Autotrophic nitrogen removal at low temperature
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ABSTRACT
In this work the autotrophic nitrogen removal was carried out at moderately low temperatures using two configurations: a) two-units one comprising a SHARON reactor coupled to an Anammox SBR and b) single-unit one consisting of a granular SBR performing the CANON process. At 20 °C the two-units system was limited by the Anammox step and its nitrogen removal capacity was around ten times lower than the CANON system (0.08 g N/(L d) versus 1 g N/(L d)). When the CANON system was operated at 15 °C the average removed nitrogen loading rate decreased to 0.2 g N/(L d). The CANON system was operated in order to limit the ammonia oxidation rate to avoid nitrite inhibition of Anammox bacteria. Since both, temperature and dissolved oxygen (DO) concentration regulate ammonia oxidizing bacteria activity, once the temperature of the reactor is decreased the DO concentration must be decreased to avoid the deeper oxygen penetration inside the granule which could cause inhibition of Anammox bacteria by oxygen and/or nitrite.

Key words | aerobic granule, ammonia oxidizing bacteria (AOB), anammox, CANON, SBR

INTRODUCTION
The ongoing research works on the anammox based process are focused on the improvement of the nitrogen removal in WWTPs. The application of the combined partial nitrification/anammox processes to remove nitrogen presents several advantages in comparison to the conventional nitrification/denitrification pathway: 1) saving up aeration costs since only half of the ammonium has to be oxidized to nitrite; 2) no organic matter is needed which makes this strategy interesting to treat wastewater without biodegradable organic carbon source or with low COD/N ratio; 3) less sludge is produced in comparison to the nitrification/denitrification strategy and the partial nitrification/denitrification process, which involves a cost reduction taking into account that the treatment of the sludge generated represents up to 50–60% of the WWTP budget; 4) less CO₂, N₂O, etc. are released to the atmosphere when using anammox based processes; 5) the introduction of anammox based processes drives the WWTPs closer to the energy autarky by removing the nitrogen from reject water, which can contain up to 25% of the total nitrogen load, and leaving more COD available to produce methane in the anaerobic digester (Siegrist et al. 2008).

The crucial aspect of the autotrophic nitrogen removal is the development of a critic mass of anammox bacteria due to their slow growth rate. To overcome this drawback good biomass retention capacity of the reactors and the optimization of the operational conditions favoring the Anammox process are mandatory. Two different configurations can be applied to combine partial-nitrification and anammox processes: a two reactor configuration (Hellinga et al. 1998) and a one reactor configuration (Sliekers et al. 2003). Since the growth rate of both ammonia oxidizers and anammox bacteria is very low, the use of granular systems is an interesting alternative to maintain a high sludge retention time in order to enhance the performance of the reactors (Fernández et al. 2008).

These processes were mainly applied to effluents with temperatures around 30 °C (van der Star et al. 2007). Such temperatures allow an optimal growth of anammox bacteria (Strous et al. 1999) and also the achievement of partial nitrification (Hellinga et al. 1998), due to the higher activation energy of ammonia oxidizing bacteria (AOB) compared to nitrite oxidizing bacteria (NOB) which makes the former
grow faster than the latter at temperatures above 25 °C. However, recent works showed that both, partial nitrification and anammox processes (Dosta et al. 2008; Vázquez-Padín et al. 2009; Qiao et al. 2010) the deammonification process (Wett et al. 2010) could be successfully operated at temperatures around 20 °C or even at lower temperatures in marine environments (Rysgaard et al. 2004).

This observation will open the possibility to apply autotrophic processes to remove nitrogen from effluents of psychrophilic anaerobic digesters and landfill leachates. Furthermore Anammox based processes could be used in the main stream of WWTPs to remove all the nitrogen from the wastewater. In this way almost all the COD could be used to produce methane.

The aim of this work was to carry out the autotrophic nitrogen removal at low temperatures using both, a single-unit and two-units configurations. In the two-units configuration the partial nitrification process was carried out in a nitrifying granular SBR and its effluent was fed to an Anammox SBR. In the case of the single unit configuration, the previous granular SBR was used after the development of Anammox biomass in the inner core of the nitrifying granules.

**MATERIALS AND METHODS**

**Reactors description**

**Granular SBR**

A SBR with a working volume of 1.5 L was used. Dimensions of the unit were: height of 465 mm and inner diameter of 85 mm, the height to the diameter ratio being 5.5. The exchange volume was fixed at 50%. The hydraulic retention time was fixed at 0.25 d. The duration of the operational cycles was of 3 hours distributed according to: 173 minutes of aeration and feeding, 1 minute of settling time and 6 minutes of effluent withdrawal. A thermostated bath was installed to control the temperature. Air was supplied to the bottom of the reactor by using an air pump to promote the transfer of oxygen into the bulk liquid and to reach a suitable mixing. The concentration of dissolved oxygen (DO) in the liquid phase was regulated by changing the ratio of fresh air to recycled air injected in the reactor. A pH controller CRISON PH28 maintained the pH value between 7.5 and 8.0.

The nitrifying granular biomass operated in this reactor was obtained from heterotrophic aerobic granules by the stepwise decrease of the COD/N ratio in the influent (Mosquera-Corral et al. 2005).

The nitrifying granular reactor was fed with the supernatant of an anaerobic sludge digester of the WWTP of Lugo (Spain) which was collected every month in 20 L containers and stored in a cold room (4 °C). The composition of the supernatant was: pH 7.5–8.3; NH₄⁺ 400–700 mg N/L; total inorganic carbon 300–505 mg IC/L and total organic carbon 20–50 mg TOC/L. The reactor operation was divided into nine different stages (Table 1) by varying the dilution of the supernatant with tap water from 90% to 0%. The effluent of the granular SBR was fed to the Anammox reactor during stage II.

**Anammox reactor**

A SBR with an effective volume of 1 L was used to carry out the Anammox process. The SBR was provided with a thermostatic

<table>
<thead>
<tr>
<th>Stage</th>
<th>Configuration</th>
<th>Days</th>
<th>DO (mg O₂/L)</th>
<th>T (°C)</th>
<th>Influent dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>——-</td>
<td>0–64</td>
<td>2.2±0.4</td>
<td>20</td>
<td>50%</td>
</tr>
<tr>
<td>II</td>
<td>Two-units</td>
<td>65–120</td>
<td>2.7±0.3</td>
<td>20</td>
<td>50%</td>
</tr>
<tr>
<td>III</td>
<td>Single-unit</td>
<td>121–243</td>
<td>2.7±0.5</td>
<td>20</td>
<td>50%</td>
</tr>
<tr>
<td>IV</td>
<td>Single-unit</td>
<td>244–287</td>
<td>3.5±0.5</td>
<td>20</td>
<td>50%</td>
</tr>
<tr>
<td>V</td>
<td>Single-unit</td>
<td>288–360</td>
<td>3.5–4.6</td>
<td>20</td>
<td>50–0%</td>
</tr>
<tr>
<td>VI</td>
<td>Single-unit</td>
<td>361–400</td>
<td>2.7±0.5</td>
<td>20</td>
<td>0%</td>
</tr>
<tr>
<td>VII</td>
<td>Single-unit</td>
<td>401–452</td>
<td>2.8–0.5–3.1</td>
<td>20</td>
<td>0–90% → 50%</td>
</tr>
<tr>
<td>VIII</td>
<td>Single-unit</td>
<td>453–1009</td>
<td>3.1±0.9</td>
<td>20</td>
<td>50%</td>
</tr>
<tr>
<td>IX</td>
<td>Single-unit</td>
<td>1010–1120</td>
<td>2.1±1.0</td>
<td>15</td>
<td>50%</td>
</tr>
</tbody>
</table>
jacket to keep the temperature at 20 ± 1°C. The pH value was not controlled and remained around 7.5. Complete mixture inside the reactor was achieved with a mechanical stirrer at 100 rpm. The SBR was operated in cycles of 6 hours according to Dapena-Mora et al. (2004) with 300 minutes of mixed feeding, 30 minutes of mixing, 20 minutes of settling and 10 minutes of effluent withdrawal. The exchange volume was of 25% and the hydraulic retention time was fixed at 1 d. The effluent from the nitrifying granular SBR was collected and stored in a cold room (4°C) prior to be feed the Anammox reactor.

The used Anammox biomass was developed inside the reactor at 30°C and fed with synthetic wastewater (Dapena-Mora et al. 2004). The biomass concentration inside the reactor was of 1.5 g VSS/L and its Anammox specific activity was of 0.28 g N/(g VSS d) at 30°C.

The operational strategy followed in this work was similar to that reported by Dosta et al. (2008). The temperature of the Anammox reactor was initially 30°C and it was stepwise decreased down to 20°C to allow the acclimatization of the biomass. Once the temperature of the reactor was decreased to 20°C and the operation stabilized, the effluent of the granular SBR was fed to the Anammox reactor as aforementioned.

Calculations

**Nitrogen loading rates**

The values of the nitrogen loading rate applied to the reactor (NLR_{applied}) and the nitrogen loading rate removed by Anammox bacteria (NLR_{removed}) were estimated as g N/(L d) based on nitrogen balances and the stoichiometry of the Anammox process [1, 3]:

\[ NLR_{applied} = \frac{\text{NH}_4^+ - N_{inf}}{\text{HRT}} \]  

(1)

\[ \Delta N = (\text{NH}_4^+ - N_{inf}) - ((\text{NH}_4^+ - N_{eff}) + (\text{NO}_2^- - N_{eff})) \]  

(2)

\[ NLR_{removed} = \frac{\Delta N}{\text{HRT}} \]  

(3)

being \text{NH}_4^+ - N_{inf} the ammonium concentration in the influent (mg N/L) and \text{NH}_4^+ - N_{eff}, \text{NO}_2^- - N_{eff}, \text{NO}_3^- - N_{eff} the ammonium, nitrite and nitrate concentrations in the effluent (mg N/L), respectively.

**Ammonia oxidation rate**

The ammonia oxidation rate in the single-unit system was always limited by the DO concentration and it can be calculated according to Equation (4):

\[ (-r_{NH_4}) = \frac{1}{r_{O_2/NH_4}} \cdot \frac{2}{R_p} \cdot \frac{\sqrt{D_{O_2} \cdot C_{O_2} \cdot r_{max} \cdot O_2}}{\rho_b} \]  

(4)

where \((-r_{NH_4})\) is the specific consumption rate of ammonia (g NH_4^+ -N/(g VSS d)); \(v_{O2/NH_4}\) the ratio of stoichiometric coefficients of both oxygen and ammonia for partial nitrification (3.42 g O_2/g NH_4^+ -N); \(R_p\) the particle radius (m); \(D_{O2}\) the diffusion coefficient of oxygen through the biofilm (m^2/d); \(C_{O2}\) the oxygen concentration in the bulk liquid (g O_2/L); \(r_{max}O_2\) the maximum specific consumption rate of oxygen (g O_2/(g VSS d)); and \(\rho_b\) the biomass density (g VSS/L_granule).

Therefore, when the operational conditions such as particles radius, dissolved oxygen concentration and/or temperature change, the ratio between the rate corresponding to the initial operational conditions and that that corresponding to the final conditions is given by Equation (5):

\[ \frac{(-r_{NH_4})_1}{(-r_{NH_4})_2} = \frac{R_{p1}}{R_{p2}} \cdot \frac{C_{O2,2}}{C_{O2,1}} \cdot e^{\frac{-E_a}{R} \cdot \frac{1}{T_1} - \frac{1}{T_2}} \]  

(5)

where \(E_a\) is the activation energy (kJ/mol); \(R\) the ideal gas constant (kJ/(mol K)); and \(T\) the temperature (K).

**Analytical procedures**

The pH and the concentrations of DO, ammonia, nitrite, nitrate, biomass concentration as volatile suspended solids (VSS), inorganic suspended solids, total suspended solids and sludge volumetric index were determined according to the Standard Methods (Standard Methods for the Examination of Water and Wastewater 1998). FISH analyses were carried out according to the methodology described by Figueroa et al. (2006).

**RESULTS AND DISCUSSION**

"Two-units" configuration

The granular SBR was operated as a partial nitrifying system during 120 days at a temperature of 20°C. Up to now when partial nitrification was developed to be combined to the anammox process, the SHARON process was selected which operates at around 30°C. Comparing the operation of the granular SBR to that of a SHARON reactor it is expected that the biomass activity decreases when the operation
temperature goes from 30°C in a SHARON reactor to 20°C in the present case. If the maximum activity value obtained is assigned to that corresponding to 30°C, the biomass activity values of Table 2 are obtained.

The fact that the granular reactor is able to accumulate 50 times the biomass concentration of the SHARON reactor allows the operation of the partial nitrification system treating higher NLRs even working at 10°C lower. Regarding the DO concentration if this value is enough to satisfy the oxygen requirements of the process no detrimental effect should be observed from the temperature change. Furthermore DO concentration affects population dynamics between AOB and NOB because of different DO half-saturation coefficients meaning that at low temperatures AOB are favored.

In the case of the anammox reactor the effect of the temperature has been previously studied and it was observed that a decrease of activity of around 50% occurs when it changes from 30°C to 20°C (Dosta et al. 2008) as it was observed in the present study with the decrease from 0.28 to 0.15 g N/(g VSS d).

In this way operating both processes in separated units will mean that they perform their activity under the maximum achievable value when they operate at 30–35°C (Wiesmann 1994; Dosta et al. 2008). From the balance calculation to the two-units system it was obtained that when the temperature was fixed at 20°C the nitrogen removal in the combined system was of 0.08 g N/(L d).

"Single-unit" configuration: CANON system

From day 120 on, the nitrogen mass balance indicated that nitrogen losses were occurring spontaneously in the nitrifying granular SBR (Figure 1) while the color of the biomass changed to the typical reddish of anammox populations. This possible presence of anammox biomass was later confirmed by FISH analysis with the Amx820 FISH probe. The stimulation of the development of this population can be attributed to the appropriated conditions inside the reactor due to the simultaneous presence of ammonia and nitrite and of an anoxic zone inside the granules. The nitrogen losses increased during Stage III until reaching a value of NLR removed of 0.6 g N/(L d). Since the effluent still contained ammonia but not nitrite, nitrogen removal rate was limited by the ammonia oxidation step. In order to increase the rate of this step, the dissolved oxygen concentration was increased from 2.7 up to 3.5 mg O2/L (Stage IV).

This strategy was successful and the NLR removed increased up to an average value of 0.8 g N/(L d). At the end of this stage, nitrite was almost not detected in the effluent but ammonium concentrations between 30 and 120 mg NH4+-N/L still remained in the effluent. For this reason, the DO concentration was progressively increased up to 4.6 mg O2/L during Stage V. No improvement on the removed NLR was observed in this case which would indicate that the ammonia oxidation rate did not increase. However, the presence of nitrite oxidation was observed in spite of the free ammonia levels (calculated according to

![Figure 1](https://iwaponline.com/wst/article-pdf/63/6/1282/445583/1282.pdf)
Anthonisen et al. (1976)) which during this period ranged between 5 and 10 mg NH$_4$-N/L. In Stage VI, the DO concentration was restored to 2.8 mg O$_2$/L in order to avoid nitrite oxidation (Stage VI). This action completely stopped nitrite oxidation but also caused a strong decrease of the AOR to 0.4 g N/(L d). On day 393, the pH controller failed and the pH value reached values higher than 9 during several days which provoked the loss of the system stability. To recover the capacity of the system the applied NLR was decreased (Stage VII). During Stage VIII, the long term stability of the CANON process was tested at an applied NLR of 0.9±0.2 g N/(L d) and a DO level of 3.1±0.2 mg O$_2$/L. Under these conditions the system was able to remove 0.5±0.2 g N/(L d), the ammonia oxidation being always the limiting step. In a last stage, the temperature of the system was decreased to 15°C and the DO concentration to 2.2 mg O$_2$/L. The average NLR removed in this stage was of 0.2 g N/(L d).

Up to now the reported CANON systems in the literature were mostly operated at 30–35°C (Sliékers et al. 2002, 2005). In the present study the fact that the granular reactor, where the CANON process is performed, was operated at 20°C, allowed the nitrogen removal rates up to 1.1 g N/(L d) which are in the range of 0.075–1.5 g N/(L d) reported for CANON systems operated at higher temperatures (Sliékers et al. 2002, 2005). This high capacity of system even working at 20°C could be related to the applied start-up strategy. The minimum temperature at which Anammox activity is detected in wastewater treatment systems has been found to be of around 10°C while in marine environments was of −2°C (Rysgaard et al. 2004). Generally, CANON systems are developed from anammox reactors inoculated with nitrifying biomass and operated under microaerobic conditions. This strategy seems to be not suitable because an important decrease of the anammox activity is observed during the start-up probably due to the fact that the nitrifying activity was not high enough to maintain microaerobic conditions (Sliékers et al. 2002, 2005; Liu et al. 2008). In this case the CANON system was developed from a granular nitrifying reactor operated under oxygen limiting conditions to promote the suitable conditions for the anammox biomass: 1) the presence of ammonia and nitrite in equimolar ratio; 2) high biomass retention capacity; and 3) an anoxic zone in the inner part of the granules.

Considerations about the suitable conditions for the CANON process

During the operation of a CANON process, the activity of AOB in the external layers of the granules should be high enough, to protect Anammox bacteria from the penetration of dissolved oxygen, but it must be also controlled to avoid inhibition of Anammox bacteria by high nitrite concentrations. This inhibition can occur when the diffusion rate of nitrite to the anoxic zone is higher than the nitrite consumption by Anammox bacteria.

The activity of ammonia oxidizing bacteria is directly related to the fraction of the granule which is under aerobic conditions. Since the oxygen penetration depth depends on the dissolved oxygen concentration (Equation (6)), this parameter can be used to control the activity of ammonia oxidizing bacteria.

\[ \delta_{O_2} = \sqrt{\frac{2 \cdot D_{O_2} \cdot C_{O_2}}{r_{max_{O_2}} \cdot \rho_b}} \]  

where \( \delta_{O_2} \) is the oxygen penetration depth (m).

The ammonia oxidation is also strongly affected by temperature changes. This can be a problem when the temperature of the influent suffers fluctuations. Since the ammonia oxidation and Anammox processes have a similar activation energy value (around 70 kJ/mol (Wiesmann 1994; Dosta et al. 2008)), a decrease of temperature would affect to the intrinsic rate of both processes in a similar way. Then, it would be expected that the balance between nitrite production and consumption was not affected. However, a decrease of temperature would also cause an increase of the oxygen penetration depth even with the same dissolved oxygen concentration in the bulk liquid is set (Equation (6)). Therefore, the proportion of aerobic zone would increase. Then, the Anammox process rate would be affected by both, the decrease of temperature and a lower proportion of anoxic zone while, in the case of the ammonia oxidation rate, the effect of a temperature decrease would not be so strong due to the increase of the aerobic zone. In order to maintain a balance between ammonia oxidation and Anammox rates when the temperature decreases, the oxygen concentration in the bulk liquid has to be reduced in such way that the oxygen penetration depth is maintained constant, i.e., the \( C_{O_2}/r_{max} \) ratio has to be kept constant.

The capacity of the system was always limited by the ammonia oxidation rate in order to avoid an excessive nitrite production which could inhibit the Anammox process because high concentrations of this substrate cause a detrimental effect of this activity. Taking into account that the ammonia oxidation rate was limited by the internal mass transfer rate and oxygen was the limiting substrate of the process, the overall rate can be determined by Equation (4). Then, the effects of the changes of operational conditions...
during different stages on the overall rate could be estimated according Equation (5). Taking as reference the average activity observed during Stage II, Equation (5) predicts activity values close to those experimentally observed (Table 3).

Comparing the nitrogen removal capacities of the two-units and the single reactor systems it can be stated that the latter allows the treatment of a nitrogen loading rate around ten times higher (1 g N/(L d) versus 0.08 g N/(L d)) than the former at the same operational temperature (20°C).

**CONCLUSIONS**

- The single-unit system seems to be more suitable than the two-units one to carry out autotrophic nitrogen removal at moderately low temperatures. This system was able to treat NLRs of 1 and 0.2 g N/(L d) at 20 and 15°C, respectively, due to its high biomass retention capacity which allows the accumulation of 50 times more biomass containing AOB and Anammox bacteria.
- To maintain the stability of the CANON process, the ammonia oxidation rate should be controlled to avoid a possible inhibition of the Anammox process by nitrite. The DO concentration in the bulk liquid can be used as the control parameter to fit the ammonia oxidation rate to the changes of the operational conditions.
- The application of autotrophic nitrogen removal processes at low temperatures opens the possibility to remove nitrogen in the main stream of WWTPs, i.e., the COD could be mainly used to produce methane in the anaerobic digester which would make those WWTPs energetically self-sufficient.

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