Biofilm and granular systems to improve Anammox biomass retention

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Abstract
Appropriate biomass retention in reactor systems is a crucial factor for the accurate operation of the Anammox process due to the slow growth rate of this bacterial population. In the present study two different approaches were studied and compared to improve Anammox biomass retention minimizing wash-out events: 1) formation of granular biomass using influents with high inorganic salts concentrations by production of saline precipitates acting as promoters for biomass aggregation (reactor SBR1); 2) use of zeolite particles as carrier material for Anammox biofilm formation (reactor SBRZ). Both alternatives allowed the reduction of biomass wash-out in the effluent to values as low as 18 mg VSS/L (SBR1) and 6 mg VSS/L (SBRZ). As a consequence the biomass concentration increased significantly inside each reactor. In the case of the SBRZ the specific Anammox activity (SAA) of the biomass was also enhanced increasing from 0.33 to 0.54 g N/(g VSS·d). Although both approaches allow the improvement of the biomass retention the precipitation option is recommended for waters with high salt content and for the rest the zeolite addition gives even better results than the previous one.

Keywords
Anammox; biofilm; granule; precipitation; sequencing batch reactors; solids retention; zeolite.

INTRODUCTION

The removal of the nitrogen present both in municipal and industrial wastewaters (basically as ammonium) is carried out conventionally by means of nitrification and denitrification processes. This procedure is suitable for the treatment of strong nitrogenous wastewaters rich in biodegradable carbon, but it results expensive for the treatment of wastewaters with low carbon to nitrogen (C/N) ratios, like rejection waters from anaerobic sludge digestion. The treatment of these effluents involves important amounts of dissolved oxygen for nitrification and addition of an external source of organic matter for the denitrification step with the consequent increase of operational costs. The use of a combining system composed by a process of partial nitrification of ammonium to nitrite and the Anammox process leads to a reduction of costs. The Anammox (ANaerobic AMMonium Oxidation) is an autotrophic process which consists in a combination of ammonium and nitrite by Planctomycete-type bacteria under anoxic conditions to generate nitrogen gas (Strous *et al*., 1999).

The application of the Anammox process is limited by its long start-up periods due to very low growth rates and biomass yields of the involved biomass (Jetten *et al.*, 1997). Minimizing the wash-out of biomass in the effluent by improving its retention becomes critical when biomass with so long duplication time (0.003 h^{-1}, Strous *et al.*, 1998) is used. Systems where the improvement of biomass retention is achieved reduce the duration of start up period and provide better conditions to implant the Anammox process at industrial scale. In reactors where biomass grows in form of biofilms or granules the formation of compact aggregates increases the settling velocity of the biomass and improves its retention and the amount of biomass growing in suspension is minimized.

One of the contributing factors to the development of biofilms from suspended sludge is the presence of nuclei or bio-carriers for microbial attachment. The attachment of cells to these particles has been proposed as the initiation step for biofilm formation. Since the second step was the formation of a dense and thick biofilm on the cluster of the inert carriers. To enhance sludge aggregation the inert materials must have some properties, such as, a high specific surface area, good hydrophobicity and spherical shape (Yu *et al*., 1999). The addition of zeolite particles as support material in reactors containing suspended biomass seemed to be effective in promoting the formation of anaerobic granules (Hulshoff-Pol, 1989). Besides, natural zeolites (as clinoptilolite) are widely used around the world as selective ionic exchangers capable of removing ammonium ion from waste waters. Recently some researchers (Lahav and Green, 2000; Lee *et al*., 2001; Yang, 1997) have demonstrated that zeolite particles improve the operation of nitrification reactors. This improvement affects to both the sedimentation properties of the biomass and the performance of ammonium removal. Considering these facts and the similarities between nitrification and Anammox processes regarding the slow growth of both groups of bacteria, it is supposed that using clinoptilolite as carrier material for attached growth of Anammox biomass could improve the start up and operation of an Anammox reactor.

The aggregation of biomass can be promoted also by means of favouring the precipitation of inorganic salts. According to the DLVO (Derjaguin, Landau, Verwey, and Overbeek) theory when the two surfaces carry a charge of the same sign, there is a free energy barrier between them which acts as a repulsive force. This force seriously prevents the approach of one cell to another. The increase of the ionic strength of the medium would decrease the electrical repulsion, by compressing the double layer, and could initiate cell-to-cell interaction. Therefore, an increase of the salinity can favour the granulation. Moreover, the solubility product is also influenced by the salinity of the medium and its increase would enhance the precipitation of the salts less soluble which could act as nuclei for granulation.

In the present work two approaches to improve the retention of Anammox biomass in sequencing batch reactors were tested to promote the biomass aggregation using:

a) high inorganic salt concentrations in feeding medium to provoke precipitation,
b) zeolite particles as carrier material.

MATERIALS AND METHODS

Experimental set-up

Experiments were carried out in two Sequencing Batch Reactors of 2 L (SBR1) and 5 L (SBRZ) of useful volumes, respectively. Temperature was controlled at 33 ºC by using a thermostatic jacket and pH was not controlled and ranged between 7 and 8. The complete mixture inside the reactors was achieved using mechanical stirrers with a rotating speed of 100 rpm. The control of the pumps and different periods of the operational cycles was performed with a PLC system.

Zeolite (10 g/L) was added as support material to SBRZ. The added zeolite was clinoptilolite (ZeoCat, Spain) with a 96 % degree of purity and initial particle sizes between 0 and 1 mm. A separation by size was performed by sieving and particles with sizes between 0.5 and 1.0 mm were collected. Smaller sizes are useless due to its slow settling rate.

Feeding media and operational strategy

Both reactors were fed with a synthetic autotrophic medium described by Dapena-Mora *et al*. (2004a). The ammonium to nitrite molar ratio in the feeding media was fixed at 1.

The SBR1 was feed according to the media described in Table 1 in three different operational periods. The Nitrogen Loading Rate (NLR) applied was kept constant at 0.4 g N/(L·d) and the salinity of the feeding media was increased during the operation (Table 1). The settleable solids present in the effluent were returned to the reactor. In the case of SBRZ, the applied NLR was constant during periods I and II and in period III it was stepwise increased according to the biomass removal capacity. No biomass from the effluent was returned to the reactor.

Both reactors were operated in cycles of 6 hours distributed in four periods: mixed fill (300 min), mix (30 min), settle (15 min) and draw (15 min) according to Dapena-Mora *et al*. (2004b). The exchange volume was fixed at 12.5 and 25% for SBR1 and SBRZ, respectively. The Hydraulic Retention Time (HRT) was maintained at 2 days in the SBR1 and 1 day in the SBRZ.

Table 1. Characteristics of the feeding media used for both reactors in the different operational periods.

Reactor	Periods	Days	NaCl (g/L)	Zeolite	NH_4^+-N (mg/L)	NO_2^--N (mg/L)	NLR (g N/(L·d))
SBR1	I	0-18	0	---	400	400	0.4
	II	18-50	5	---	400	400	0.4
	III	50-80	10	---	400	400	0.4
SBRZ	I	0-13	---	No	30	30	0.06
	II	13-50	---	Yes	30	30	0.06
	III	50-160	---	Yes	30-300	30-300	0.06-0.60

Inocula

Both reactors were inoculated with enriched Anammox sludge from a laboratory scale SBR operated at the University of Santiago of Compostela (Dapena-Mora *et al*., 2004a). The initial concentrations of biomass were 1.0 and 0.24 g VSS/L for SBR1 and SBRZ, respectively. The initial specific Anammox activities were 0.4 g N/(g VSS·d) and 0.35 g N/(g VSS·d) for the biomass of SBR1 and SBRZ, respectively.

Specific Anammox Activity (SAA) tests

Batch experiments to determine the SAA were performed according to the methodology described by Buys *et al.* (2000) and the modification made by Dapena-Mora *et al.* (2004c), based on the measurement along time of the overpressure generated in closed vials by the nitrogen gas produced.

Analytical methods

Nitrate, nitrite and ammonium concentrations were determined spectrophotometrically (APHA, 1985). The concentrations of solids, determined as Total Suspended Solids (TSS), the fraction corresponding to the biomass as Volatile Suspended Solids (VSS), and the sludge volumetric index (SVI) of the sludge were determined according to standard methods (APHA, 1989).

The distribution of particle size was measured using an Image Analysis procedure. Images of the granules were taken with a digital camera (Coolsnap, Roper Scientific Photometrics) combined with a stereomicroscope (ZMZ-2T, Nikon). For the digital image analysis the programme Image ProPlus was used. Specifically the programme served to calculate the average feret diameter of the granules. The elemental analysis of the surface of the biomass aggregates was made using a transmission electron microscope (TEM) (PHILIPS CM12).

RESULTS AND DISCUSSION

Reactors operation

The experiments in SBR1 were performed during 72 days. During the three operational periods the nitrite (limiting substrate) was almost fully depleted along the cycle and the concentration of ammonium in the effluent was practically constant at a value of 100 mg NH_4^+-N/L. The nitrogen loading rate (NLR) applied during the whole period was around 0.4 g N/(L·d). No significant effect was observed in the effluent composition due to the presence of tested NaCl concentrations (5 and 10 g NaCl/L) in the feeding media.

The SBRZ was initially operated during 13 days (Period I) to obtain stable operation conditions and complete nitrite removal. On day 13, zeolite (10 g/L) was added to the reactor and the applied NLR was maintained constant at a value of 0.06 g N/(L·d) (Period II). From day 50, the applied NLR rate was stepwise increased up to 0.6 g N/(L·d) (Period III).

In order to prove that both systems were not overloaded the Specific Nitrogen Loading Rate (SNLR) applied to the reactor and the Specific Anammox Activity (SAA) of the biomass were compared following the same procedure as Dapena-Mora *et al*. (2004d). The SNLR applied to SBR1 during the operational period was always below the maximum specific activity of the biomass which ranged between 0.35 and 0.45 g N/(g VSS·d) (Figure 1A). This fact indicates the system was operated close to be overloaded only during Period I. In the rest of the operational periods not only no biomass wash-out was observed but also an important increase of the biomass concentration was measured. The SAA of the biomass decreased from 0.4 to 0.35 g N/(g VSS·d) when 5 g/L of NaCl were added but when the concentration of salt was increased to 10 g/L the SAA increased to 0.45 g N/(g VSS·d) which is slightly higher than the initial value (Figure 1A). These changes of the SAA values are not correlated to the salinity of the medium and could be due to the necessity of an adaptation period.

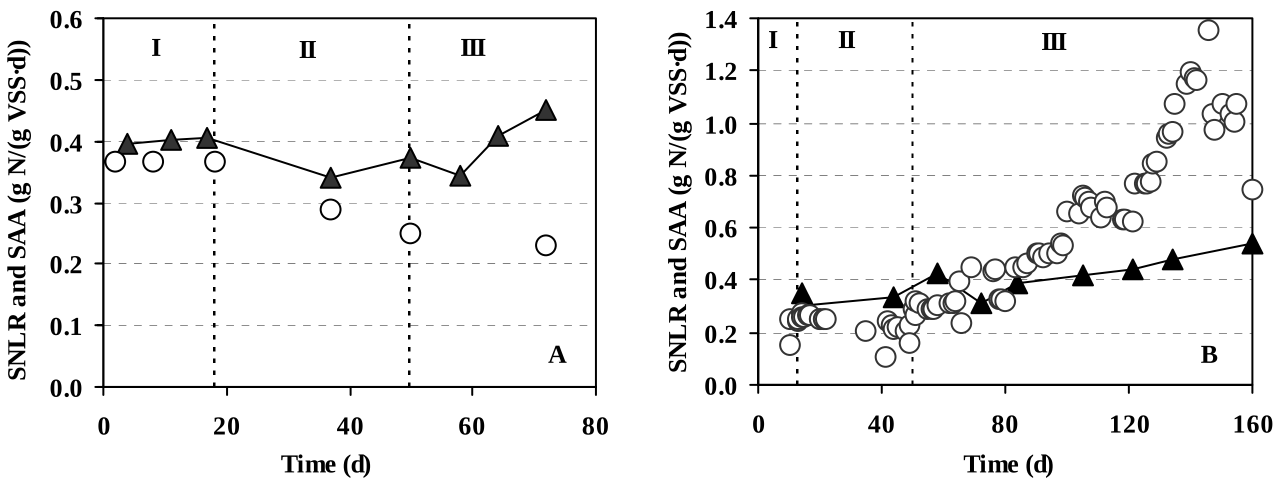


Figure 1. Specific Nitrogen Loading Rate (SNLR) applied (○) and Specific Anammox Activity of the biomass (SAA) (▲) in reactors SBR1 A) and SBRZ B).

In case of SBRZ the SAA measured of the biomass was lower than the SNLR applied (Figure 1B) which could be an indication of overload stages. But this could be attributed to the difficulty of collection of homogeneous biomass samples due to biomass stratification inside the reactor, which led to not realistic values of SNLR. The SAA experienced an increase from 0.33 to 0.54 g N/(g VSS·d) without and with zeolite presence in the reactor, respectively. On day 80, the stirring speed was increased from 100 to 180 rpm in order to improve the fluidization of zeolite particles. This fact increased the shear forces on biomass which might be related to the decrease of the SAA.

Biomass retention

The biomass concentration was constant in reactor SBR1 (Figure 2A) during Period I which was an indication of the existing balance between production and wash-out of biomass. Once the NaCl was added in the feeding (Periods II and III) the biomass concentration increased steadily in 0.6 g VSS/L within 54 days. The productivity of Anammox sludge obtained from the stoichiometry of the process given by Strous was 0.038 g VSS produced/g NH_4^+-N consumed (Strous *et al.*, 1998). Therefore, 0.82 g VSS would have been produced during this interval of operation of the reactor. Assuming that the decay of the biomass is neglected the amount of biomass retained in the reactor was the 73 % of the stoichiometrically produced amount.

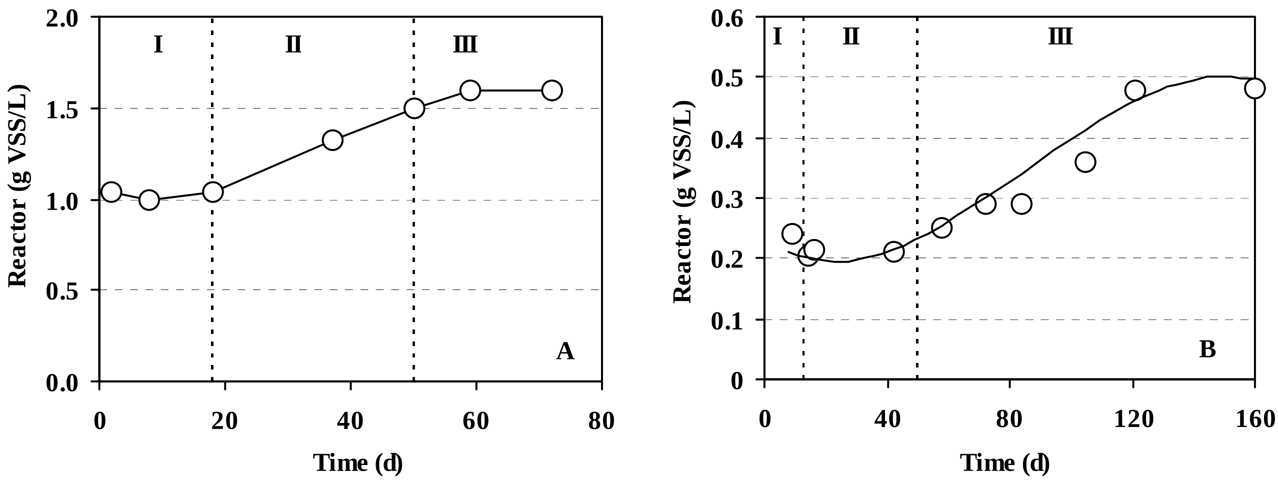


Figure 2. A) Concentration of biomass (g VSS/L) (○) in SBR1 A) and SBRZ B).

In case of reactor SBRZ the biomass concentration was maintained constant (around 0.2 g VSS/L) during Periods I and II but an increase of the applied NLR during Period III caused an increase up to 0.48 g VSS/L (Figure 2B). As it was mentioned before these results must be read with care due to the difficulty of biomass sampling.

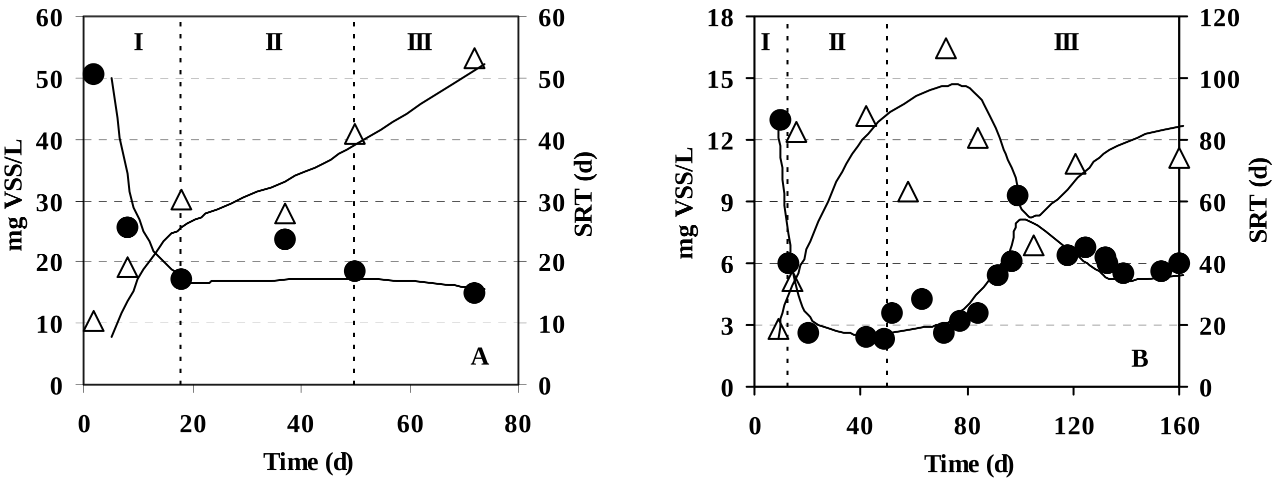


Figure 3. Biomass concentration in the effluent (mg VSS/L) (●) and sludge retention time (SRT, d) (Δ) in reactors SBR1 A) and SBRZ B).

In both cases after NaCl addition in SBR1 and zeolite addition in SBRZ the concentration of solids in the effluent decreased significantly and as a consequence SRT values of 50 and 75 days, respectively were obtained (Figures 3A and 3B). The addition of zeolite caused a decrease on the biomass concentration in the effluent from 13 to 3 mg VSS/L which supposed an increase of the SRT from 19 to 90 days.

Reasons for the improvement of biomass retention

In order to establish the different phenomena involved in the improvement of the biomass in each reactor the characteristics of the obtained biomass aggregates were analysed.

In the SBR1 the percentage of inorganic fraction in the biomass increased from the 10% in the period I until 21% in period III. This inorganic fraction is attributed to the precipitation of inorganic salts due to the NaCl addition. The precipitation was achieved increasing the salinity of the media and therefore varying the equilibrium and the solubility of the products. Elemental analysis of the precipitates present in the surface of the granules indicated that they were mainly composed by Ca_3PO_4 which is characterized by its very low solubility. These precipitates could easily act as precursors for the formation of Anammox granules, being a support material where the biomass could attach, or this precipitation could also occur directly on the surface of already formed aggregates. Thus the improvement of the settleability of the granules can be directly related to this inorganic precipitation which caused an increase of the density of the aggregates producing sludge with good sludge volumetric index (SVI) values. Although the precipitates play an important role as support material, in the conditions described it can also represent a problem if it occurs in a large extension on the surface of already formed aggregates. In such a way, if the amount of formed precipitates is too high the whole surface of the aggregates can be covered and the activity of the biomass reduced due to the increase of diffusional resistance. Concerning the biomass physical characteristics, the feret diameter increased from 0.47 to 0.58 mm (24% of increase) and the Sludge Volume Index (SVI) decreased from 120 to 50 mL/g VSS indicating the compacting of the biomass (Figure 4A). Therefore, the capacity of the system to retain biomass increased.

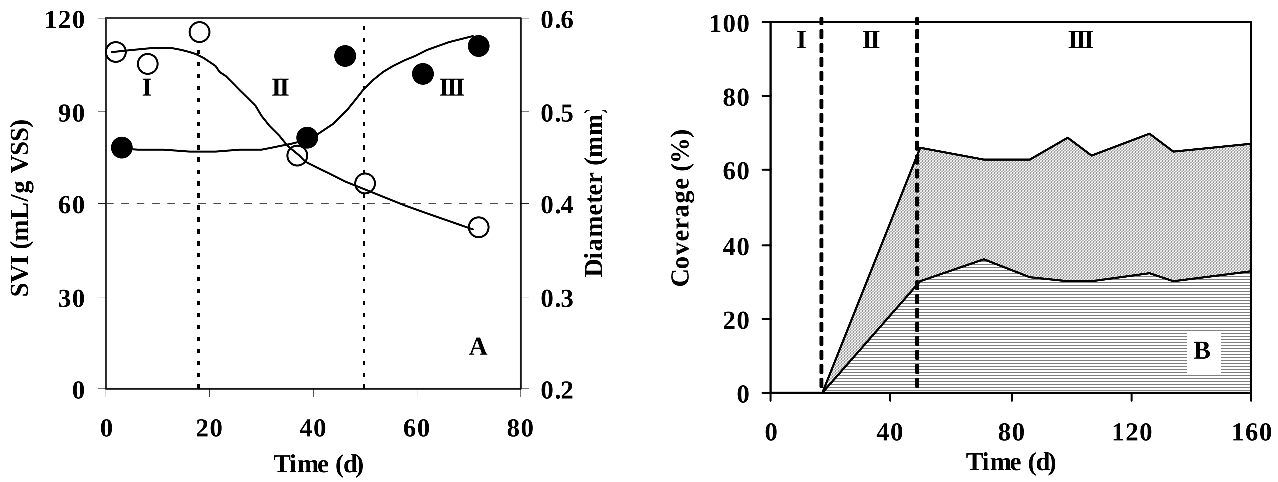


Figure 4. A) Sludge volumetric index (SVI) (○) and average feret diameter (●). B) Percentages of zeolite fully covered, partially covered and no covered.

In the case of SBRZ once the particles of zeolite were added, a biofilm started to growth attached to their surfaces. Percentages of 30 and 36% for fully and partially covered particles, respectively were achieved (Figure 4B). Nevertheless, biofilm formation on the zeolites surfaces was only observed during Period II and the increase of the applied NLR seemed to favour the formation of granules without the use of zeolite as nuclei. The formation of an incipient biofilm around the carrier particles was observed.

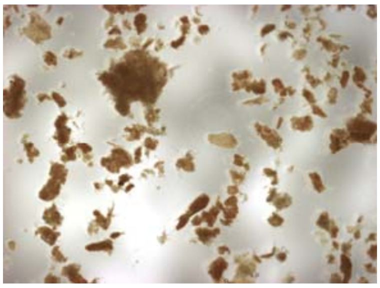
Day 1 1x	Day 65 1x	Day 72 1x
		

Figure 5. Stereomicroscope images of biomass in the reactor SBR1 in different operational days.

Visual observation of the samples of reactor SBR1 indicated that during period I the biomass forming small flocs and in suspension was predominant (Figure 5). During periods II and III no biomass in suspension was observed and the small flocs became aggregates. This behaviour was already observed by Campos *et al*. (2002) working with nitrifying sludge. In the case of SBRZ the amount of suspended biomass was neglected and the formation of biofilm growth on the surfaces of the zeolite particles was observed (shown with arrows in Figure 6). In this reactor SBRZ the particle size measured as average feret diameter was almost constant around 0.6 mm.

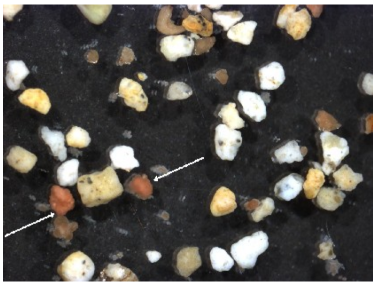
Native. 50 x	Operation day 175. 10 x
	

Figure 6. Stereomicroscopic observation of a native zeolite particle and zeolite particles after 150 days inside the reactor SBRZ.

Application of both strategies

The external addition of salt to enhance granulation of Anammox biomass might not be feasible at industrial scale due the cost of reagents. But the use of wastewater containing high salt concentrations is a possibility. In the industry effluents with high salt content are relatively common, as those produced in the fish canning industry, due to the use of sea water during the manufacturing processes. These effluents are firstly treated in an anaerobic digestion stage and then a treatment for nitrogen removal is necessary. Therefore, the application of the Anammox process for their treatment could be advisable together with the use of the salt content to promote precipitates and to produce good settling aggregates. Although the promotion of precipitates must be carefully controlled to avoid the precipitation of extremely high amounts of salts, which could diminish the activity of the Anammox biomass as it was previously observed in the case anaerobic sludge (Van Langerak *et al.*, 1998).

The use of support materials for biomass growth is relatively common. Zeolite particles have been already used in nitrifying and anaerobic systems as carrier material due to its low cost. Zeolites are very suitable for biomass attachment but they must be treated with caution due to their high density. When particles are not fully covered (start-up of the process) the homogeneous distribution of biomass, without stratification in height in the reactor, requires the application of strong mixture forces. Furthermore this important mixture effort involves the use of high liquid or gas flows or high stirring speeds which can cause negative effects over the biomass attachment, due to the high shear stress provoked by the abrasion produced by the impact between bare zeolite particles, or the biomass specific activity. Once the particles are fully covered with biomass this problem disappeared due to the decrease of global particle density.

From the results obtained in the present work, the addition of zeolite particles to an Anammox SBR seems to have some advantages compared to the operation of a similar SBR with addition of salts to promote the formation of inorganic precipitates: 1) The SBRZ allowed a higher enrichment of the Anammox biomass (higher SAA) and 2) the averaged estimated SRT was around 80 days while the SBR with salt addition achieved a maximum SRT value of around 50 days. Moreover, the addition of zeolite had a clear effect on the decrease of biomass concentration in the effluent which is a crucial matter when the start up is carried out in a reactor inoculated with biomass containing a very low fraction of Anammox population.

CONCLUSIONS

Improvement of the Anammox biomass retention, indicated by an increase of biomass concentration inside SBR, was achieved following two alternative paths: via dissolved salt (NaCl) addition for precipitate production and via addition of an external support material (zeolite).

The increase of biomass retention was correlated to an improvement of biomass settling properties (lower SVI values and bigger average diameters) in SBR1 while in the SBRZ was correlated to biomass attachment. In both cases reduction of biomass growth in suspension was observed.

The SAA of the biomass experienced slight variations in the presence of NaCl concentrations but it increased due to the addition of zeolite particles.

It is demonstrated that both alternatives are valid and the choice of one or another depends on the characteristics of the water to be treated.

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REFERENCES

APHA-AWWA-WPCF. (1989). Standard Methods for examination of water and wastewater. 17th Ed. Washington.

Buys B.R., Mosquera-Corral A., Sánchez M. and Méndez R. (2000). Development and application of a denitrification test based on gas production. *Water Science and Technology*, **41**(12), 113-120.

Campos J.L., Mosquera-Corral A., Sánchez M., Méndez R. and Lema J. M. (2002). Nitrification in saline wastewater with high ammonia concentration in an activated sludge unit. *Water Research*, **36**(10), 2555-2560.

Dapena-Mora A., Van Hulle S., Campos J.L., Méndez R., Vanrolleghem P.A. and Jetten M.S.M. (2004a). Enrichment of Anammox biomass from municipal activated sludge: experimental and modelling results. *Journal of Chemical Technology and Biotechnology*, **79**, 1421-1428.

Dapena-Mora A., Arrojo B., Campos J.L., Mosquera-Corral A. and Méndez R. (2004b). Improvement of the settling properties of Anammox sludge in an SBR. Journal of *Chemical Technology and Biotechnology*. **79**, 1417–1420.

Dapena-Mora A., Campos J.L., Mosquera-Corral A. and Méndez R. (2004c). Development and application of an Anammox activity test based on gas production. *European Symposium on Environmental Biotechnology, ESEB 2004*, ISBN 90 5809 653 X, 649-652.

Dapena-Mora A., Campos J.L., Mosquera-Corral A., Jetten M.S.M. and Méndez R. (2004d). Stability of the ANAMMOX process in a gas-lift reactor and a SBR. *Journal of Biotechnology* **110**, 159-170.

Hulshoff-Pol LW.(1989). The phenomenon of granulation of anaerobic sludge. Ph.D. Thesis, Agricultural University of Wageningen, The Netherlands: Wageningen.

Jetten M.S.M., Horn S.J. and van Loosdrecht M.C.M. (1997). Towards a more sustainable municipal wastewater treatment system. *Water Science and Technology*, **35**, 171-180.

Lahav O. and Green, M. (2000). Ammonium removal from primary and secondary effluents using a bioregenerated ion-exchange process. *Water Science and Technology*, 42(1-2), 179–185.

Lee J.C., Kim J.S., Kang I.J., Cho M.H., Park P.K. and Lee C.H. (2001). Potential and limitations of alum or zeolite addition to improve the performance of a submerged membrane bioreactor. *Water Science and Technology*, **43**(11), 59–66.

Strous M., Heijnen J.J., Kuenen J.G. and Jetten M. (1998). The sequencing batch reactor as a powerful tool for the study of slowly growing anaerobic ammonium-oxidizing microorganisms. *Applied Microbiology and Biotechnology,* **50**, 589-596.

Strous M., Kuenen J.G. and Jetten M. (1999). Key physiological parameters of anaerobic ammonium oxidation. *Applied Microbiology and Biotechnology,* **65**, 3248-3250.

van Langerak E.P.A., Gonzalez-Gil G., Van Aelst A., Van Lier J.B., Hamelers H.V.M. and Lettinga G. (1998). Effects of high calcium concentration on the development of methanogenic sludge in upflow anaerobic sludge bed (UASB) reactors. *Water Research*, **32**(4), 1255-1263.

Yang L. (1997). Investigation of nitrification by co-inmobilized nitrifying bacteria and zeolite in a batchwise fluidized bed. *Water Science and Technology,* **35**(8), 169–175.

Yu H.Q., Tay J.H. and Fang H.H.P. (1999). Effects of added powdered and granular activated carbons on start-up performance of UASB reactors. *Environmental Technology,* **20**, 1095–101.