Coupled anaerobic ammonium oxidation and hydrogenotrophic denitrification for simultaneous NH$_4$-N and NO$_3$-N removal

Tatsuru Kamei, Rawintra Eamrat, Kenta Shinoda, Yasuhiro Tanaka and Futaba Kazama

ABSTRACT

Nitrate removal during anaerobic ammonium oxidation (anammox) treatment is a concern for optimization of the anammox process. This study demonstrated the applicability and long-term stability of the coupled anammox and hydrogenotrophic denitrification (CAHD) process as an alternative method for nitrate removal. Laboratory-scale fixed bed anammox reactors (FBR) supplied with H$_2$ to support denitrification were operated under two types of synthetic water. The FBRs showed simultaneous NH$_4$-N and NO$_3$-N removal, indicating that the CAHD process can support NO$_3$-N removal during the anammox process. Intermittent H$_2$ supply (e.g. 5 mL/min for a 1-L reactor, 14/6-min on/off cycle) helped maintain the CAHD process without deteriorating its performance under long-term operation and resulted in a nitrogen removal rate of 0.21 kg-N/m$^3$/d and ammonium, nitrate, and dissolved inorganic nitrogen removal efficiencies of 73.4%, 80.4%, and 77%, respectively. The microbial community structure related to the CAHD process was not influenced by changes in influent water quality, and included the anammox bacteria ‘Candidatus Jettenia’ and a Sulfuritalea hydrogenivorans-like species as the dominant bacteria even after long-term reactor operation, suggesting that these bacteria are key to the CAHD process. These results indicate that the CAHD process is a promising method for enhancing the efficiency of anammox process.

Key words | anammox, hydrogen gas, hydrogenotrophic denitrification, nitrate

INTRODUCTION

The anaerobic ammonia oxidation (anammox) process relies on the activity of chemoautotrophic bacteria (so called anammox bacteria), which converts ammonium nitrogen (NH$_4$-N) and nitrite nitrogen (NO$_2$-N) into nitrogen gas (N$_2$) and some nitrate nitrogen (NO$_3$-N) (Strous et al. 1998). The anammox process promotes aeration and electron donor addition cost savings of 50% and 100%, respectively, compared to the current nitrification and denitrification processes for NH$_4$-N removal. To exploit this high cost effectiveness, numerous studies have attempted to apply the anammox process to treat wastewater containing high NH$_4$-N concentrations, such as landfill leachate, digested wastewater, and industrial wastewater (e.g. Zhang et al. 2011; Isaka et al. 2017). Based on recent efforts, a commercial model of an anammox plant has been developed and widely applied at the plant-scale at over 100 sites (Lackner et al. 2014). However, the anammox process cannot readily remove NO$_3$-N due to the physiological characteristics of anammox bacteria. Moreover, NO$_3$-N, an industrial byproduct found in effluents, is a major concern in the anammox wastewater treatment process design, more so in the case of high nitrogen loadings. This is because, as revealed in the stoichiometry of the anammox process (Equation (1)), approximately 26% of NH$_4$-N is converted into NO$_3$-N (Sliekers et al. 2002), and this could lead to the concentration of NO$_3$-N exceeding the allowed limit in wastewater if the concentration of NH$_4$-N is high in the untreated wastewater. Thus, residual NO$_3$-N removal should be conducted in the effluent from anammox process. Further, the effluent from anammox process usually contains NO$_3$-N and residual NH$_4$-N, resulting in fluctuations in the NO$_2$-N generation efficiency in partial nitrification.
units, which are commonly installed in anammox treatment facilities (Xie et al. 2018). Therefore, secondary treatment is required after the anammox process to simultaneously remove residual NH$_4$-N and NO$_3$-N. As such, both NO$_3$-N removal by the anammox process and simultaneous NH$_4$-N and NO$_3$-N removal are necessary to enhance the treatment efficiency of the anammox process.

$$\text{NH}_4^+ + 1.32\text{NO}_2^- \rightarrow 1.02\text{N}_2 + 0.26\text{NO}_3^- + 2.03\text{H}_2\text{O} \quad (1)$$

Coupling the anammox process with heterotrophic denitrification is an option to enable NO$_3$-N removal and simultaneous NH$_4$-N and NO$_3$-N removal. This has been developed for the treatment of municipal wastewater containing organic carbon (Sumino et al. 2006; Takekawa et al. 2014). However, the influent of the anammox treatment system generally do not contain organic carbon, because the anammox process is inhibited by the presence of organic carbon (Ni et al. 2012). Activation of heterotrophic denitrification requires the addition of organic carbon, and applying this technology to mainstream anammox treatments could cause further water quality deterioration. Therefore, coupled anammox and denitrification that can be operated under inorganic conditions must be developed.

We previously evaluated the concept of simultaneous NH$_4$-N and NO$_3$-N removal in inorganic wastewater via anammox and autotrophic hydrogenotrophic denitrification (HD), which drives the denitrification process via NO$_3$-N and NO$_2$-N reduction (Lee & Rittmann 2002). This combination of the anammox and HD processes (hereafter referred to as the CAHD process) uses hydrogen gas (H$_2$) as an electron donor in the NO$_3$-N reduction step (Figure 1).

The estimated equation for the simultaneous removal of NH$_4$-N and NO$_3$-N via the CAHD process was calculated by combining the anammox and HD processes from a previous study (Kamei et al. 2015) (Equation (2)).

$$\text{NH}_4^+ + 1.06\text{NO}_3^- + 1.32\text{H}_2 \rightarrow 1.02\text{N}_2 + 3.32\text{H}_2\text{O} \quad (2)$$

This equation reveals that almost the same amount of NH$_4$-N and NO$_3$-N could be removed via the CAHD process. Since both microbial processes are autotrophic reactions (Smith et al. 1994; van de Graaf et al. 1996), excess sludge production could likely be minimized. H$_2$, unlike other electron donors (e.g. methanol, ethanol), does not inhibit the anammox process, and can be utilized in the coupling anammox and denitrification process for the simultaneous removal of NH$_4$-N and NO$_3$-N (Waki et al. 2015). Supplying H$_2$ can remove residual oxygen in influent water, which supports and stabilizes the anammox and HD processes in the CAHD process. Including this, the deterioration of the anammox process induced by electron donor addition may be neglected in the CAHD process.

Furthermore, H$_2$ utilization as an electron donor in this process can provide several advantages. Since HD activity can be controlled by changing the dose of H$_2$, the denitrification performance of CAHD process can be controlled and changed easily. On the other hand, a secondary treatment system for residual electron donor can be eliminated in the CAHD process, since H$_2$ is naturally released from treated water to the atmosphere.

Recently, complete oxidation of NH$_4$-N to NO$_3$-N by nitrification and subsequent coupling denitrification and anammox is focused as a new concept of anammox treatment, since complete nitrification is not required, sensitive system control compared to partial nitrification (Si et al. 2018). According to current research work, the CAHD process can also be applied to the treatment of inorganic wastewater containing only NH$_4$-N, by combining nitrification for oxidizing NH$_4$-N to NO$_3$-N. The CAHD process could be a promising technology for wastewater treatment of anammox process based on the several advantages mentioned above. To develop the CAHD process, the key factors for combining the two processes, the performance stability, and the microbial structure, should be evaluated comprehensively. However, limited information is available on the nitrogen removal efficiency of the CAHD process, although many studies have been conducted on the anammox and HD processes independently. Therefore, in this study, we evaluated (1) the performance stability of a synthetic inorganic wastewater treatment using CAHD process to estimate the long-term performance of the

![Figure 1](https://iwaponline.com/wst/article-pdf/79/5/975/562135/wst079050975.pdf)
system and (2) the major microbial species related to the CAHD process to help clarify the mechanism.

**METHODS**

**Experimental design**

We conducted two independent experiments to evaluate the performance of the CAHD process using synthetic inorganic wastewater (Experiment I), and the long-term stability of the nitrogen removal performance (Experiment II) in fixed-bed reactors (FBRs). The FBRs were inoculated with anammox sludge immobilized on non-woven fabric as a microbial seed. The anammox sludge used in this research was obtained via long-term cultivation of activated sludge. The initial biomass concentration was set to ∼12.5 g wet weight (w.w)/L in each experiment. Beside this, seed sludge for HD was not inoculated in these experiments, since we had found the concurrent occurrence of anammox and HD processes after cultivation of anammox sludge under H2 supplying condition as reported in previous research (Kamei et al. 2015). The FBRs were operated at a water temperature of 35 °C. H2 (more than 99.99% purity) produced from a water electrolytic H2 generator (HG-260; GL Science, Tokyo, Japan) was supplied through a commercial air stone diffuser with a 15×30-mm diameter. The H2 flow rates were set at 2.5 to 5 mL/min for a reactor volume of 1 L, and were changed based on the experimental setup. The dissolved oxygen (DO) concentration of the influent in each experiment was maintained below 0.3 mg/L to minimize the inhibition of anaerobic processes in the presence of oxygen.

Details of the experimental conditions are summarized in Table 1. In Experiment I, two sets of FBRs with 4.2-L working volumes were prepared and operated under both pH-controlled and pH-uncontrolled conditions to compare the nitrogen removal efficiencies. Figure 2 summarizes the overall experimental set up. The pH inside the reactor was maintained at 7.8–8.3 via the periodic supply of carbon dioxide gas. The FBRs were operated under four operational conditions (Runs 1–4) to evaluate the CAHD process through sequencing operations (Run 1) and batch-mode operations to elucidate the presence of the anammox process (Run 2), the HD process (Run 3), and the effect of reduced H2 on the CAHD process under the sequencing reactor operation (Run 4). The batch experiments of Run 2 and 3 were conducted only to pH controlled reactor after Run 1. Subsequently, the reactor was operated again in the sequencing mode setting for Run 4. In Experiment I, synthetic inorganic wastewater was prepared using tap water and adding reagents (Table 2). (NH4)2SO4, NaNO2, and NaNO3 were added as sources of NH4-N, NO2-N and NO3-N. The dose pattern was varied based on each run with the initial concentrations set at 40 mg-N/L for each of the nitrogen sources (Table 1).

In Experiment II, the long-term performance stability was evaluated under the conditions of Run 5. The FBRs were restarted and operated with intermittent H2 gas supply with the pH controlled at 7.8–8.3 via periodic carbon dioxide supply. A synthetic medium for anammox bacterial enrichment (Strous et al. 1998) was used as the influent water to assess the effect of intermittent H2 supply on the CAHD process (Table S1, available with the online version of this paper). In this experiment, (NH4)2SO4 and NaNO3 were added to obtain NH4-N and NO3-N concentrations of 40 mg-N/L each. The H2 was supplied in the same volume as that in Experiment I, although the reactor volume was changed to 2 L. To compare the performance changes and intermittent gas patterns, the H2 supply rate was calculated using Equation (3):

\[
\text{H2 supply ratio (%) } = 100 \times \left( \frac{T_{\text{H2}}}{T_A} \right) \tag{3}
\]

**Table 1** | Details of the experimental set up

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Reactor volume (L)</th>
<th>HRT* (hour)</th>
<th>Initial sludge (g w.w/L)</th>
<th>Runs</th>
<th>Operational mode</th>
<th>NH4-N (mg-N/L)</th>
<th>NO2-N (mg-N/L)</th>
<th>NO3-N (mg-N/L)</th>
<th>H2 flow rate (mL/min/L-reactor)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>4.2</td>
<td>7</td>
<td>12.5</td>
<td>Run 1</td>
<td>Continuous</td>
<td>40</td>
<td>40</td>
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<td>5</td>
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<td></td>
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<td>Run 2</td>
<td>Batch</td>
<td>40</td>
<td>–</td>
<td>40</td>
<td>–</td>
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<td></td>
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<td></td>
<td>Run 3</td>
<td>Batch</td>
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<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Run 4</td>
<td>Continuous</td>
<td>40</td>
<td>40</td>
<td>–</td>
<td>2.5, 3.8, 5</td>
</tr>
<tr>
<td>II</td>
<td>2</td>
<td>7</td>
<td>12.5</td>
<td>Run 5</td>
<td>Continuous</td>
<td>40</td>
<td>40</td>
<td>–</td>
<td>5 (Intermittent supply)</td>
</tr>
</tbody>
</table>

*HRT: Hydraulic retention time.
where $T_{H2}$ represents the H2 supply time during one cycle and $T_A$ is the duration of one cycle (20 min).

### Water sampling and analysis

The DO concentration was measured with a portable DO meter (Multi 3410; WTW, Weilheim, Germany). Influent and effluent water samples were collected and filtered through 0.45-μm membrane filters (Merck Millipore, Darmstadt, Germany). The samples were frozen and stored until the water quality analysis. To measure inorganic nitrogen concentrations, we used the phenate method to determine the NH4-N concentration (JWWA 1996) and the colorimetric and ultraviolet methods to determine the NO2-N and NO3-N concentrations (APHA 1998). Based on the results of the water quality analysis, the nitrogen loading rate (NLR) and nitrogen removal rate (NRR) were calculated using Equations (4) and (5).

$$\text{NLR (kg-N/m}^3\text{d)} = C_{in} \times Q / V \quad (4)$$

$$\text{NRR (kg-N/m}^3\text{d)} = \Delta C \times Q / V \quad (5)$$

where $C_{in}$ represents the dissolved inorganic nitrogen (DIN) concentration as the sum of NH4-N, NO2-N, and NO3-N in influent water and $\Delta C$ denotes the difference in DIN difference between influent and effluent water from the reactor. Meanwhile, $Q$ and $V$ represent the water flow rate and volume of the FBRs in each experimental condition, respectively.

### Microbial community analysis using bacterial 16S rRNA amplicon sequences

Samples for the microbial community analysis were collected from the FBRs of both Experiments I and II, and were collected from the surface of the microbial carriers (day 0) and suspended solids (on each operational day). Total nucleic acids were extracted from 0.1 g w.w of sample using a FastDNA SPIN Kit for Soil (MP-Biomedicals, Santa Ana, CA, USA). A partial region (the V4 hypervariable region) of the bacterial 16S rRNA gene was amplified by polymerase chain reaction (PCR) using the primers Eub-515f (5'-GTGCCAGCMGCGGCGGTAA-3') and Eub-806r (5'-GGACTACSVGGGTATCTAA-3') (Caporaso et al. 2011). The total bacterial concentration in each sample collected from the FBRs ranged from $1.8 \times 10^8$ to $2.4 \times 10^9$ copies/g w.w biomass. To minimize the PCR biomass, the PCR reaction comprised 20 cycles. The amplified products were subjected to next-generation sequencing using an Illumina MiSeq gene sequencer by a commercial sequencing service (FASMAC, Atsugi, Japan). In total, 48,105–87,786 sequence reads were obtained and classified into operational taxonomical units (OTUs) based on a 3%
divergence (i.e. 97% similarity). In total, 116–155 OTUs in each biomass sample were obtained after classification.

RESULTS AND DISCUSSION

CAHD process in synthetic inorganic wastewater treatment

Figure 3 presents the NH$_4$-N and NO$_3$-N removal efficiencies and NO$_2$-N concentration in the reactors. Under pH controlled conditions NH$_4$-N and NO$_3$-N concentrations decreased and NO$_2$-N concentrations slightly increased after supplying H$_2$. The pH-controlled reactor maintained simultaneous NH$_4$-N and NO$_3$-N removal for more 30 days continuously, with NH$_4$-N and NO$_3$-N removal efficiencies of 46% and 80%, respectively, at the end of the experiment. The NO$_2$-N concentration was maintained at around 5 mg-N/L throughout the experiment. By contrast, although NO$_3$-N removal and NO$_2$-N accumulation were observed in the FBR with uncontrolled pH, no significant NH$_4$-N removal was observed. Since simultaneous removal of NH$_4$-N and NO$_3$-N were detected, enhanced NRR and stability were only observed under the pH-controlled condition (Figure 4). The NRR in this reactor reached 0.18 kg-N/m$^3$/d for an NLR of 0.32 kg-N/m$^3$/d and achieved a DIN removal rate of 56% after 30 days of operation. By contrast, the reactor with uncontrolled pH showed a two-fold lower NRR, reaching 0.09 kg-N/m$^3$/d by day 30. In addition, the pH inside the reactor reached 8.7 after 5 days of operation, and then remained at around 8.6–8.9 until end of the operation. Since the reactor pH exceeded the suitable growth range for anammox bacteria (6.5–8.5) (Ali et al. 2018), the anammox process was inhibited in the pH uncontrolled reactor. Further, the NH$_4$-N and NO$_2$-N removal via the anammox process was suppressed, resulting in NO$_2$-N accumulation after NO$_3$-N reduction by the HD process in this reactor. In the CAHD process, denitrification mainly occurs via the anammox reaction. Thus, the pH uncontrolled reactor showed a lower NRR than the pH controlled reactor.

The results of the batch experiments in Runs 2 and 3 showed the simultaneous occurrence of the anammox and HD processes under pH-controlled conditions (Figure 5). Simultaneous removal of NH$_4$-N and NO$_3$-N with slight increments in NO$_2$-N were detected under conditions conducive to the anammox process (Run 2), where NO$_2$-N was added instead of NO$_3$-N (Figure 5(a)). The NO$_2$-N to NH$_4$-N removal ratio throughout the experimental period was 1.21, which was similar to the theoretical value based on the anammox process. In addition, NO$_3$-N removal with NO$_2$-N accumulation occurred only under H$_2$ supply in the batch experiment of Run 3 (Figure 5(b)). The occurrence of NO$_3$-N reduction and NO$_2$-N accumulation depended on the supply of H$_2$, which is suggestive of the concurrent occurrence of the HD process in the reactor and might support simultaneous NH$_4$-N and NO$_3$-N removal along with the anammox process. Further, pH control should be maintained to avoid anammox process deterioration and achieve simultaneous NH$_4$-N and NO$_3$-N removal via the CAHD process.

However, NH$_4$-N degradation was insignificant under H$_2$ supply in Run 3. Additionally, the NO$_3$-N removal was dominant under continuous operation in Run 1, as shown in the
performance results detected at day 30 (NH₄-N removal efficiency: 48%, NO₃-N removal efficiency: 80%) and the higher NO₃-N to NH-N removal ratio (NO₃-N/NH₄-N = 1.3–2.1), which was greater than the theoretical ratio for the CAHD process (NO₃-N/NH₄-N = 1.06), after achieving stable denitrification (Figure S1, available with the online version of this paper). These results suggest the predominance of NO₂-N reduction via the HD process even after achieving simultaneous NH₄-N and NO₃-N removals.

Insufficient NO₂-N supply for the anammox process would limit the denitrification efficiency of the CAHD process. Adequate H₂ supply induced NO₂-N reduction in the HD process, as reported by Lee & Rittmann (2002) (e.g. 0.18 g H₂ per 1 g NO₂-N reduction and 0.21 g of H₂ per 1 g NO₂-N reduction). Thus, the pH-controlled FBR was operated with reduced H₂ flow rates in Run 4 to minimize NO₂-N reduction and achieve NO₂-N accumulation in the HD process. However, the results revealed suppressed NO₃-N reduction in the HD process due to a lack of H₂, resulting in less NO₂-N accumulation and a decrease in the NH₄-N removal via the anammox process (Table S2, available online). To minimize NO₂-N reduction in the HD process and maintain the long-term operation of the CAHD process, we considered decreasing the contact time between H₂ and HD bacteria as an alternative method according to the results of Run 3 (Figure 5(b)). In Run 3, NO₃-N reduction and NO₂-N accumulation occurred under H₂ supply. Moreover, NO₂-N might have been consumed in the anammox process without H₂ supply where NH₄-N was removed; this is indicative of the suppression of NO₂-N reduction in the HD process without H₂ supply. Thus, intermittent H₂ supply can control the suppression of NO₂-N reduction in the HD process. Hence, the FBR was restarted and successfully operated with intermittent H₂ supply for another 180 days continuously in Experiment II, supporting the long-term maintenance of the CAHD process.

Long-term nitrogen removal performance under intermittent H₂ supply

After 30 days of operation of the CAHD process under continuous H₂ supply, intermittent H₂ supply was started under the conditions for Run 5 in Experiment II. The denitrification performance of the FBR was maintained, and simultaneous removal of NH₄-N and NO₃-N was detected continuously for more than 180 days (Figure 6). FBR
operation under a H2 supply rate of 70% (14/6 min on/off cycle) seemed to be much more stable than the other conditions (75%: 15/5 min on/off cycle; 60%: 12/8 min on/off cycle). Although the performance was occasionally fractured under these conditions, the effluent concentrations of NH4-N and NO3-N were below 15 and 10 mg-N/L in most samples, respectively (Figure 6(a)). At the end of the run, the NH4-N, NO3-N, and DIN removal rates reached 73.4%, 80.4%, and 77%, respectively, while achieving an NRR of 0.21 kg-N/m$^3$/d under an NLR of 0.27 kg-N/m$^3$/d (Figure 6(b)). Compared to the results of Experiment I, the NO3-N to NH4-N removal ratio was less than 1.6 after beginning intermittent H2 supply, which was closer to the theoretical value of the HD process than the results of Experiment I (Figure S2, available online). Therefore, intermittent H2 supply could avoid NO2-N reduction via the HD process while maintaining NO3-N reduction, resulting in good performance of the anammox process for NH4-N removal without a lack of NO2-N.

High gas production costs and lower gas use efficiencies are major bottlenecks of H2 use in microbiological nitrogen removal systems, including the CAHD process, since H2 has a low solubility in water (i.e. standard-state saturation concentration of 1.6 mg/L). Recent studies have attempted to overcome these bottlenecks with newly developed applications, for instance the development of closed and pressurized reactor operations (Epsztein et al. 2011), special gas diffusers such as hollow fiber membranes, and microbubble diffuser systems (Eamrat et al. 2016). Moreover, Epsztein et al. (2017) developed a sealed and pressurized reactor that yielded an H2 use efficiency of 100% during the denitrification process. Meanwhile, Eamrat et al. (2016) reported that the use of a microbubble diffuser with intermittent H2 supply enhanced the gas bubble retention time, resulting in H2 savings of 75% compared to continuous gas supply during NO3-N removal (Eamrat et al. 2016). Although further optimization of this process is necessary, the CAHD process could be maintained by controlling the reduction of NO3-N and accumulation of NO2-N via intermittent H2 supply. The H2 volume required to support NO3-N reduction and NO2-N accumulation in the CAHD process may be further minimized by applying this newly developed technology, making the CAHD process more feasible for application to the simultaneous removal of NH4-N and NO3-N from inorganic wastewaters, such as secondary effluent from anammox treatment and wastewater containing NH4-N after oxidizing of NH4-N to NO3-N by nitrification process.

Analysis of the microbial community in the CAHD process

We analyzed the changes in the microbial community structure of pH-controlled FBRs during Run 1 of Experiment I and Run 5 of Experiment II, and summarized these at the phylum level although a Proteobacteria abundance was
described based on subclass classification (Figure 7(a)). The major taxa in each sample included Planctomycetes, β-Proteobacteria, Chlorobi, and Armatimonadetes. However, the relative abundances of Planctomycetes and β-Proteobacteria changed markedly after beginning H2 supply. Consequently, β-Proteobacteria became the predominant taxon in each condition, accounting for 40.6% (day 30, Run 1, Experiment I) and 70.6% (day 171, Run 5, Experiment II) of the total bacteria. Moreover, it occurred in the presence of NO3-N reduction in both experiments. By contrast, although the relative abundance decreased in the presence of H2, Planctomycetes was the second-most dominant taxon over the course of the operational period in both experiments.

The major bacterial genera were ‘Candidatus Jettenia’ in Planctomycetes and Dok59 in β-Proteobacteria in both Experiments I and II (Figure 7(b)), indicative of similar microbial communities in the inoculum and suspended sludge in both FBRs, even though the wastewater types and operational periods differed. The genus ‘Candidatus Jettenia’ is known for its activity in the anammox process, including several proposed species (Ali et al. 2015). In Experiment II, this genus still accounted for ~15.2% of the total bacteria after 180 days of operation. In addition, another anammox genus, ‘Candidatus Brocadia’, appeared and accounted for 1.5% of the total bacteria on day 171, indicating that the reactor conditions were still conducive to anammox bacterial growth, although the overall relative abundance of anammox bacteria decreased in the suspended sludge.

The changes in sludge appearance also suggested the existence of the anammox process in the reactor during long-term reactor operation in Experiment II (Figure S3, available online). Brownish-colored sludge accumulated in the reactor, and most of the sludge became brownish over the course of Experiment II. However, the microbial carrier as the inoculum maintained a reddish color with tiny anammox granules in the suspended sludge. Since the water quality analysis results revealed NH4-N removal, these results indicated that the anammox process, and thus nitrogen removal activity, continued in the reactor even after long-term operation with intermittent H2 supply in Experiment II.

After starting H2 supply, the genus Dok59 increased and became the dominant genus, ahead of the genera Thauera and Hydrogenophaga, which are hydrogen-dependent denitrifiers (Kämpfer et al. 2005; Mao et al. 2013). After long-term system operation in Experiment II, the relative abundance of this genus reached 65%, representing the dominant taxon. Dok59 is an unclassified genus in the family Rhodocyclaceae. Detailed information of Dok59 is very limited, although several studies have reported its
detection in biomass from anammox reactors (Chang et al. 2011). The homology of the Dok59 sequence from the next-generation sequencing analysis was compared to that in the BLAST database provided by the National Center for Biotechnology Information (NCBI) (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and showed 96% similarity to the Sulfuritalea hydrogenivorans strain sk43H1T (family: Rhodocyclaceae). This species is an autotrophic bacterium isolated from fresh lake sediment, and can perform nitrate reduction using thiosulfate, elemental sulfur, and H2 as electron donors (Kojima & Fukui 2011). The Sulfuritalea hydrogenivorans-like species could exist as members of the microbial community of enriched anammox sludge by using NO3-N produced via the anammox process and alternative electron donors, and may have become highly enriched after beginning the experimental run by using H2 as the major electron donor. These results indicate that anammox bacteria and the Sulfuritalea hydrogenivorans-like species might perform simultaneous NH4-N and NO3-N reduction in the CAHD process.

**CONCLUSIONS**

Coupled denitrification via anammox and HD removed NH4-N and NO3-N simultaneously from synthetic inorganic wastewater, indicating that this coupled denitrification process could be applied to inorganic wastewater treatment. Intermittent H2 supply (e.g. 14/6 min on/off cycle; H2 flow rate: 5 mL/min/lreactor) might be a critical factor in maintaining the CAHD process for stable long-term performance, resulting in NH4-N, NO3-N, and DIN removal efficiencies of 73.4%, 80.4%, and 77%, respectively, after 180 days of operation. The Sulfuritalea hydrogenivorans-like species and the anammox bacteria ‘Candidatus Jettenia caeni’ coexisted in the FBRs, indicating that simultaneous NH4-N and NO3-N removal was achieved by these two microbes. Further studies should be conducted to optimize the H2 volume for NO3-N reduction and NO2-N accumulation in H2-dependent denitrification and evaluate the treatment performance and microbial community changes through experiments using actual wastewater.

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