Influence of dissolved oxygen concentration on the start-up of the anammox-based process: ELAN®

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ABSTRACT

The anammox-based process ELAN® was started-up in two different sequencing batch reactor (SBR) pilot plant reactors treating municipal anaerobic digester supernatant. The main difference in the operation of both reactors was the dissolved oxygen (DO) concentration in the bulk liquid. SBR-1 was started at a DO value of 0.4 mg O_2/L whereas SBR-2 was started at DO values of 3.0 mg O_2/L. Despite both reactors working at a nitrogen removal rate of around 0.6 g N/(L d), in SBR-1, granules represented only a small fraction of the total biomass and reached a diameter of 1.1 mm after 7 months of operation, while in SBR-2 the biomass was mainly composed of granules with an average diameter of 3.2 mm after the same operational period. Oxygen microelectrode profiling revealed that granules from SBR-2 where only fully penetrated by oxygen with DO concentrations of 8 mg O_2/L while granules from SBR-1 were already oxygen penetrated at DO concentrations of 1 mg O_2/L. In this way granules from SBR-2 performed better due to the thick layer of ammonia oxidizing bacteria, which accounted for up to 20% of all the microbial populations, which protected the anammox bacteria from non-suitable liquid media conditions.

Key words | anammox, autotrophic nitrogen removal, dissolved oxygen, granule, oxygen microprofiles

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INTRODUCTION

In the last decade, the development of anammox (anaerobic ammonium oxidation) processes has contributed to an important reduction in the energy and resources consumption required for the removal of nitrogen from highly loaded streams with a low chemical oxygen demand/nitrogen (COD/N) ratio (Siegrist *et al.* 2008). The application of anammox-based processes to the main stream lines of wastewater treatment plants (WWTPs) can save 28% of costs and reduce the greenhouse gas emissions (Morales *et al.* 2015). Anammox processes are operated combined to a partial autotrophic nitritation process in which the oxidation of approximately half of the ammonium to nitrite is carried out by ammonia oxidizing bacteria (AOB). The low biomass yield of AOB and anammox bacteria (Strous *et al.* 1999) is at the same time an advantage, due to its low sludge production, and a disadvantage, due to the need for good biomass retention capacity of the reactor systems. In consequence, systems with a high sludge retention time are needed, such as those based in a biofilm-based setup: rotating contactors (Pynaert *et al.* 2003), moving bed reactors (Cema *et al.* 2006), fixed bed reactors (Furukawa *et al.* 2006) and granular biomass reactors (Fernández *et al.* 2008); or based in membrane bioreactors (Trigo *et al.* 2006).

The ELAN® process (acronym of 'ELiminación Autótrofa de Nitrógeno', autotrophic nitrogen removal in Spanish) is an anammox-based process carried out with granular biomass in one single reactor which has stood out as an efficient way to remove nitrogen from highly loaded streams with a low COD/N ratio (Vázquez-Padín *et al.* 2014). The system is based on the establishment of aerobic and anoxic zones within the granule.

The oxygen penetration depth within the granule defines the existence of a borderline between AOB and anammox bacteria, as oxygen inhibits the activity of the latter bacteria.

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In this way, AOB can grow in the outer part of the aggregates, where they produce nitrite and consume oxygen to provide anoxic conditions in the inner part of the granule. In the anoxic zone, ammonium, left from the incomplete AOB activity, and nitrite, produced during partial nitrification, has to be present in order to allow the growth of anammox bacteria. Experimental research performed by Vlaeminck *et al.* (2010), Vázquez-Padín *et al.* (2010b) and mathematical simulations by Volcke *et al.* (2010) confirmed that AOB are located at the more external layers of the granule, while anammox bacteria are situated in the anoxic part of the granule, but still close to the bulk liquid and to the layer of the AOB. The activity of AOB is directly related to the fraction of the granule which is under aerobic conditions (Vázquez-Padín *et al.* 2011). In addition, oxygen limitation regulates the amount of nitrite produced and as a consequence also influences the anammox activity.

The granulation process depends on different operational parameters, such as hydrodynamic shear force, mixing characteristics or dissolved oxygen (DO) concentration (Campos *et al.* 2009; Khan *et al.* 2013). The size of the granules has an important effect on the activity of AOB, as different granules have distinct surface/volume ratios and consequently, different oxygen depth penetrations (Volcke *et al.* 2010). Since the oxygen penetration depth depends on the DO concentration, this parameter can be used to control the activity of AOB, control the aerobic/anoxic ratio and therefore, to control the balance of AOB and anammox activities (Vázquez-Padín *et al.* 2011).

In this research work, the start-up of two different ELAN® reactors with different configurations and operational characteristics was compared in terms of nitrogen removal performance and biomass characteristics focusing on the effect of the DO concentration, its effects over the oxygen penetration depth in the granules and its relation to the average diameter of the granules.

MATERIAL AND METHODS

The ELAN® process was carried out at pilot scale in two cylindrical sequencing batch reactors (SBRs). The dimensions of the pilot scale reactor SBR-1 were: total height of 1.75 m, working height of 1.55 m and inner diameter of 1.0 m. The reactor had a working volume of 1,200 L and a height to diameter (H/D) ratio of 1.5. The dimensions of pilot scale reactor SBR-2 were: total height of 0.9 m, working height of 0.7 m and inner diameter of 0.6 m. The reactor had a working volume of 200 L and an H/D ratio of 1.2. The volume exchange ratio of both reactors was 20%.

The operational cycle of the reactors was divided into feeding, reaction, settling and withdrawal stages. The operational strategy was based on the control of the hydraulic retention time (HRT) and the DO concentration in the bulk liquid, in order to avoid ammonium and nitrite limitations and/or inhibitions as described by Vázquez-Padín *et al.* (2010b), and following the 'conductivity versus time slope' as a method for reactor surveillance as detailed by Vázquez-Padín *et al.* (2014). This conductivity drop is mainly related to the conversion of ammonium and bicarbonate ions, both present in the anaerobic digester supernatant, into nitrogen gas and biomass, respectively. The temperature of the reactors was not controlled and reached an average of 30.3 °C ±1.0 °C and 29.6 °C ±0.6 °C in SBR-1 and SBR-2, respectively.

Both reactors were fed with the supernatant of anaerobic sludge digesters of two different municipal WWTPs located in the northwest of Spain with similar characteristics: Guillarei and Vigo WWTPs for SBR-1 and SBR-2, respectively. The characterization of the influents is summarized in Table 1. Both reactors were inoculated with flocculent nitrifying biomass. In both reactors the minimum settling velocity for the biomass imposed by the settling time was 1 m/h.

The concentrations of total and volatile suspended solids (TSS and VSS), sludge volume index (SVI), and alkalinity content were determined according to *Standard Methods* (APHA-AWWA-WPCF 2005). Ammonium, nitrite, nitrate, total nitrogen (TN), and COD concentrations were determined with 'Dr Lange' kits; sulfanilamide was added to the nitrate kit in order to avoid its interference with nitrite. DO concentration, pH and conductivity in the bulk liquid of the reactors were measured using a luminescent DO sensor (LDO sc, Hach Lange), a digital differential pH/ORP sensor

Table 1 | Characterization of the anaerobic sludge digester supernatant

Parameter	Units	SBR-1	SBR-2
NH_4^+	mg N/L	540–1,045 (938 ± 101)	410–810 (590 ± 85)
Total COD	mg COD/L	130–390 (149 ± 26)	170–450 (285 ± 101)
Conductivity	mS/cm	6.0–8.9 (7.4 ± 1.0)	4.6–8.8 (6.1 ± 0.8)
pH	–	8.0 ± 0.05	8.1 ± 0.15
VSS	g/L	(0.19 ± 0.07)	(0.05 ± 0.02)

(In parentheses average and standard deviation.)

(pHD sc, Hach Lange) and a digital inductive conductivity sensor (3798-S sc, Hach Lange), respectively. The average size of the granules was determined using the Image Pro Plus software and analyzing the digital photographs of at least 200 granules. The settling velocity of the granules was measured by recording the time taken for a set of individual granules to descend from a certain height to the bottom in a measuring cylinder. Fluorescence *in situ* hybridization (FISH) analyses were carried out according to the methodology described by Figueroa *et al.* (2006).

Specific air fluxes in the reactors were calculated by dividing the air flux applied by the reactor volume. Specific anammox activity tests were performed according to the methodology described by Dapena-Mora *et al.* (2007). The nitrogen loading and removal rates (NLR and NRR, respectively), the ammonium and nitrite oxidation rates (AOR and NOR, respectively), and the nitrogen removal by anammox (ANR) in g N/(L d) were calculated by means of nitrogen mass balances according to Vázquez-Padín *et al.* (2010b). From the stoichiometry of the combination of the ammonia oxidation by AOB and anammox processes, the ratio AOR/ANR = 0.637 is obtained which indicates an ideal ratio between AOB and anammox activities, with the nitrite produced by AOB completely consumed by the anammox bacteria.

OX-10 Unisense A/S (Aarhus, Denmark) Clark-type O_2 microelectrodes were used to perform the microprofiles within the granules formed in both reactors (Revsbech 1989). When profiling, the conditions of the experimental chamber were kept as similar as possible to the reactor operating conditions. To simulate the hydrodynamic conditions from the reactor, the aeration flow in the chamber was regulated to maintain a k_{La} value of 1 min^{-1} which was the same value as in the SBR-2. The liquid medium inside the chamber simulated the reaction medium (around 140 mg N/L in order to avoid ammonium limitation). The DO concentration in the microelectrode chamber was controlled by changing the air/Ar ratio. At least three microprofiles for each granule and DO concentration were measured to perform the oxygen depth penetration figures.

RESULTS

Start-up comparison

A good biomass accumulation was achieved in both sequencing batch reactors (Figure 1). In reactor SBR-1, the biomass concentration increased constantly from an average

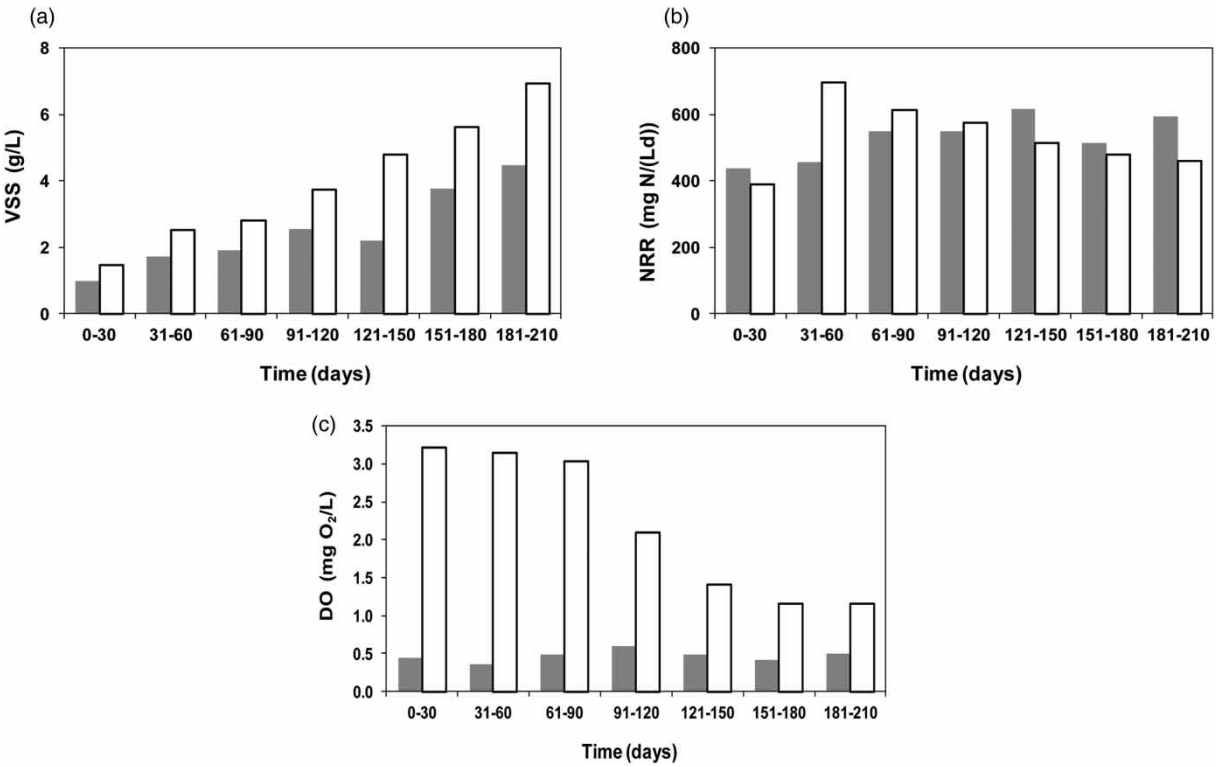

Figure 1 | (a) Solids concentration in g VSS/L, (b) nitrogen removal rate (NRR) in mg N/(L d) and (c) DO concentration in mg O_2/L, in the reactor SBR-1 (■) and SBR-2 (□) throughout 7 months of operation. The values represented in bars are average values for a period of 1 month.

concentration of 1.0 g VSS/L during the first month to an average of 4.5 g VSS/L after 7 months, then the system needed around 200 days to multiply the biomass concentration by a factor of 5. In reactor SBR-2 the final concentration after 210 days of operation was close to 7.0 g VSS/L, while that reactor was inoculated with around 0.7 g VSS/L of flocculent biomass, then the system needed approximately 200 days to multiply the biomass concentration by a factor of 10. The average VSS/TSS ratio of the biomass in the reactor was 0.81 ± 0.07 and 0.91 ± 0.03 for SBR-1 and SBR-2, respectively.

The settling properties of the biomass formed in both reactors were excellent. In this way, the average SVI_5 of the biomass in the reactor SBR-1 during the experimental operation was 139 ± 25 mL/g TSS, while the average SVI_5 of the biomass in SBR-2 during the same time was 47 ± 18 mL/g TSS. The formed granules in SBR-2 had a settling velocity of 6 m/h. The mean biomass productivity for the first 210 days was 60 g VSS/kg $N_{removed}$, a fairly high observed yield taking into account the low productivity of the autotrophic microorganisms (AOB and anammox bacteria).

Similar nitrogen removal rates were achieved in both reactors (Figure 1(b)); despite the biomass characteristics and the average DO concentration being notably different during the operation of the reactors (Figure 1(c)). In this way, a NRR of around 0.6 g N/(L d) was achieved in both reactors after only 3 months of operation. In the case of the reactor SBR-1, the appropriated balance between AOB and anammox was not reached during the first months of operation, which caused an accumulation of nitrite in the system during the first month; with an average of 100 mg N/L. This nitrite accumulation was overcome by the development of new anammox biomass in the system and did not seem to cause any growth inhibition.

The HRT variations were regulated in order to avoid ammonium and nitrite limitations and/or inhibitions. The 'conductivity vs. time slope' controlled the duration of the reaction phases in the sequencing reactors. Consequently, the average HRT values in reactors SBR-1 and SBR-2 were 1.2 ± 0.2 d and 1.0 ± 0.5 d, respectively. Conductivity and pH values at the end of the operational cycles were 2.24 mS/cm and 6.95 for SBR-1 and 3.00 mS/cm and 6.88 for SBR-2, respectively.

The DO concentration needed to achieve these removal rates was around 0.4 mg O_2/L for SBR-1, while a value of 3.1 mg O_2/L was used in SBR-2. The reduction in the DO concentration applied in the SBR-2 after day 90 led to a small reduction in the removal efficiency in this period (Figure 2(b)). NLR applied to reactor SBR-2 was increased from around 0.2 g N/(L d) to around 1.0 g N/(L d) during the first month of operation. The initial nitrogen removal rate by the anammox bacteria in the reactor was 0.13 g N/(L d) and, after 1 month of operation, it increased up to 0.85 g N/(L d), while the ammonia oxidation rate reached 0.54 g N/(L d). This implied that the ratio between AOB and anammox activities was 0.635, close to the ideal ratio for the autotrophic nitrogen removal. This quick increase was due to both the increase in the biomass concentration (Figure 1(a)) and the increase of its anammox specific activity (up to 0.36 g N/(g VSS d)). It demonstrated the success of the ELAN® process to establish the right conditions for achieving a good balance between AOB and anammox bacteria.

To evaluate the present results it is interesting to take into account that the start-up of the first full-scale anammox plants treating the supernatant of a sludge digester lasted for several years, due to the slow growth rate of the involved microorganisms (van der Star *et al.* 2007). In this way, the first start-up of the DEMON process took a period of 2.5 years,

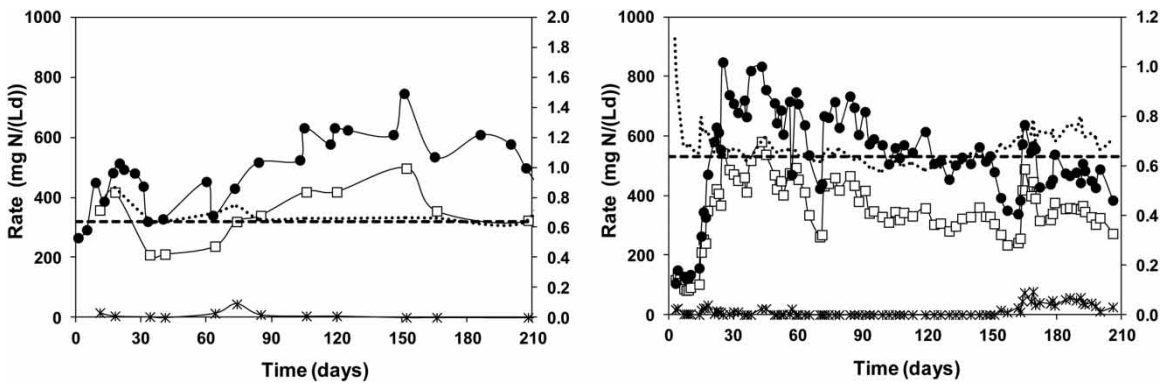

Figure 2 | Nitrogen removal rate (NRR) by anammox (●), ammonia oxidation rate (AOR) (□) and nitrite oxidation rate (NOR) (*) in mg N/(L d) and AOB/anammox ratio (···). The line at 0.637 represents the stoichiometric value of the AOB/anammox ratio for the autotrophic nitrogen removal. (a) SBR-1, (b) SBR-2.

while the transfer of substantial amounts of seed sludge permitted reduction of this time to 50 days in the next start-up processes (Wett 2007). With the knowledge gathered throughout this time (Vázquez-Padín *et al.* 2010a, b, 2014) the start-up of anammox-based reactors (such as ELAN®) was reduced notably, and around 100 full-scale plants based on different anammox processes were in operation in 2014 (Lackner *et al.* 2014).

The increase of biomass concentration (floccular and granular) in SBR-1 made it necessary to increase the specific air flux to the reactor, from 2 to 5 $L_{air}/(L_{reactor}$ h), in order to maintain constant the DO concentration in the bulk liquid and to achieve a good biomass mixing (Figure 3(a)). Conversely, the aeration rate was maintained approximately constant at around 5.5 $L_{air}/(L_{reactor}$ h) in the reactor SBR-2. Simultaneously to the biomass concentration increase in SBR-2, the DO concentration in the bulk liquid decreased (Figure 1(c)).

In ELAN® reactors, oxygen supplied to the reactor has to limit the nitrogen removal rate in order to avoid unstable periods due to oxygen and/or nitrite inhibition effects on anammox biomass. Since the effluent of the pilot reactors still contained ammonia but not nitrite, nitrogen removal rate was limited by the ammonia oxidation step.

The oxygen penetration depth determines the thickness of the external aerobic layers, where AOB grow. Consequently, it determines the size of the granule (Figure 3(b)–3(d)). In this way, in SBR-1, granules had a final diameter after 7 operational months of 1.1 mm and represented roughly 20% of the total biomass. Conversely, in SBR-2, the average diameter of the granules reached 2.3 mm and the granules represented approximately 90% of the total biomass.

Oxygen microprofiles and microbiology

Granules with an average diameter of 1.4 ± 0.4 mm were selected from SBR-1 from days 175 to 181, while granules with an average diameter of 4.8 ± 0.4 mm were selected from SBR-2 from days 165 to 170, to perform the

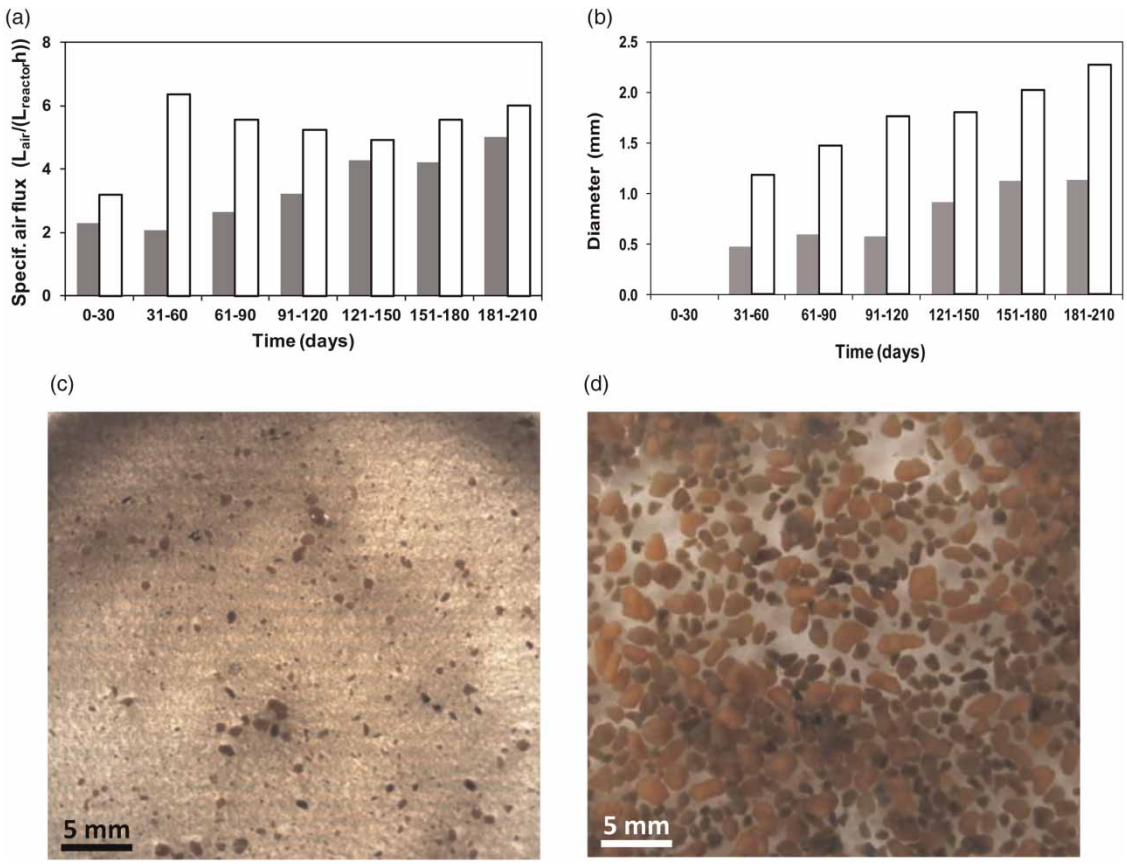

Figure 3 | (a) Specific air flux in $L_{air}/(L_{reactor}$ h) and (b) average diameter of the granules in mm, in the reactor SBR-1 (■) and SBR-2 (□) throughout 7 months of operation. The values represented in bars are average values for a period of 1 month. (c) and (d) Biomass photographs from SBR-1 on day 97 and SBR-2 on day 100 of operation, respectively.

microprofiles and determine the oxygen depth penetration at different DO concentrations in the bulk liquid.

Small granules obtained in SBR-1 were fully penetrated by oxygen, except when DO concentrations below 1 mg O_2/L were applied in the bulk liquid. In this case, the oxygen penetrated around 150 μm in the outer layers of the granule (Figure 4(a)). Thus, anammox bacteria in these granules were more susceptible to suffering inhibition by oxygen, for example when temperature and ammonia oxidation rate decreased. NOB out competition of anammox bacteria can be produced in small granules, where oxygen penetration depth increases the aerobic fraction of the biomass.

Conversely, the oxygen penetration depth for large granules such as the ones obtained in SBR-2 varied from 50 to 150 μm, when DO varied from 1 to 4 mg O_2/L, while full penetration was only obtained when DO concentration was increased to 8 mg O_2/L (Figure 4(b)). Vázquez-Padín *et al.* (2010b) determined that the oxygen penetration depth varied from 100 to 350 μm, when the DO concentration varied from 1.5 to 35.2 mg O_2/L by flushing a mixture of pure O_2 or N_2 and air. Vlaeminck *et al.* (2009) estimated a maximum oxygen penetration depth of 120 μm in granules with a diameter of 1.8 mm operated at a DO concentration of 1.1 mg O_2/L. Systems with bigger granules are easier to control and more robust towards disturbances in bulk oxygen concentration (Volcke *et al.* 2012).

According to Li & Liu (2005) when aerobic granules are larger than 0.5 mm, DO became a major limiting factor of metabolic activity of aerobic granules over substrate. Vlaeminck *et al.* (2010) showed that the activity and abundance of AOB decreases and the activity and abundance of anammox bacteria increases with the increase of the size of the granules creating a dynamic behavior of the systems in terms of the properties of the granules.

Regarding the bacterial populations present in the granules microbiology analysis by FISH of the biomass in SBR-1 and SBR-2 showed that the most abundant microorganism was anammox bacteria of the order *Planctomycetales*, more concretely *Brocadia fulgida*, accounting for around 80–90% in SBR-1 and 65–75% in SBR-2. An estimation performed by Figueroa *et al.* (2012) determined that half of the identified bacteria in their autotrophic nitrogen removal reactor were also *Brocadia fulgida*. The ammonia oxidizers involved in the ELAN® process belonged to the genus *Nitrosomonas* and accounted for around 5% in the biomass of SBR-1 and between 5 and 20% in SBR-2. NOB were detected in the biomass and belonged to the *Nitrospirae* phylum. Their concentrations were notably reduced from the start-up period, where it accounted for 5–10%, to less than 2% at the end of the experimental period. The protection provided by the oxic layer with a high density of *Nitrosomonas*, which hinder and lower the variations in the bulk liquid composition, confers on the anammox process high resistance to temperature, pH or oxygen changes (Vázquez-Padín *et al.* 2009). In the case of SBR-2, where large granules were obtained at high DO concentrations, the abundance of AOB was higher than in SBR-1. Vázquez-Padín *et al.* (2010b) determined the stratification in depth of the AOB and anammox bacteria inside similar granules to those obtained in the present research work. In the outermost 200 μm layer of their granules almost all the biomass consisted of *Nitrosomonas*, while anammox bacteria (belonging mainly to the genus *Candidatus* Kuenenia stuttgartiensis) were located between 400 and 1,000 μm

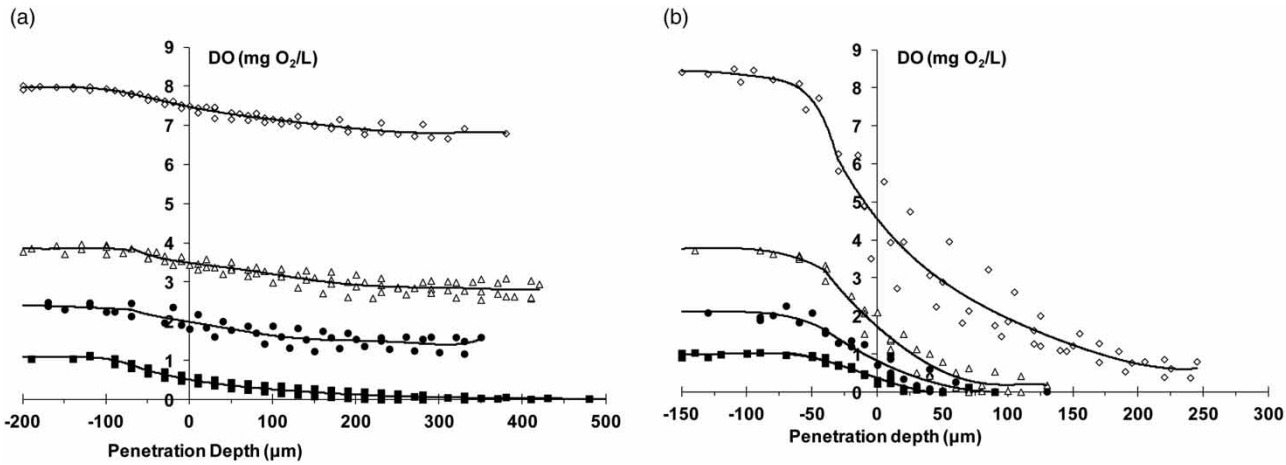

Figure 4 | DO profiles performed at different DO concentrations in the bulk liquid (around 8 mg O_2/L (◇), 4 mg O_2/L (Δ), 2 mg O_2/L (●) and 1 mg O_2/L (■)) in (a) granules from SBR-1 and (b) granules from SBR-2.

in depth inside the granules, where DO was absent during normal reactor operation. DO concentration in their reactor was larger than that used in the SBRs of the present research study, consequently the anoxic layer and the anammox bacteria in the granule were found in deeper layers.

Microsensors together with microbiological analyses provide useful information about the oxygen penetration depth, the distribution of the aerobic and anoxic zones within the granules and the present bacterial populations, useful to understand the operation of the ELAN® process at macroscopic scale.

CONCLUSIONS

Oxygen mass transfer determines the balance between AOB and anammox bacteria in ELAN® systems. Comparable nitrogen removal rates of 0.6 g N/(L d) were obtained in reactors started-up with completely different DO concentrations in the bulk liquid (0.4 and 3.0 mg O_2/L).

Despite obtaining similar nitrogen removal rates, the biomass aspect and morphology was completely different. While in SBR-1 the biomass was mainly composed by flocs with a small proportion of granules (with 1.1 mm of diameter); SBR-2 was composed mainly of granules with an average diameter of 2.3 mm.

The biomass of SBR-2 provides the system with more stability and robustness since the outer aerobic layer, where AOB *Nitrosomonas* are present, is thicker and might cope with loads and oxygen supply variations throughout operation. Microsensor measurements corroborate these results indicating that the granules were not oxygen fully penetrated and an anoxic core, where anammox *Brocadia fulgida* were present, was guaranteed in the granules' interior.

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