High rate nitrogen removal by the CANON process at ambient temperature

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ABSTRACT

Completely autotrophic nitrogen removal over nitrite (CANON) is a cost-effective nitrogen removal process. Implementation of the CANON process relies on the cooperation of ammonium-oxidizing and Anammox bacteria, as well as the inhibition of nitrite-oxidizing bacteria. Strict limitations on dissolved oxygen (DO) concentration in the reactor, and the addition of sufficient inorganic carbon in the influent, were adopted as the main operational strategies. The reactor was fed with synthetic inorganic wastewater composed mainly of NH$_4$$^+$-N, and operated for 106 days. Stable nitrogen removal rates (NRR) of around 1.4 kg N m$^{-3}$ d$^{-1}$ were obtained at ambient temperature. Morphological characteristics and analysis of bacterial community confirmed the formation of functional outer aerobic and inner anaerobic granular sludge, providing evidence of stable nitrogen removal.

Key words | ambient temperature, anammox, CANON, DO limitation, nitrogen removal, SEM, sufficient inorganic carbon, 16S r RNA

INTRODUCTION

In municipal and industrial systems, wastewater discharge that contains large amounts of nitrogen can be toxic to aquatic life, deplete dissolved oxygen (DO), cause eutrophication in receiving water bodies, and affect the suitability of wastewater for reuse (Wang et al. 2010). Conventional wastewater treatment systems for nitrogen removal are currently limited by their high operational cost, which is due to the large amount of energy required to create aerobic conditions for bacterial nitrification, and is also due to the use of organic carbon to help remove nitrate by bacterial denitrification (Kartal et al. 2010). Practical and cost-effective nitrogen removal technologies are needed, especially for older plants with limited space for expansion (Downing & Nerenberg 2008). An innovative and promising direction which meets the above requirements has been proposed recently, requiring only a single oxygen-limited step to remove ammonium from wastewater (Zhu et al. 2008). The two sequential reactions reported rely on the simultaneous interaction of two groups of autotrophic bacteria under oxygen-limiting conditions (Third et al. 2001) (Equation (1)).

$$\text{NH}_4^+ + 0.85\text{O}_2 \rightarrow 0.435\text{N}_2 + 0.13\text{NO}_3^- + 1.3\text{H}_2\text{O} + 1.4\text{H}^+$$  

(1)

These microbiological processes for completely autotrophic nitrogen-removal over nitrite (CANON) can, in an autotrophic manner, convert ammonium into dinitrogen gas (Sliekers et al. 2003). The implementation of nitrogen removal from wastewater using the CANON process relies on the cooperation of ammonium-oxidizing bacteria (AOB) and Anammox bacteria, as well as the inhibition of nitrite-oxidizing bacteria (NOB). Conditions that favor AOB over NOB include low DO (Munich et al. 1996), high pH (Villaverde et al. 1997), and high free ammonia (FA) (Anthonisen et al. 1976). Yang et al. (2001a) reported that under strictly controlled DO concentrations the activity of AOB was not inhibited, but NOB activity was effectively inhibited. A better understanding of oxygen-limited conditions may be essential for improving and optimizing the CANON process to obtain effective nitrogen removal. It is also widely accepted that carbon limitation may be the main reason for the dramatic decrease in the growth and activity of nitrifying bacteria below neutral pH (Wett & Rauch 2005). Yang et al. (2001b) documented that sufficient influent inorganic carbon (IC) may play a positive role in the enrichment of Anammox microorganisms. For these reasons, further investigation is necessary.
to gain insight into the function and mechanism of available IC on the CANON process.

Here, the feasibility of first cultivating Anammox bacteria under progressively increasing DO concentrations, and subsequently inoculating nitrifying bacteria, is investigated as a novel start-up method for the CANON process. Sufficient IC and limited DO concentrations were used as operating conditions at room temperature, to initiate nitrogen removal in this study. In addition, the microbial community structure of AOB and anammox bacteria in the CANON reactor were analyzed by the denaturant gel gradient electrophoresis (DGGE) of PCR-amplified 16S rRNA gene.

**MATERIALS AND METHODS**

**Experimental setup and operational strategy**

An upflow column reactor was used in this study (Figure 1). The reactor had an effective volume of 2.5 L and was operated in upflow mode with the influent introduced from the bottom by a peristaltic pump (Cassette Tube Pump SMP-21, Japan). Mixing was provided by a centrally located axial mixer with a 9 cm mixing blade, operated at 150 rpm. Given the high mixing rate, the system approximated a completely mixed tank reactor, where the bulk liquid concentrations are equal to the effluent concentrations. A settling tank was used for sludge sedimentation and recycling. A scraper arm was centrally located in the settler and allowed to rotate, and solids were recycled to the reactor from the bottom of the settler. The recirculation flow of solids was 100% of the influent flow rate.

From the point of view of process operation, the 106-day study can be divided into three periods (Table 1). In period I, the reactor was inoculated with enriched Anammox granular sludge, which was taken from an upflow column reactor with a novel spiral style used as a gas solid separator (GSS) (Yang et al. 2006c), and the initial concentration of suspended solids (MLSS) was 8 g/L. The DO concentration in the influent tank was increased progressively from 1 to 5 mg/L to cultivate bacteria attached to the outer layer of the Anammox granular sludge and capable of consuming DO in the reactor. In period II, nitrifying bacteria taken from a partial nitrification (PN) reactor (Yang et al. 2005a) were inoculated into the CANON reactor, with an initial MLSS concentration of around 3 g/L. Air was supplied to the reactor at a low flow rate of around 0.5 L/min, chosen to be effective for NH$_4^+$-N oxidation, but inadequate for the further oxidation of NO$_2^-$-N. In period III, the stable and high-rate CANON process was realized, with the DO concentration in the reactor maintained below 0.1 mg/L.

Experiments were conducted at ambient temperature, and the pH in the reactor was kept at 7.5–7.7 by the addition of acidic (1N HCl) and alkaline (1N NaOH) solutions. An aquarium air pump and a porous stone provided aeration in the form of fine bubbles. During the operational period, synthetic wastewater with a composition of NH$_4^+$-N 40–140 mg/L, NO$_2^-$-N 0–130 mg/L, KHCO$_3$ 1,500–3,000 mg/L, KH$_2$PO$_4$ 54 mg/L, FeSO$_4$·7H$_2$O 9 mg/L and EDTA 5 mg/L was used.
Analytical methods

The pH and DO values in the reactor were determined using a pH meter (B-211, Horiba, Kyoto, Japan) and a DO meter (D-55, Horiba, Kyoto, Japan), respectively. MLSS analysis was performed by drying the contents of the reactor at 105 °C in an evaporating dish. Influent and effluent samples were either analyzed immediately or stored in a refrigerator at 4 °C until analyses could be carried out. The NH4-N concentration was analyzed by the modified phenate method, using o-phenylphenol (OPP) (Kanda 1995). The NO2-N and NO3-N concentrations were measured using a colorimetric method. IC and total nitrogen (TN) were analyzed using a TN meter (Shimadzu, Japan).

SEM observation

The surface and inner parts of the Anammox granules were observed using scanning electron microscopy (SEM). Samples were first washed in a 0.1 M phosphate buffer solution (pH 7.4) for 5 min. The samples were then hardened for 90 min in a 2.5% glutaraldehyde solution prepared with the phosphate buffer solution. Next, samples were washed in the buffer solution three times for 10 min each, and fixed for 90 min in a 1.0% OsO4 solution prepared using the phosphate buffer solution. After washing samples three times for 10 min each in the buffer solution, the samples were dehydrated for 10 min each in serially graded solutions of ethanol at concentrations of 10, 30, 50, 70, 90 and 95%. SEM observations were conducted using a scanning electron microscope (JEOL, JSM-5310LV, Japan).

Cloning and sequencing of 16S rRNA gene

The purified fragments were ligated into the EcoRV site of pBluescript II KS+ (Stratagene, USA), and Escherichia coli DH 5α was transformed using the constructed plasmids. White colonies including the insert were randomly chosen and the plasmids were extracted by the alkaline method. The nucleotide sequences were determined with a 3130×1 genetic analyzer and BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems, USA). The sequences determined in this study were compared with the sequences in the nr-database using the basic local alignment search tool (BLAST) program available on the NCBI web site.

RESULTS AND DISCUSSION

Nitrogen removal performance of the CANON reactor

In this study, the CANON reactor was continuously fed with synthetic wastewater for 106 days. Performance of the CANON system in terms of various nitrogen removal rates (NRR) is shown in Figure 2.

Period I

To start up the CANON reactor, Anammox granules were first introduced into the reactor. We cultivated Anammox in the presence of progressively higher DO concentrations, from 1 to 5 mg/L. During the first 15 days of reactor operation, the NLR was increased gradually, to 3 kg N m⁻³ d⁻¹, with the effluent NO2-N concentration ranging from 5 to 35 mg L⁻¹, indicating unstable reactor performance. The increasing DO concentration, ambient temperature and high NLR were considered to be responsible for the unsteady performance of the reactor. However, Liu et al. (2008) pointed out that the cultivation of Anammox is not restricted to completely anaerobic conditions. In addition, Yang et al. (2006) documented that a stable NRR of 17.5 kg N m⁻³ d⁻¹ can be obtained at operating temperatures of 23 ± 2 °C. Therefore, Anammox granules were expected to adapt to the environmental conditions of high DO concentration and ambient temperature. From day 16 to day 30, Anammox sludge seemed to gradually adapt to the operational conditions, with the DO values in the reactor always under 0.05 mg/L, indicating the existence of some kinds of bacteria that could consume oxygen and make the reactor anoxic. During this period, the maximum NLR of 4.8 kg N m⁻³ d⁻¹ was obtained with a corresponding NRR of 3.6 kg N m⁻³ d⁻¹ under an influent DO concentration of around 5 mg/L. The rapid adaptation to experimental conditions should also be attributed to the cultivation of Anammox sludge under a high NLR prior to this study, resulting in sludge with elevated activity.

Period II

The CANON reactor was inoculated with nitrifying sludge on day 31, to convert suitable NH4-N to NO2-N. Air was supplied to the reactor at a flow rate of around 0.5 L/min, to cultivate AOB. We progressively increased the influent NH4-N concentration from 40 to 100 mg/L, with a constant influent NO2-N concentration of 20 mg/L, to ensure the
consumption of NH$_4$-N and to prevent potential inhibition by high FA. The TN removal rate was around 73%, with an average effluent NO$_2$-N concentration below 10 mg/L during this period, except for some occasional spikes, indicating the establishment of stable cooperation between AOB and Anammox bacteria. The adaptation of Anammox bacteria to a high DO concentration in period I was considered one of the primary reasons for the reactor avoiding experiencing DO shock in some areas.

**Period III**

A maximum NLR of 1.6 kg N m$^{-3}$ d$^{-1}$ was achieved on day 71, with an influent NH$_4$-N concentration of 140 mg/L and an HRT of 2.1 h. Although aeration was supplied at rates as high as 0.8 L/min, the DO concentration in the reactor was maintained near to 0 mg/L during this period. Between day 71 and day 106, the reactor experienced a sharp increase in NRR, from 0.98 to 1.44 kg m$^{-3}$ d$^{-1}$, under a constant NLR of 1.6 kg N m$^{-3}$ d$^{-1}$. An important performance characteristic is the nitrogen removal efficiency. A high NLR did not lead to a significant decrease in nitrogen removal efficiency during this period, indicating the feasibility of operating the CANON reactor at ambient temperature and under conditions of limited DO.

**Effects of limited oxygen concentration**

A requirement of the stable performance of the CANON process is that challenges encountered during reactor operation must be addressed. Oxygen is a co-substrate in the nitrification reaction, and its concentration greatly affects the reaction rate of both ammonium and nitrite oxidizers (Chuang et al. 2007). The high oxygen demand of the surface layer created an anoxic zone in the internal part of the granule, where the Anammox process could take place (Vázquez-Padín 2010). During the stable running period of the reactor, the DO concentration was always maintained below 0.1 mg/L, which was advantageous in that it delayed the reaction until the formation of NO$_3$-N, instead of NO$_2$-N.
Thereafter, the appropriate amount of produced NO₂⁻N reacted with the residual NH₄⁺-N under anoxic conditions in the inner granules, resulting in a high rate of nitrogen removal during the CANON process.

In this study, strictly limiting the DO concentration in the reactor was adopted as one of the main control strategies, through which the AOB are allowed to be active in the outer layers of granule, thereby producing a suitable amount of NO₂⁻N for the Anammox organisms that are active in the inner layers. A substantial amount of NO₃⁻-N tends to accumulate as a by-product when sufficient DO concentrations are supplied to the reactor. Currently, a limited oxygen concentration plays an integral role in the inhibition of NOB. Although NOB are very likely to co-exist in the community, they are out-competed at low DO concentrations, due to their weaker affinity for oxygen as compared with AOB, leading to the limited accumulation of NO₃⁻-N (Chuang et al. 2007). In accordance with CANON equilibrium, a lower NO₃⁻-N/ NH₄⁺-N ratio of 0.08 ± 0.01 (theoretically 0.13) was obtained in this study, leading to a nitrogen removal efficiency of as high as 90%. One of the reasons for this phenomenon is the sufficient addition of IC. Yang et al. (2010b) assumed that some types of enzyme activity in the Anammox process increase under high IC concentrations, and further promote the occurrence of different types of nitrogen-transformation. The second explanation is the probable coexistence of heterotrophic denitrifiers. Due to the presence of anoxic zones in the reactor and inside the sludge, as well as the organic carbon provided by bacterial lysis, heterotrophic denitrification was very likely to occur. Third, the probable emission of N₂O is also responsible for the high rate of nitrogen removal observed, as N₂O is more likely to be formed under lower DO concentrations found during nitrification (Park et al. 2000). Based on the above conclusions, the simultaneous existence of AOB, Anammox and denitrifiers in the microbial community of the reactor played an important role in the high rate of nitrogen removal achieved through the CANON process. However, to develop environmentally conscious operational practices, a realistic understanding of how the bacterial community is affected is required in the near future.

**Effects of sufficient inorganic carbon**

Several studies have documented the importance of IC as a facilitating factor for NOB and a limiting factor for AOB (Guisasola et al. 2006; Torà et al. 2010). Yang et al. (2010b) pointed out that sufficient IC concentration in the influent may be indispensable for Anammox bacteria. Due to the fact that the stable performance of the CANON process relies on the cooperation of PN and Anammox processes, the IC is very likely to play an integral role in the start-up of the CANON process. From day 0 to day 30, sufficient IC was supplied to the reactor, with the corresponding IC/TN ratio maintained at 0.67 ± 0.05. Significant positive effects of IC on nitrogen removal performance in the Anammox process were observed, with a maximum NLR of 4.8 kg N m⁻³ d⁻¹ obtained even under a high influent DO concentration of 5 mg/L. Additionally, as nitrification is an alkalinity-consuming process, the PN process in the reactor was responsible for IC loss, and sufficient alkalinity enabled the obvious and stable accumulation of NO₂⁻ during the PN process. From day 51 to day 106, a stable NH₄⁺-N removal rate of 1.2 ± 0.2 kg N m⁻³ d⁻¹ was obtained with a sufficient influent IC concentration of 400 ± 10 mg/L and a corresponding influent IC/TN ratio of 2.75 ± 0.05. Potential explanations for the contribution of IC to the improvement of the CANON process are proposed as follows. First, IC has proven to be a key parameter for controlling the effluent NH₄⁺-N to NO₂⁻-N molar ratio in the PN process, which is indispensable to the subsequent Anammox process (Ganigüé et al. 2009). In addition, sufficient IC serves as a pH buffer, providing the Anammox bacteria with a degree of protection from changes in pH (Yang et al. 2000a). However, the relationship between the IC/NH₄⁺-N ratio and the cooperation of PN and Anammox in the CANON process is still poorly understood and requires further investigation.

**Comparison of nitrogen removal performance among CANON reactors**

The results obtained in this study are most likely due to the suitable operation strategies applied herein. Traditionally, two strategies were generally applied to start-up the CANON process: (1) inoculating an Anammox reactor with nitrifying biomass; and (2) operating a PN reactor under oxygen-limited conditions and then inoculating with an Anammox biomass (Liu et al. 2008). An obvious decreasing trend in Anammox activity was observed when the first strategy was applied (Sliekers et al. 2003). With respect to the second strategy, the PN reactor is not conducive to full contact between the Anammox sludge and the substrate, leading to inefficient nitrogen removal. The operational strategy applied in this study was based on an improvement of the first strategy, as referenced above. Prior to the inoculation of an Anammox reactor with a nitrifying biomass, we cultivated Anammox in the presence of progressively
increasing DO concentrations, leading to the adaptation of Anammox to high DO concentrations. The oxygen-limited strategy is also of primary importance for both producing a suitable ratio of NH$_4$-N/NO$_2$-N and keeping the Anammox sludge from producing inhibition effects. Furthermore, we cannot exclude positive effects from the reactor, which may have helped to strengthen the efficiency of the CANON process to some extent and to improve NRR.

**Sludge characteristics**

Investigation of the physical properties of nitrifying biomass could allow for greater understanding of the stable nitrogen removal achieved by the CANON process. Morphological characteristics of the biomass were observed using microscope images and SEM. There appeared to be many cavities inside the granules (Figure 3(a)), resulting in the entrapment of nitrogen bubbles in the outer and inner layers of the granules. With the continuous growth and multiplication of bacteria in a granule, some dispersed bacteria in the medium may also adhere to the granule and be integrated into the bacterial aggregate (Liu et al. 2005). Trigo et al. (2006) also reported that the Anammox aggregates tend to grow together. After the 90-day enrichment, the granules had a relatively well-defined and dense inner structure (Figure 3(b)), compared with the seed sludge. Once the initial aggregates form, subsequent granulation could be regarded as a mere phenomenon resulting from an increase in biofilm thickness. The shear stress provided by a centrally located axial mixer led to a glandular-like flock formation. Microscope images showed that Anammox bacterial cells were surrounded by activated sludge (Figure 3(c-f)), providing evidence of the formation of functional outer aerobic and inner anaerobic sludge. With the increase in the biofilm thickness of the surrounding sludge, coexistent bacteria capable of nitrification reach a suitable ratio, and the subsequent nitrogenous concentration can be treated by Anammox sludge in the inner part of the granule. In addition, the formation of an anoxic zone inside the granule provided a better environment for the Anammox bacteria. As shown in Figure 3(c-f), the insides of the Anammox granules were red, rather than black, which is a feature of active biomass (Chen et al. 2010). However, the authors maintain that the adaptation of Anammox to a high DO concentration was of importance in protecting Anammox from potential DO shock, especially during the period in which the functional outer aerobic and inner anaerobic sludge was immature.

**Bacteria community analysis for CANON reactor**

We analyzed the bacterial community by using universal eubacterial primers to investigate whether these bacteria are coexisting in this reactor after 102 days of cultivation with synthetic wastewater. Table 2 shows the main results of homology search for 16S rRNA gene sequences in the

![Figure 3](https://iwaponline.com/wst/article-pdf/65/10/1826/441974/1826.pdf)
community existing in the CANON reactor. Five clones had 97% sequence identities with ammonia-oxidizing bacterium *Nitrosomonas europaea strain ATCC 25978*. 5 of 25 had 100% sequence identities with *Candidatus Kuenenia stuttgartiensis* and uncultured anoxic sludge bacterium KU2, which was detected in the Anammox reactors of our previous studies and known to be an Anammox bacterium. Additionally, we detected thirteen clones having 97% sequence identities with *Thermomonas haemolytica isolate S6*, which was thought to be the denitrifying and phenol-degrading organism (Baek et al. 2005). The bacterial species were responsible for the stable performance of the reactor. 16S rRNA gene analysis revealed that *Nitrosomonas, Candidatus Kuenenia*, and *Thermomonas*-related bacteria were enriched during the 102 days of cultivation with the synthetic wastewater. In this study, Anammox bacteria were enriched during the 102 days of cultivation with the synthetic wastewater. In this study, Anammox bacteria were enriched during the 102 days of cultivation with the synthetic wastewater. In this study, Anammox bacteria were enriched during the 102 days of cultivation with the synthetic wastewater. In this study, Anammox bacteria were enriched during the 102 days of cultivation with the synthetic wastewater. In this study, Anammox bacteria were enriched during the 102 days of cultivation with the synthetic wastewater. In this study, Anammox bacteria were enriched during the 102 days of cultivation with the synthetic wastewater. In this study, Anammox bacteria were enriched during the 102 days of cultivation with the synthetic wastewater. In this study, Anammox bacteria were enriched during the 102 days of cultivation with the synthetic wastewater. In this study, Anammox bacteria were enriched during the 102 days of cultivation with the synthetic wastewater. In this study, Anammox bacteria were enriched during the 102 days of cultivation with the synthetic wastewater. In this study, Anammox bacteria were enriched during the 102 days of cultivation with the synthetic wastewater.

### Table 2 | Homology search results for 16S rRNA gene sequences of the main bacterial members in the CANON reactor

<table>
<thead>
<tr>
<th>OTU</th>
<th>Taxon</th>
<th>Accession</th>
<th>Identity</th>
<th>Number of clones</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Uncultured anoxic sludge bacterium KU2</td>
<td>AB054007</td>
<td>100</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td><em>Candidatus Kuenenia stuttgartiensis</em></td>
<td>CT573071</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Uncultured <em>Nitrosomonas</em> sp. clone G67</td>
<td>GQ891825</td>
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<td>5</td>
</tr>
<tr>
<td></td>
<td><em>Nitrosomonas europaea strain ATCC 25978</em></td>
<td>GQ451713</td>
<td>97</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Nitrosomonas</em> sp. R5c47</td>
<td>AF386749</td>
<td>97</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td><em>Thermomonas haemolytica isolate S6</em></td>
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<tr>
<td></td>
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<td>AJ786786</td>
<td>97</td>
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</tr>
</tbody>
</table>

Note: Operational taxonomic units (OTUs) were defined by a 1% distance level in nucleotide sequences.

are needed to quantify each activity of ammonia oxidizing, Anammox and denitrifying, respectively.

### CONCLUSION

In this study, we confirmed that the CANON process is reliable for treating NH4\(^+\)-N contained in wastewater. A stable NRR of 1.44 kg N m\(^{-3}\) d\(^{-1}\) was obtained, which is the highest level ever reported at ambient temperatures. Limited oxygen concentration and a sufficient addition of IC were found to be a proper operational strategy for the start-up of the CANON process. The formation of functional outer aerobic and inner anaerobic sludge was observed by microscopy, providing evidence of stable CANON performance. 16S rRNA gene analysis revealed that *Nitrosomonas, Candidatus Kuenenia* and *Thermomonas*-related bacteria were enriched during the 102 days of cultivation with the synthetic wastewater.

### REFERENCES


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