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**Start-up strategies of membrane-aerated biofilm reactors (MABR) for completely autotrophic nitrogen removal**

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**Abstract** Completely autotrophic nitrogen removal, coupling aerobic and anaerobic ammonium oxidation, can be achieved via redox stratified biofilms growing on gas-permeable membranes. These sequential reactions are mediated by aerobic and anaerobic ammonium oxidizing bacteria (AOB and AnAOB). The major downside of this process stems from a long start-up period due to the slow growth rate of AnAOB. Therefore, two different start-up strategies, i.e., continuous inoculation of AnAOB and sequential batch inoculation of AOB and AnAOB, were tested in two laboratory scale membrane-aerated biofilm reactor (MABRs). Results indicate that the continuous inoculation strategy was more rapid and effective to achieve nitrogen removal than the sequential inoculation approach. Nitrogen loss in the reactor continuously inoculated with AnAOB was observed after 120 day operation, with an average NH$_4^+$-N and TN removal rate of 3.41 and 1.95 g-N/m$^2$-membrane/day, respectively. On the other hand, nitrogen loss was hardly observed in the MABR with the sequential inoculation strategy.

**Keywords** Anammox; autotrophic nitrogen removal; membrane-aerated biofilm reactor; nitrification

**INTRODUCTION**

Discharge control of ammonium-rich wastewater streams has gained importance over the last decades, because of their ability to severely deteriorate water quality into the receiving water bodies. Many processes and technologies have been developed since then to treat these streams, but those relying on the completely autotrophic nitrogen removal coupling aerobic and anaerobic ammonium oxidation, have gained the most recent attention because of its lower energy requirements, lower emission of greenhouse-effect gasses and the avoidance of needing to add an external carbon source as electron donor to complete the process (Lackner et al., 2008).

Membrane-aerated biofilm reactors (MABR) have lower footprints and are easier to control than suspended growth processes, being an attractive technology for the wastewater treatment industry.
In a MABR a biofilm is developed on an aeration membrane. In this way, different functional microbial populations can be optimally engineered by independent control of the intra-membrane gas pressure and the flux of dissolved substrates, creating oxic-anoxic zones within the biofilm depth (Syron and Casey, 2008).

This principle could be applied to achieve completely autotrophic nitrogen removal. The NH$_4^+$-N present in the influent stream could diffuse into deeper aerobic biofilm regions where would be partially oxidized it to NO$_2^-$-N by AOB by making use of the oxygen supplied through the membrane as electron acceptor. The adjusted gas pressure could potentially allow NOB outcompetition in the reactor, in case they were present in the inoculation sludge. By doing so, the produced NO$_2^-$-N and the remaining NH$_4^+$-N would be utilized by AnAOB growing in anoxic biofilm zones, converting them into dinitrogen gas. (Gong et al., 2007; Lackner et al., 2008; Terada et al., 2007).

Unfortunately, one of the main drawbacks associated to this process is the long start-up time, due mainly to the slow growth rate of AnAOB (doubling time is approximately 8-11 days), as well as the high sensitivity of AnAOB towards oxygen and nitrite concentrations and changes in their growth environment (0.8 mg-O$_2$/L and 60 mg-NO$_2^-$-N/L are enough to inhibit their activity in batch operation) (Pynaert et al., 2004; Tsushima et al., 2007; van der Star et al., 2007). Therefore, the development of a reliable and relatively fast start-up procedure seems to be a clear need for the optimization of single-stage completely autotrophic nitrogen removal processes.

The aim of the present study was to investigate and compare two different start-up strategies for the inoculation of AnAOB, i.e. continuous seeding of AnAOB versus their inoculation in batch mode, in lab-scale MABRs performing partial nitrification of ammonium to nitrite.

**MATERIALS AND METHODS**

**Experimental setup**

Experiments were conducted in two laboratory-scale MABRs, each with a working volume of about 2.41 L. The installed membrane modules consist of 10 hollow-fiber membrane bundles with 128 hollow-fibers each and an inner/outer fiber diameter of 200/280 μm (Model MHF3504, Mitsubishi Rayon Co., Ltd., Tokyo, Japan). The total membrane surface area is about 0.34 m$^2$ for each reactor. Air at 15 KPa (relative pressure) was used as oxygen source, which was supplied to the membrane module from the bottom of the reactor and vented in the upper end (flow-through configuration). The pressure and gas flow rate in the membrane lumen, which can be adjusted by means of a set of valves, were monitored by using a pressure and gas flow gauge. The reactor was kept completely mixed by recirculating reactor medium by making use of an aquarium pump working at a flow rate of 10 L/h. Medium temperatures ranged between 23 and 32°C. The main elements of the setup are represented in Figure 1. R1 and R2 only differed on the presence of the tubular reactor with immobilized AnAOB used to inoculate R1.

Synthetic wastewater was continuously supplied as feed water in this study by diaphragm pumps (DME 2-18, Grundfors, Denmark). The influent flow rates varied from 200 to 300 ml/h in R1 and
from 175 to 290 ml/h in R2. The prepared wastewater consisted of (NH₄)₂SO₄ (2.86 g/L), KH₂PO₄ (0.058 g/L), MgSO₄·7H₂O (0.128 g/L), CaCl₂·2H₂O (0.039 g/L), NaCl (0.262 g/L), KHCO₃ (2.48 g/L) and NaHCO₃ (1.603 g/L), and 1ml/L feed of trace element solution I (EDTA (5 g/L) and FeSO₄·7H₂O (5 g/L)) and solution II (EDTA (15 g/L), ZnSO₄·7H₂O (0.43 g/L), CoCl₂·6H₂O (0.24 g/L), MnCl₂·4H₂O (0.99 g/L), CuCl₂·2H₂O (0.25 g/L), Na₂MoO₄·2H₂O (0.22 g/L), NiSO₄·6H₂O (0.19 g/L), NaSeO₄·10H₂O (0.21g/L) and H₃BO₄ (0.014 g/L)).

**Figure 1**: Experimental setup. (1): substrate bottle; (2): gas bag; (3): diaphragm pump; (4): reactor body and membrane module; (5): needle valve; (6): pressure gauge; (7): ball valve; (8): flow meter; (9) centrifugal pump; (10): inoculation reactor; (11): overhead electronic stirrer.

**Inoculation strategies**
Two different inoculation strategies were applied for the start-up of the presented MABRs. In first place, R1 was inoculated with enriched nitrifying biomass. After one month of operation, a 0.8 L tubular reactor with attached AnAOB was placed in the recirculation line (continuous AnAOB inoculation strategy). By proceeding this way, AnAOB were supposedly continuously fed into the MABR with the recirculated medium and could eventually adhere on the already existing biofilm. R2 was also inoculated with enriched nitrifying biomass and operated for one month. However, in
this case AnAOB were added directly during batch operation of the reactor, which lasted for 10 days (sequential AnAOB inoculation strategy). Nitrifying and AnAOB inocula were obtained from the Lundtofte WWTP (Denmark) and a laboratory-scale reactor with immobilized AnAOB which was operated for more than 400 days.

Analytical methods

Influent and effluent samples were filtered though 0.45 μm pore size syringe filter before analysis. During the first 75 days of operation, the concentrations of the relevant nitrogen species, i.e. NH4+-N, NO2--N and NO3--N, were analyzed by a colorimetric autoanalyzer (AA3/AAce System, Bran+Luebbe, Germany). Due to the great uncertainty associated with the proposed analytical method from day 120 on, concentration results were obtained manually by spectrophotometry using commercially available test kits (Spectroquant 00683, Spectroquant 14776 and Spectroquant 14773; Merck, Germany). pH values and DO concentrations were determined by pH (SenTix 41, WTW, Germany) and DO meters (Oxi340i, WTW, Germany).

RESULTS AND DISCUSSION

The performances of the two described MABRs are shown in Figure 2. This figure collects all data gathered after inoculation of the AnAOB biomass. As appreciated, samples were not taken from day 75 to day 100, however, the reactor remained in operation. The data collected during the first 75 days of operation was subjected to a high level of uncertainty and cannot be fully trusted.

The reactors were operated trying to target the optimum oxygen to ammonium surface loading ratio (J_O2/J_NH4) proposed by Terada et al. (2007) in their model-based study for the optimization of counter-diffusion MABRs. According to the cited paper, a J_O2/J_NH4 ratio of 1.73 is necessary to maximize the nitrogen removal in these types of system.

It can be observed in the figure that no nitrogen loss was observed until day 120 in both R1 and R2. From that point on, the presented concentrations remained practically constant. In R1 the average ammonium concentration in the influent (after day 120) was 545 mg-N/L. As refers to the effluent, the average concentrations of NH4+-N, NO2--N and NO3--N were 322, 0.56 and 106 mg-N/L, respectively. For R2, the average concentration of NH4+-N in the influent was 537 mg-N/L, while the average concentrations of NH4+-N, NO2--N and NO3--N in the effluent were 300, 24 and 196 mg-N/L. This suggests that more than 40% of NH4+-N (3.41 g-N/m2-membrane/day) can be removed in both of our reactors. However, low NO2--N and high NO3--N concentration in both reactors indicates that the produced NO2--N was further oxidized by NOB, which is an unwanted operation situation. This points out the difficulty to remove all NOB at the start-up stage, in which they thrive with AOB at the bottom of the biofilm where oxygen and NO2--N are the highest.

Figure 3 shows the TN removal efficiency in both reactors. After 120 days of operation, the average TN removal efficiency achieved 22.51% in R1. Please note that despite the fact that the Anammox inoculation reactor was detached from R1 on day 183, the TN removal efficiency was always kept above 20%, demonstrating that the proposed autotrophic nitrogen removal strategy is feasible. On the other hand, the TN removal observed in R2 during the same period was not stable and the average TN removal efficiency was a poor 5.25%. The average specific TN removal rates were 1.95
and 0.44 g-N/m²-membrane/day for R1 and R2 respectively (0.28 and 0.06 g-N/l/d).

Figure 2. Reactor performances in R1 and R2 for completely autotrophic nitrogen removal

Figure 3. Comparison of the TN removal in R1 and R2

As said, the AnAOB inoculation reactor was kept in the recirculation line until noticeable nitrogen removal in R1 was observed (day 183). This start-up time is high, but comparable to the one reported by Pynaert et al. (2004) in a similar system and much lower than the one from our previous experiences with operation of reactors with AnAOB. Once the inoculation reactor was detached it still presented AnAOB activity, making it eligible for being used at another location.
It is obvious from the presented removal efficiencies that the continuous inoculation strategy resulted more effective than the sequential one. The fact that nitrite is not present during most of R1 operation and the lower nitrate production in comparison to R2 despite of the very similar ammonium conversion suggests that AnAOB were able to colonize R1 and compete with NOB for the available nitrite. On the contrary, the very low TN removal efficiency and the accumulation of nitrite on R2 during the last period of the data presented leads to think that AnAOB were washed out and that the reported nitrogen removal could be easily attributed to experimental errors or ammonium bacterial uptake for maintenance purposes.

If \( J_{O2} \) is calculated based on the theoretical Nitrogenous Oxygen Demand (NOD) from the averaged data presented, the average \( J_{O2}/J_{NH4} \) is 1.28, quite similar to the value of 1.73 suggested previously as optimal. According to the above mentioned model-based study, such a slight deviation does not compromise very much removal efficiencies (less than 10%). However, as seen, the reactor performance stayed far away from being considered optimal suggesting that the optimal operational \( J_{O2}/J_{NH4} \) ratio can be highly influenced by the biokinetics or the configuration of the system. Some kinetic studies and simulations would have been valuable to support and test this affirmation. Unfortunately, it was not possible to carry them out due to time limitations given the completion date of the project. Three more actions were unsuccessfully attempted to enhance the performance of the system. The first one involved operation at an increased pH (value) in the reactor medium to enhance free ammonia formation and the subsequent inhibition of NOB. The second one involved further lowering the oxygen concentration on the membrane wall by decreasing the oxygen pressure in the membrane lumen to make tougher the competition for oxygen between AOB and NOB. Finally, an increase on the temperature of the reactor medium (to 33°C) did not lead to significant changes either.

A closer observation of Figure 3 gives another interesting hint on the processes going on in R1. About day 190, it is observed a peak on the TN removal efficiency in R1. This peak corresponds with the presence of nitrite in the influent solution (data not shown), suggesting that the factor that keeps the reactor from achieving higher TN removal efficiencies is the unavailability of nitrite to AnAOB and not the low AnAOB density. Perhaps inoculation of AnAOB at an earlier stage would have favoured the competition between AnAOB and NOB for nitrite given the higher affinity of AnAOB for this substrate.

### Table 1. Benchmarking table of systems for completely autotrophic nitrogen removal

<table>
<thead>
<tr>
<th>Processes Configuration</th>
<th>T (ºC)</th>
<th>TN removal rate (g-N/l/day)</th>
<th>TN specific removal rate (g-N/ m²/day)</th>
<th>TN removal efficiency (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBR</td>
<td>20</td>
<td>1.00</td>
<td>-</td>
<td>60</td>
<td>Vazquez-Padin et al. (2009)</td>
</tr>
<tr>
<td>Airlift</td>
<td>18</td>
<td>1.5</td>
<td>-</td>
<td>42</td>
<td>Slieckers et al. (2003)</td>
</tr>
<tr>
<td>UGBR</td>
<td>30</td>
<td>0.06</td>
<td>-</td>
<td>76</td>
<td>Ahn and Choi (2006)</td>
</tr>
<tr>
<td>RDC</td>
<td>&gt;20</td>
<td>1.7</td>
<td>7</td>
<td>100</td>
<td>Schmidt et al. (2003)</td>
</tr>
<tr>
<td>MBBR</td>
<td>25</td>
<td>0.5</td>
<td>1.92</td>
<td>62</td>
<td>Szatkowska et al. (2007)</td>
</tr>
<tr>
<td>MABR</td>
<td>28(^a)</td>
<td>0.28(^b)/0.47(^b)</td>
<td>1.95(^b)/3.3(^b)</td>
<td>22(^b)/39(^b)</td>
<td>This study</td>
</tr>
</tbody>
</table>
Even though the average removal efficiency in R1 was only about 20%, the TN specific removal rate is not too far behind from the one of other one-reactor systems appearing in the literature (Table 1). This gives us the chance to believe on the possibilities of this kind of configuration performing complete autotrophic nitrogen removal. The main benefit (and also the main problem) of this system compared to the ones in Table 1 is the higher oxygen transfer efficiency. Unlike the rest of configurations presented in the cited table, oxygen is supplied directly to the biofilm through an aeration membrane at a high efficiency, making the system more robust against possible oxygen limitations (oxygen supply can be increased by increasing the pressure on the membrane lumen). The con of such an efficient aeration system in a reactor with immobilized biomass is that oxygen is also supplied to NOB, lowering the performance of the process.

CONCLUSION
Two different start-up strategies for MABR performing completely autotrophic nitrogen removal were tested in two laboratory-scale MABRs. Results showed that the continuous inoculation of AnAOB (R1) was more effective than the sequential one (R2). A TN removal efficiency of 20% was achieved after 120 days at an average specific removal rate of about 1.95 g-N/m²-membrane/day in R1 while both parameters can be considered negligible in R2. The AnAOB reactor used for the inoculation was still active after inoculation. Operation of the reactors, by setting the load ratio based on the optimum $J_{O2}/J_{NH4}$ defined by Terada et al. (2007) was not successful. The competition for nitrite was identified as the limiting factor for obtaining higher removal efficiencies. Operation at higher pH, lower oxygen concentration at the membrane walls or increase of the medium temperature did not result in an increase of the removal efficiency. However, the relatively high specific removal rates even at removal efficiencies as low as 20% point out the big potential of this type of configuration. Therefore, innovative methods to operate these reactors, which allow for higher accumulation of nitrite, are necessary to enhance their performance.

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