Integration of nitrification and denitrification by combining anoxic and aerobic conditions in a membrane bioreactor
Jianfeng Li, Fenglin Yang, Dieudonné-Guy Ohandja and Fook-Sin Wong

ABSTRACT
A membrane bioreactor (MBR) was developed to achieve nitrogen removal by combining nitrification and denitrification conditions in one reactor. The activated sludge was alternated between aerobic and anoxic conditions using peristaltic pump. The biomass concentration and floc morphological properties were observed to be similar in anoxic and aerobic compartments. However, the homogeneous properties of the activated sludge did not lead to the failure of oxygen gradient formation in the reactor. Due to the position of the air diffuser, an anoxic compartment at the bottom and an aerobic compartment in the upper part of the reactor were formed after 40 days. The average total nitrogen (TN) removal efficiency was then observed to increase to 77%. The microbial characterization using polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) analysis, as well as the specific nitrogen utilization rate measurements, indicated that the nitrogen removal in the reactor occurred via nitrification and denitrification processes.

Key words | denitrification, membrane bioreactor, nitrification, nitrogen removal, PCR-DGGE, specific nitrogen utilization rate

INTRODUCTION
Wastewater discharge containing nitrogen can be toxic to aquatic life, causes oxygen depletion and eutrophication in the receiving water, therefore the reduction of nitrogen levels in such discharge is necessary. Indeed, nitrogen removal has become an important aspect of present-day wastewater treatment (Metcalf & Eddy 2003). In general, conventional biological nitrogen removal in wastewater treatment is carried out via nitrification and denitrification, which are achieved by different groups of microorganisms (Ahn 2006). Nitrification is an aerobic process, in which nitrifying bacteria oxidize ammonia to nitrite and then to nitrate, whereas in denitrification, nitrate and nitrite are reduced to nitrogen gas by heterotrophic denitrifying bacteria in the absence of oxygen (Metcalf & Eddy 2003). A large number of studies have focused on proprietary combinations of anaerobic, anoxic, and aerobic compartments or multiple compartments for nitrogen removal (Jun et al. 2003; Yang et al. 2003; Hiras et al. 2004; Wang et al. 2005). However, the aerobic system with separate anoxic tank for denitrification (Hiras et al. 2004; Wang et al. 2005) has the disadvantage of large footprint. The simultaneous nitrification and denitrification process (SND) associated with carriers or granules (Jun et al. 2003; Yang et al. 2003) may have the stability problem due to the scarcity of clear understanding for SND process.

As a modification of the conventional activated sludge process for the separation of biomass from water by membrane filtration instead of sedimentation, the membrane bioreactor (MBR) has been proven to have less land requirement and less sludge production than activated sludge process (Judd 2006). MBRs can be operated with a long solid retention time (SRT) because SRT can be
controlled independently from the hydraulic retention time (HRT) by membrane separation (Rosenberger et al. 2002; Pollice et al. 2006). Moreover, through the process of the membrane separation, MBRs have the ability to enhance the nitrification process, as the slow-growing autotrophic nitrifiers can be retained due to the long SRT (Sofia et al. 2004).

In these regards, anoxic and aerobic conditions were combined in a submerged MBR by setting the air diffuser in the middle of the reactor to form stable anoxic and aerobic compartments. Inducing a vertical dissolved oxygen (DO) gradient in the MBR would lead to the development of an anoxic compartment downward the diffuser whereas the aerobic compartment upward the diffuser. Therefore, the objective of this study is to investigate the feasibility of integrating nitrification, denitrification and membrane filtration in a single reactor unit, and the mechanism of nitrogen removal in this MBR accordingly.

**MATERIALS AND METHODS**

**Experimental configuration**

As shown in Figure 1, a 12l (effective working volume) laboratory-scale reactor was set up in this study. The dimensions of the reactor were 800 × 170 × 100 mm (height × length × width), giving a 5:1 H/D (height to equivalent diameter) ratio. The influent was introduced from the bottom, and the air diffuser was located in the middle of the reactor to provide air bubbles for the oxidation of organics and ammonia, and the reduction of membrane fouling. Two flat sheet membrane modules with an area of 0.1 m² were submerged in the aerobic compartment. The flat sheet membrane used in this work was a hydrophilic PVDF membrane with a pore size of 0.22 μm (Millipore, USA). The feed pump, recycle pump, suction pump and air diffuser were operated continuously throughout the entire experimental period.

The bioreactor was inoculated with the activated sludge obtained from the Ulu-Pandan Wastewater Reclamation Plant (Singapore). The synthetic wastewater used in this study consisted of 200 mg/l glucose and 250 mg/l sodium acetate as the carbon source; other components included 160 mg/l NH₄Cl, 40 mg/l peptone, 50 mg/l meat extract, 7.5 mg/l KH₂PO₄, 7.5 mg/l FeSO₄ and 2.5 mg/l MgSO₄. The operating conditions are shown in Table 1.

**Analytical methods**

Mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) were measured in accordance to the standard method (Clesceri et al. 1997). Dissolved oxygen (DO) concentrations at the different levels (0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75 cm) of the reactor were measured using a DO meter with a four-meter cable (YSI200, USA). The DO concentration distribution curves were plotted based on the DO concentrations at the different levels of the reactor. Total Organic Carbon (TOC) and Total Nitrogen (TN) concentrations were determined by a TOC analyzer equipped with a total nitrogen measuring unit (TNM-1, Shimadzu, Japan).

**Table 1 | Operating conditions of the MBR**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Operating time (days)</td>
<td>140</td>
</tr>
<tr>
<td>Reactor temperature (°C)</td>
<td>24–26</td>
</tr>
<tr>
<td>TOC/TN</td>
<td>4:1</td>
</tr>
<tr>
<td>Aeration rate (l/min)</td>
<td>3</td>
</tr>
<tr>
<td>Recirculation rate/ feed rate</td>
<td>4:1</td>
</tr>
<tr>
<td>HRT (h)</td>
<td>12</td>
</tr>
<tr>
<td>SRT (days)</td>
<td>60</td>
</tr>
</tbody>
</table>
NH$_4$-N, NO$_2$-N and NO$_3$-N concentrations were measured using the DR/2400 spectrophotometer (HACH ODYSSEY) and the standard HACH testing kits.

**Particle size distribution and fractal dimension**

The floc size distribution was determined using the Malvern Mastersizer 2000 instrument with a detection range of 0.02–2,000 μm. Each sample was measured three times with a standard deviation of 0.1–4.5%. The value of d (0.5) was quoted as a factor of particle size distribution.

The structure of the flocs was quantified in terms of fractal dimension ($D_f$), which is often used to describe the geometric characteristics of activated sludge flocs (Waite 1999). The $D_f$ was calculated from the raw light-scattering data using the Malvern Mastersizer 2000, which was in accordance with the method proposed by Guan et al. (1998) on the basis of a power law relationship between the total scattering intensity of the light from the aggregates and the magnitude of the scattering vector. The scattering intensity at each detector was calculated from the raw scattering data by using information from the Malvern Mastersizer 2000. By plotting the log of the light scattering intensity as a function of the log of the light scattering vector, the linear slope is given as the $D_f$, which ranges from 1 to 3. A high value of $D_f$ is related to compact and dense flocs.

**Microbial observation**

Activated sludge samples were taken from the middle of the aerobic compartment and anoxic compartment in order to ensure a representative sample of the biomass. The sludge samples were examined using light microscopy and the images were captured on a Keyence VH-Z75 (Japan) microscope attached to a PC based charge-coupled device. The microbial composition of activated sludge was observed with a scanning electron microscope (SEM) (JSM-5310LV, JEOL Ltd, Japan). The samples were gently washed with clean water and fixed with 2% glutaraldehyde for 4 h. The samples were then washed 3 times with 0.1 M sodium cacodylate buffer, dehydrated through a series of 10 min washing with 50, 70, 85, and 95% ethanol, and were finally stored in 100% ethanol. The samples stored in ethanol were dried using a Critical Point Dryer (E3000) (VG Microtech, UK) and sputter coated using an Au–Pd sputter coater (SPI-Module, Structure Probe Inc. USA) before they were observed with SEM.

**DNA extraction, polymerase chain reaction (PCR) amplification and denaturing gradient gel electrophoresis analysis (DGGE)**

The sludge samples were centrifuged (13,000 rpm, 10 min) and 100–200 mg of sludge (wet weight) was mixed with 50 ml of 20% sodium dodecyl sulfate, 800 μg baked glass beads (0.1 mm) and 500 ml of saturated phenol in a 2 ml tube. The samples were lysed in a BeadBeater machine (Biospec products, USA) for 5 min (5,000 oscillations/s). DNA extraction was carried out according to the protocols previously described (Watanabe et al. 1998). The amplification of the V3 region of the 16S rRNA sequence from region 357 to 517 was achieved by the polymerase chain reaction using the following primers 357F (5'-GCC CGC CGC GCG CGG GCG GCG GGG CGG GGG CAC GGG GGG CCTCCT ACG GGA GGC AGC AG-3') and 517R (5'-ATT ACC GCG GCT GCT GG-3') (Muyzer et al. 1993). The PCR was conducted in a thermal cycler (Eppendorf, Germany) and a touchdown thermal profile technique using a 2 min activation and 10 min extension (Watanabe et al. 1998). The DGGE was conducted in a DGGE system (BIORAD Decode, USA) using the method described by Muyzer et al. (1993). Electrophoresis was conducted in a 1 × TAE buffer solution at 90 V and 60°C for 15 h.

**Specific nitrogen utilization rate**

Batch experiments were conducted to determine the specific activities of the nitrifiers and denitrifiers. These activity tests measured the maximum rates of nitrogen utilization per biomass unit for the nitrifiers and denitrifiers, expressed as mg NH$_4$-N/g MLVSS h and mg NO$_3$-N/g MLVSS h. The assembly used for the determination of the activities of nitrifiers and denitrifiers was similar to that described previously (Guo et al. 2005). 100 ml substrates solution and 20 ml mixed liquor sample withdrawn from one of the compartments of the reactor were introduced to a flask. The initial concentrations of substrates were 100 mg/l NH$_4$-N and 400 mg/l alkalinity for...
nitrifiers and 100 mg/l NO₃-N and 800 mg/l COD for denitrifiers. Any DO in the denitrifying feed was removed by purging it with nitrogen gas after adding to the flasks. During the culture, periodic sampling and measurement of the nitrogen compounds (NH₄-N and NO₃-N) were carried out.

RESULTS AND DISCUSSION

Performance of the MBR

Throughout the entire period, the effluent TOC was below 3 mg/l, indicating that an average removal efficiency of 98% was achieved. Figure 2 shows the TN concentration in the influent and effluent and the TN removal efficiency of the reactor. It can be seen in Figure 2 that the TN concentration in the influent was 41.9 ± 2.2 mg/l throughout the entire period. From Day 1 to Day 40, which was considered as a start-up phase, the TN concentration in the effluent was 17.0 ± 2.7 mg/l, indicating a TN removal efficiency of 58.7 ± 7.5%, this was apparently not as good as that in the remainder of the experiment, in which the TN concentration was 9.6 ± 3.2 mg/l and TN removal efficiency was 77.2 ± 7.6% respectively.

Considering the ratio of recirculation rate to feed flow rate (4:1), the activated sludge alternated fast between aerobic and anoxic conditions. However, this did not lead to the failure of the domination of different types of bacteria in different compartments. The integration of nitrification and denitrification in this reactor indicated that the microorganisms had great adaptation to the environment. The aerobic compartment and anoxic compartment in this reactor might act as the active centers for nitrification and denitrification respectively, in which ammonia nitrogen was converted to nitrate and nitrate was then converted to nitrogen simultaneously.

Characteristics of the activated sludge in the reactor

Table 2 shows the morphological properties of the activated sludge in both the aerobic compartment and anoxic compartment. From Table 2, it can be seen that $D$ (0.5) (both volume and number) as well as the inner structure of the activated sludge, expressed as the fractal dimension ($D_f$) in the different compartments, were similar. As an internal property of self-similar flocs, the fractal dimension has a relationship with the activated sludge morphology. Compact aggregates have been found to have higher $D_f$ values, while fractal dimensions are lower for “loose” aggregates (Guan et al. 1998). The similarity of the average particle size and the fractal dimension demonstrated the morphological similarity of the activated sludge in different compartments, which was probably caused by the recirculation between two compartments.

Figure 3 shows the MLSS concentrations in the aerobic compartment and anoxic compartment during the experimental period of 140 days. The MLSS concentrations in the aerobic compartment and the anoxic compartment of the MBR stabilized after day 40 at 8,168 ± 385 mg/l and 8,529 ± 493 mg/l, respectively. MLSS was building up in both compartments during start-up period (day 1–40). The MLSS concentration in the anoxic compartment was slightly higher than that in the aerobic compartment, which may be due to settling of the microorganisms by gravity.

Figure 4 illustrates the evolution of the dissolved oxygen profiles in the reactor at different days. It can be observed that the DO concentration was always the highest at the

<table>
<thead>
<tr>
<th>$D$ (0.5) (µm)</th>
<th>Volume</th>
<th>Number</th>
<th>$D_f$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aerobic compartment</td>
<td>239.28 ± 20.57</td>
<td>4.30 ± 0.24</td>
<td>2.076 ± 0.025</td>
</tr>
<tr>
<td>Anoxic compartment</td>
<td>233.32 ± 23.85</td>
<td>4.84 ± 0.76</td>
<td>2.079 ± 0.024</td>
</tr>
</tbody>
</table>

Table 2 | Comparison of the morphological properties in the aerobic compartment and anoxic compartment after Day 40 ($n = 30$)
position where the diffuser was located. On Day 5, the DO concentration below 1 mg/l was only in the region of from 10 cm to the bottom. However, as sludge accumulated, the DO concentration became lower throughout the whole reactor. It is widely accepted that increasing biomass concentration will decrease the oxygen transfer coefficient (Holakoo et al. 2007). Therefore, the decrease in the DO concentration in the reactor was likely due to the increasing biomass concentration. The decrease of DO concentration resulted in a larger volume below the diffuser becoming anoxic. It can also be observed from Day 40, when the biomass concentration stabilized, DO concentration was unchangeable.

It can be concluded that although the morphological properties as well as the MLSS concentration appeared to be similar in different compartments, the increasing sludge concentration would lead to the formation of a vertical oxygen gradient in the reactor, which in turn separated the reactor into two different compartments: an aerobic compartments at the top and an anoxic compartments at the bottom of the reactor.

Figure 5 shows the photographs of the activated sludge within the different compartments taken through microscope observation. It can be seen in Figure 5(a, b) that the filamentous bacteria and a large number of epistyli protozoa co-existed in the sludge in the aerobic compartment. While there were few live protozoa in the sludge in the anoxic compartment (Figure 5(c, d)), some sole bacteria were found in this compartment (Figure 5(d)). These photographs clearly showed that the micro-ecology in the two compartments was different. It is well accepted that in addition to the nitrifying bacteria, epistyli and vorticella protozoa are present in relatively large numbers during rapid nitrification (Gerardi 2002). Therefore, the large number of epistyli protozoa presented in the aerobic compartment supported the fact that nitrification was occurring rapidly in the aerobic compartment but not in the anoxic compartment. No live protozoa were observed in the anoxic compartment, due to the low DO concentration.

Microorganisms are very important in biological wastewater treatment processes because most of contaminants are degraded by their activities. Figure 6(a) shows the microbial composition of the activated sludge in the reactor, as observed by scanning electron microscopy (SEM). It can be seen in Figure 6(a) that the rod-like bacteria and coccoid bacteria were tightly linked together in the activated sludge. LaPara et al. (2006) investigated the bacterial community structure of starch-fed MBRs and revealed that complex bacterial communities were found to coexist on simple feed media. Thus, the organics and nitrogen removal in the reactor were achieved by the activities of these different types of microorganisms.

To further investigate the structure of microbial community in the reactor, the microbial populations in the aerobic compartment and anoxic compartment were examined and compared by analyzing the denaturing gradient gel electrophoresis (DGGE) profiles of the 16S rDNA fragments. PCR-DGGE is a widely used combined technique for the structure and diversity of a bacterial community based on the extracted DNA. This technique can be used not only to estimate the genetic diversity of microbial
communities in natural habitats but also to infer the phylogenetic affiliation of the community members (Vallaeys et al. 1997; Petersen & Dahllof 2005). By using this technique, it was found that the α- and β-subclasses of Proteobacteria were the dominant groups in the MBR, while the Nitrosospira spp. and Nitrospira spp. were the predominant groups of the ammonia and nitrite oxidizing groups, respectively (Luxmy et al. 2000; Li et al. 2005). Moreover, it has also been reported that such bacterial communities are constant over long periods of time (Witzig et al. 2002). In this study, PCR-DGGE was conducted on Day 100, when the reactor was in a steady state. It can be seen in Figure 6(b) that the band pattern of the DGGE profiles for the samples from the aerobic compartment and anoxic compartment were different. This result indicates that the dominant bacterial communities in the aerobic compartment and the anoxic compartment were different, implying that the microbial metabolisms and processes were different in these compartments.

Mechanisms of nitrogen removal in the reactor

In order to better understand the mechanisms in the reactor for the nitrogen removal, the NH₄-N, NO₂-N and NO₃-N concentrations within the different compartments were measured. NO₂-N was below the detect limitation (0.2 mg/l) throughout the entire experimental period. Figure 7(a) shows the NH₄-N concentrations measured during the experimental period of 140 days. As shown in Figure 7(a), the NH₄-N concentration in the aerobic compartment was 4.1 ± 1.9 mg/l and was 3.5 ± 1.2 mg/l in the effluent, throughout the experimental period. This revealed that the NH₄-N was primarily degraded in the aerobic compartment. Figure 7(b) shows the NO₃-N
concentration throughout the experimental period of 140 days. The NO$_3$-N concentrations measured in the anoxic compartment were relatively high before Day 40. The average NO$_3$-N concentration was $11.7 \pm 4.4 \text{mg/l}$. The growth and activity of the denitrifiers were active under a DO level of 1 mg/l (Gerardi 2002). Therefore, the relatively small anoxic compartment during this period was probably the reason for the accumulation of NO$_3$-N in the anoxic compartment. In contrast, NO$_3$-N concentration in the anoxic compartment stabilized at $1.40 \pm 0.60 \text{mg/l}$ after Day 40. The low NO$_3$-N concentration in the anoxic compartment indicated that the NO$_3$-N generated from aerobic compartment was almost fully degraded in the anoxic compartment after Day 40.

However, the NH$_4$-N concentration in the anoxic compartment became higher than that in the aerobic compartment after Day 40, when the MLSS concentration in the anoxic compartment started to stabilize at $8529 \pm 493 \text{mg/l}$. Similarly, it can also be observed in Figure 8 that the NO$_3$-N began to accumulate in the aerobic compartment after Day 40. This was in accordance with the study of Holakoo (Holakoo et al. 2007), who reported that the high MLSS concentration would lead to the partial accumulation of the substrate.

**Specific nitrogen utilization rate**

To further understand the nitrogen removal mechanism in the reactor, batch experiments were conducted to study the specific activities of the sludge samples taken from the different compartments. Figure 8 shows the specific nitrogen utilization rate of the microorganisms taken from the aerobic compartment and anoxic compartment on Day 100. It can be seen in Figure 8 that the specific nitrification rate and denitrification rate of the microorganisms in the aerobic compartment were $11.61 \pm 0.70 \text{mg NH}_4\text{-N/g MLVSS h}$ and $4.37 \pm 0.26 \text{mg NO}_3\text{-N/g MLVSS h}$, respectively; while in the anoxic compartment they were $6.35 \pm 0.41 \text{mg NH}_4\text{-N/g MLVSS h}$ and $9.33 \pm 0.61 \text{mg NO}_3\text{-N/g MLVSS h}$, respectively. The specific nitrification rate and denitrification rate of the microorganisms taken from both compartments indicate that the nitrifiers and denitrifiers co-existed within the same compartment.
Comparing the specific nitrification rate with the denitrification rate of the microorganisms in the same compartment, it can be seen that the microorganisms from the aerobic compartment had a higher level of nitrifying capability, while the microbes from the anoxic compartment had a higher level of denitrifying capability. This result further confirmed the dominance of nitrifiers in the aerobic compartment and of denitrifiers in the anoxic compartment.

CONCLUSIONS

A membrane bioreactor combining anoxic and aerobic conditions was established to study the characteristics and mechanism of nitrogen removal. This study has shown that:

- The recirculation in the reactor resulted in the MLSS concentration and the morphological properties of the sludge being similar in the different compartments of the reactor; however, this did not prohibit the formation of a vertical oxygen gradient.
- As sludge accumulated in the MBR, the increased sludge concentration decreased the oxygen transfer coefficient, which in turn enhanced the formation of a vertical oxygen gradient. However, higher MLSS concentrations would lead to a partial accumulation of NH₄-N in the anoxic compartment and NO₃-N in the aerobic compartment.
- After 40 days of biomass accumulation, the vertical oxygen gradient separated the reactor into two different compartments: an aerobic compartment in the upper space and an anoxic compartment at the bottom of the reactor. The TN removal efficiency achieved in this reactor increased to 77%.
- The microbial observation, using PCR-DGGE analysis, as well as the specific nitrogen utilization rate measurements, indicated that the nitrogen removal in the reactor occurred via nitrification and denitrification processes: the aerobic compartment and anoxic compartment acted as active centers for the nitrification and denitrification reactions, in which ammonia nitrogen was converted to nitrate, and nitrate was then converted to nitrogen respectively.

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REFERENCES


