DO was controlled automatically (Oxymax W COS31 probe with Liquisis M COM 223 controller; Endress & Hauser, Switzerland). For tests 1 and 2, the DO values were set at 0.35–0.45 and 0.35–0.50 mg O₂ l⁻¹ respectively. The delay time for the online DO measurements resulted in under- and overshoot of DO values with an increased air flow rate. DO was therefore also measured more exactly with a portable digital oxygen measurement device (HACH HQ30d, Germany).

SBR cycle

The SBR were operated in cycles of 1 h. In the first phase, feeding and reaction occurred during which the reactor was mixed and the DO was controlled (test 1: 49 min; test 2: 52 min). One litre and 0.5 l was fed to the reactors of test 1 (VER: 50%) and test 2 (VER: 25%) respectively. Feeding was semi-continuous for reactors B, C and D with pauses between feeding of 1.25, 2.5 and 2.5 min respectively. Subsequently, the biomass was allowed to settle for 6 min for test 1 and 3 min for test 2, so that the MSV was 0.7 m h⁻¹. Finally, an effluent pump removed the supernatant over 4 min. The last minute was a short pause. During the course of test 2, an anoxic phase of 5 min with only mixing and no aeration was temporarily implemented.

Chemical analyses

Nitrite and nitrate were determined on a 761 Compact Ion Chromatograph (Metrohm, Switzerland) equipped with a conductivity detector. Ammonium (Nessler method) was measured according to standard methods (Greenberg *et al.*, 1992). The pH was measured with a C532 pH meter (Consort, Belgium). The nitrate balance was calculated as the nitrate produced per net ammonium consumed [NO_3^- -N production/(NH_4^+ -N consumption – net NO_2^- -N production)].

Biomass fractionation

After a test, biomass was sieved using various pore sizes (0.10, 0.25 and 0.50 mm) and washed with phosphate buffer (100 mg P l⁻¹, pH 8) removing residual dissolved (in)organic compounds. The fractions were analysed with methods described in the following sections.

Aerobic and anoxic batch activity tests

The specific activities of AerAOB and AnAOB of the different fractions were determined in aerobic (fed with ammonium) and anoxic (fed with ammonium and nitrite) batch tests, respectively, on a shaker at 30–34°C, as described in detail by Vlaeminck and colleagues (2007).

Fluorescent *in situ* hybridization

Fluorescent *in situ* hybridization was carried out on the different fractions. Hybridizations were performed on sludge

samples fixed with 4% (w/v) paraformaldehyde. Probe sequences and formamide concentrations were applied according to probeBase (Loy *et al.*, 2003): Amx820 for '*Candidatus* Kuenenia and Brocadia', and a probe mixture of NSO1225 and NSO190 for β -proteobacterial AerAOB. The AnAOB and AerAOB abundance was qualitatively evaluated by combining the mentioned probes with an equimolar mixture of EUB338I, II and III, targeting all bacteria, and 4'-6-diamidino-2-phenylindole (DAPI), targeting all DNA-containing cells. The probe fluorochromes were Cy3, FITC and Cy5 for AnAOB, AerAOB and all bacteria respectively. Image acquisition was done on a Nikon Eclipse TE2000E epifluorescence microscope (Nikon, Japan) equipped with highly sensitive ANDOR ixon3 EMCCD camera (Andor Technology, Ireland). Images were superimposed with ImageJ freeware.

Denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis was performed to compare the community structure (AerAOB, *Planctomycetes* and total bacteria). DNA extraction, nested PCR and DGGE were performed according to Pynaert and colleagues (2003), based on the phylogenetic primers CTO189ABf, CTO189Cf, and CTO653r for AerAOB, PLA40f and P518r for *Planctomycetes*, and GC338 and 518r for all bacteria. The obtained DGGE patterns were subsequently processed with BioNumerics software (Applied Maths, Belgium). To obtain an ecological and quantitative interpretation of the community fingerprints, a three-parameter analysis was performed based on the number of bands (a measure for richness), community organization (a measure for evenness) and Pearson correlation coefficient (Read *et al.*, 2011).

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Macroscopic views on the biomass of reactors A (left) and B (right) on day 19. For both reactors, brown and red granules are clearly visible.

Fig. S2. Macroscopic views on the biomass of reactors C (left) and D (right) on day 14. Reactor C contained mainly granular biomass while reactor D contained also more smaller aggregates. In both reactors, some of the granules had a distinctive reddish colour.

Fig. S3. FISH micrographs with AerAOB (probes Nso1225 and Nso190, green) and AnaAOB (probe Amx820, red) of the different size fractions of biomass from reactors A and B (subsets 1 and 2 for 0.10–0.50 mm and > 0.50 mm respectively), and C and D (subsets 1, 2 and 3 for 0.10–0.25 mm, 0.25–0.50 mm and > 0.50 mm respectively).

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