Effects of hydraulic loading rate and aeration mode on nitrogen removal and nitrogen functional gene abundances in subsurface wastewater infiltration systems

Yafei Sun, Jing Pan, Shiyue Qi and Hexin Fei

ABSTRACT

Matrix dissolved oxygen, nitrogen removal and nitrogen functional gene abundances in two artificial aeration modes, continuous aeration (CA) and intermittent aeration (IA), in subsurface wastewater infiltration systems (SWISs) under different hydraulic loading rates (HLRs) were investigated. Aeration not only successfully created aerobic conditions at 50 cm depth, but also did not change anoxic or anaerobic conditions at 80 and 110 cm depths. Meanwhile, aeration significantly enhanced chemical oxygen demand, NH₄⁺-N, and total nitrogen (TN) removal and the enrichment of nitrogen removal functional genes (amoA, nxrA, napA, nirG, nirK and qnorB) compared to the non-aerated SWIS, especially for high HLRs. IA SWIS (79.7%–85.8%) had a better performance on TN removal compared with CA SWIS (73.8%–82.2%) when the HLRs ranged from 0.06 to 0.3 m³/(m²·d). Intermittent aeration is a sensible strategy to achieve high HLR, good nitrogen removal performance and comparatively low operation cost for SWISs.

Key words | continuous aeration, denitrification, intermittent aeration, nitrification

INTRODUCTION

Subsurface wastewater infiltration systems (SWISs) have received increasing attention in recent years due to their high organics and phosphorus removal performance, and low construction and operation costs (Li et al. 2011; Pan et al. 2016). However, nitrogen removal was quite low and pollutant removal decreased obviously under high hydraulic loading rate (HLR) (0.125 m³/(m²·d)), which remain as major challenges for SWISs (Li et al. 2012; Pan et al. 2015; Wang et al. 2015a). Insufficient supply of oxygen is the major cause of limited nitrogen removal in SWISs (Song et al. 2016; Yang et al. 2016). Artificial aeration has been regarded as an effective way to control dissolved oxygen (DO) (Fan et al. 2013; Song et al. 2016). Many studies have reported that artificial aeration was the effective way to achieve high pollutant removal when dealing with high strength wastewater (Wu et al. 2015; Song et al. 2016). Fan et al. (2013) concluded that an intermittent aeration strategy could enhance organics and nitrogen removal in subsurface flow constructed wetlands. Wu et al. (2015) evaluated the removal performances of organic pollutants and nitrogen in vertical flow constructed wetlands with and without intermittent aeration fed with different strengths of influent, and Song et al. (2016) investigated the effects of chemical oxygen demand (COD) to nitrogen ratios on pollutant removal in the SWISs with/without intermittent aeration. Microbiological nitrification and denitrification are the main mechanisms responsible for nitrogen removal in a SWIS (Li et al. 2011, 2012), which involve ammonia monoxygenase (amoA), nitrite oxidoreductase (nxrA), periplasmic nitrate reductase (napA), membrane-bound nitrate reductase (nirG), nitrite reductase (nirK/nirS), nitric oxide reductase (qnorB) and nitrous oxide reductase (nosZ) functional genes (Ji et al. 2012). Unfortunately, so far there have been no reports about the effects of HLR and aeration mode on nitrogen removal and nitrogen functional gene abundances in SWISs.

The main objectives of this paper were: (1) to investigate the characteristics of DO profiles along the SWISs under different aeration modes and HLRs; (2) to evaluate nitrogen removal performance and nitrogen functional genes (amoA, nxrA, napA, nirG, nirK, nirS, qnorB and nosZ) under different aeration modes and HLRs; (3) to identify optimal operation schemes for nitrogen removal.

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MATERIAL AND METHODS

System description and operation

Three laboratory-scale parallel SWISs made of Plexiglas vertical tubes (120 cm in height and 50 cm in diameter) were operated under different conditions in a greenhouse. As shown in Figure 1, they were composed of non-aerated (NA) units, continuous aerated (CA) units, and intermittent aerated (IA) units. CA and IA SWISs were composed of aerated units which consisted of air compressors, air tubes and micro-bubble diffusers at a depth of 40 cm. A distributing pipe was installed at 50 cm depth below the surface in each infiltration system. A micro-bubble diffuser and distributing pipe were surrounded by 5 cm of gravel (10–20 mm, diameter) to protect clogging and diffuse air. Ten centimetres of gravel (10–20 mm, diameter) was placed at the bottom to support the infiltration system and evenly distribute the treated water. The treated wastewater was collected at the bottom of the column near the outlet. DO electrodes were buried at the midpoint of SWISs at 50, 80 and 110 cm depths to monitor the matrix DO of pilot systems. Each infiltration system was filled with the same matrix, which consisted of 80% brown soil and 20% coal slag by weight. The brown soil was collected from the top 20 cm from Shenyang Ecological Station, containing 31.3 ± 0.3 g/kg of total organics, and having 159.2 ± 2.1 m²/kg of surface area and (8.9 ± 0.1) × 10⁻⁵ cm/s of hydraulic conductivity. The coal slag, 4–8 mm in diameter, purchased from a local market, was used to improve the permeability and absorption area of the matrix. The mixed matrix contained total organics of 28.1 ± 0.5 g/kg, total nitrogen (TN) of 0.8 ± 0.2 g/kg, total phosphorus of 0.6 ± 0.1 g/kg and pH 7.3.

CA and IA SWISs were subjected to aeration with an airflow rate of 2.0 ± 0.2 L/min. IA SWIS had four aerated/non-aerated (A/N) cycles every day. In each A/N cycle, the system was firstly subjected to aeration for an hour and then had 5-hour interval without aeration. The aeration would begin at 0:00, 06:00, 12:00 and 18:00, respectively. Influent HLRS of 0.06, 0.18 and 0.3 m³/(m² d) were adopted in this study.

Figure 1 | Schematic diagram of three SWISs. (1) Infiltration system body; (2) high-level tank; (3) liquid flow control valve and meter; (4) gas flow meter; (5) air compressor; (6) dissolved oxygen electrode; (7) distributing pipe; (8) micro-bubble diffuser; (9) outlet; (10) sampling port.
and the corresponding hydraulic retention times were 1.5, 0.5 and 0.3 d, respectively. Wastewater from Shenyang Normal University campus was pretreated in a septic tank prior to being fed into each SWIS continuously. The concentration ranges of wastewater after pretreatment were COD 185.5–261.8 mg/L, NH$_4^+$-N 32.3–43.8 mg/L, TN 36.4–46.7 mg/L, total phosphorus 3.2–6.8 mg/L, suspended solids 123–148 mg/L; water temperature was 23.2–24.7 °C, and pH was 7.0–7.4. All SWISs were operated for 2 months before sampling to allow systems to mature.

**Sample collection and analytical methods**

Water samples were taken from influent and effluent every 10 days at 6:00, 12:00, 18:00 and 24:00. Composite samples were used in each analysis. COD, NH$_4^+$-N and TN were analyzed according to Standard Methods (APHA 2005). Statistical checks were made at significant differences of 0.05 for all analyses using SPSS 12.0 (n = 10).

Matrix samples were collected from sampling ports (samples were labeled 50 cm layer, 80 cm layer and 110 cm layer, respectively) after each experiment. Four samples were taken from each layer and mixed well (approximately 5.0 g). After each on-site collection, the samples were stored in an ice incubator, and subsequently sent to laboratory for analyses. Functional gene abundances involved in nitrogen removal were quantitated by quantitative polymerase chain reaction (qPCR) technique according to Ji et al. (2012). Soil DNA kits (Omega, D5625-01) were used to extract and purify the total genomic DNA from the samples. Extracted genomic DNA was detected by 1% agarose gel electrophoresis and preserved at −20 °C until use. Quantitative analysis was performed on the target fragments of the following functional genes: amoA, nxxA, narG, napA, nirK, nirS, qnorB and nosZ using the primers listed in Table 1. The primers were synthesized by Shanghai Invitrogen Biotechnology Co. Ltd (China). Each primer concentration was 10 pmol/μL. The plasmids containing the functional genes (amoA, nxxA, napA, narG, nirK, nirS, qnorB and nosZ) were manufactured by the Sangon Biotechnology Company (Shanghai, China). The protocol and parameters for each target gene are summarized in Table 1. The standard samples were diluted to yield a series of 10-fold concentrations, and were subsequently used for qPCR standard curves. The R$^2$ values for the standard curves were more than 0.99. qPCR was performed on a Roche Lightcycler 480 real-time PCR detection system (Roche Diagnostics, Meylan, France) in final 20 mL volume reaction mixtures containing the following components: 10 mL SYBR Green I PCR master mix (Applied Biosystems, USA), 1 mL template DNA (sample DNA or plasmid DNA for standard curves), forward and reverse primers, and sterile water. qPCR was performed in a three-step thermal cycling procedure. Each qPCR was performed in 40 cycles and followed by a melting curve analysis. Sterile water was used as a negative control and the data obtained from the qPCR were normalized to copies per gram of biological carrier in the SWISs.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer</th>
<th>Amplification size (bp)</th>
<th>qPCR programs</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>amoA</td>
<td>amoA1F</td>
<td>491</td>
<td>3 min at 95 °C, 15 s at 95 °C, 20 s at 57 °C and 30 s at 72 °C.</td>
<td>Rotthauwe et al. (1997)</td>
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<td></td>
<td>amoA2R</td>
<td></td>
<td></td>
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<tr>
<td>nxxA</td>
<td>F1norA</td>
<td>323</td>
<td>3 min at 95 °C, 15 s at 95 °C, 20 s at 57 °C and 30 s at 72 °C.</td>
<td>Attard et al. (2010)</td>
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<tr>
<td></td>
<td>R2norA</td>
<td></td>
<td></td>
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<tr>
<td>narG</td>
<td>1960m2f</td>
<td>100</td>
<td>10 min at 95 °C, 15 s at 95 °C, 45 s at 58 °C and 30 s at 72 °C.</td>
<td>Lopez-Gutierrez et al. (2004)</td>
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<tr>
<td></td>
<td>2050m2r</td>
<td></td>
<td></td>
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<tr>
<td>napA</td>
<td>napAV17F</td>
<td>152</td>
<td>10 min at 95 °C, 15 s at 95 °C, 50 s at 57 °C and 30 s at 72 °C.</td>
<td>Bru et al. (2007)</td>
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<td></td>
<td>napA4R</td>
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<tr>
<td>nirK</td>
<td>nirK876F</td>
<td>165</td>
<td>3 min at 95 °C, 15 s at 95 °C, 20 s at 57 °C and 30 s at 72 °C.</td>
<td>Henry et al. (2004)</td>
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<td></td>
<td>nirK1040R</td>
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<tr>
<td>nirS</td>
<td>nirScd3aF</td>
<td>413</td>
<td>3 min at 95 °C, 15 s at 95 °C, 20 s at 57 °C and 30 s at 72 °C.</td>
<td>Kandeler et al. (2006)</td>
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<td></td>
<td>nirSR3cd</td>
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<tr>
<td>qnorB</td>
<td>qnorB2F</td>
<td>262</td>
<td>5 min at 95 °C, 15 s at 95 °C, 20 s at 56 °C and 8 min at 72 °C.</td>
<td>Gesche &amp; James (2003)</td>
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<td>qnorB5R</td>
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<td></td>
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<tr>
<td>nosZ</td>
<td>nosZ1527F</td>
<td>250</td>
<td>3 min at 95 °C, 15 s at 95 °C, 20 s at 57 °C and 30 s at 72 °C.</td>
<td>Scala &amp; Kerkhof (1998)</td>
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<td>nosZ1773</td>
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</table>
RESULTS AND DISCUSSION

DO profiles in an A/N cycle

Anaerobic and aerobic environments can be well distinguished via the DO profile in a SWIS (Fan et al. 2015). DO concentration profiles of NA, CA and IA SWISs in an A/N cycle are shown in Figure 2. DO concentrations along NA SWIS under different HLRs were below 1.0 mg/L. For different HLRs, DO concentrations detected at 50 cm depth of NA SWIS were in the range of 0.36–0.91 mg/L, and less than 0.26 and 0.03 mg/L at 80 and 110 cm depths, which indicated that NA SWIS was under anoxic or anaerobic conditions at 80 and 110 cm depths, and aerobic conditions were not good at 50 cm depth. Wang et al. (2010) showed that the prevailing conditions in SWISs were anoxic or anaerobic below a distribution pipe because air diffusion to the soil matrix was limited. In contrast, artificial aeration enhanced oxygen of the SWISs. DO concentrations at 50 cm depth of IA and CA SWISs were significantly higher than that of NA SWIS ($P < 0.05$), but no significant differences were observed between them at 80 and 110 cm depths ($P > 0.05$). Under different HLRs, DO concentrations were more than 6.52 mg/L during aeration and as high as 2.84 mg/L when supplementary aeration was turned off for IA SWIS at a depth of 50 cm. However, at depths of 80 and 110 cm, DO concentrations were below 0.46 and 0.18 mg/L during aeration, and below 0.38 and 0.09 mg/L without aeration, respectively. High aeration-induced DO concentrations were reduced by the decomposition of nutrients and organic matter, which may explain the decreasing tendency in the NA period of IA SWIS. For CA SWIS, DO concentrations were more than 6.52 mg/L at a depth of 50 cm and less than 0.46 and 0.11 mg/L at depths of 80 and 110 cm under different HLRs, respectively. Aerobic conditions were effectively developed at 50 cm depth and anoxic or anaerobic conditions were not changed at 80 and 110 cm depths within CA and IA SWISs. Sequential aerobic and anaerobic conditions were well developed by intermittent aeration. Zhong et al. (2014) reported that aerobic conditions occurred in the top section and anaerobic or anoxic conditions occurred in the subsequent sections in the matrix, which would favor nitrification and denitrification. Moreover, DO concentrations decreased with increasing HLR in NA, CA and IA SWISs. This phenomenon could be explained by the fact that more oxygen was consumed by oxidation of excessive organic matter and nutrients.

Nitrification and nitrogen removal performance

NH$_4^+$-N removal performances are shown in Figure 3. NA SWIS achieved removal rates of NH$_4^+$-N of 72.4%, 52.9% and 27.4% under the HLR of 0.06, 0.18 and 0.3 m$^3$/(m$^2$ d), respectively. Under the HLR of 0.18 and 0.3 m$^3$/(m$^2$ d), average NH$_4^+$-N concentrations in NA SWIS were 17.94 and 27.66 mg/L, which were higher than the class I value (15 mg/L) according to the discharge standard of pollutant for municipal wastewater treatment plants in China (GB18918-2002). Nitrification, as an aerobic chemo-auto-trophic microbial process, plays an important role in nitrogen removal, which occurs only when oxygen is present in a high enough concentration to support the growth of aerobic nitrifying bacteria (Fan et al. 2015). Most conventional SWISs fail to fulfill this first step due to insufficient oxygen supply. In order to improve the DO availability in SWISs, artificial aeration appeared to be the most effective alternative to guarantee sufficient oxygen supply.
As shown in Figure 3, average effluent NH$_4^+$-N concentrations of CA and IA SWISs were significantly lower than that of NA SWIS ($P < 0.05$). There were no significant difference between CA and IA SWISs ($P > 0.05$). It is generally accepted that DO concentrations above 1.5 mg/L are essential for better nitrification (Ye & Li 2013). In NA SWIS, DO concentrations were lower than 1.0 mg/L at all depths, which led to anoxic and anaerobic environment in the matrix, thus NH$_4^+$-N removal was seriously limited and average NH$_4^+$-N removal rate was below 73% under the HLR of 0.06, 0.18 and 0.3 m$^3$/m$^2$ d). HLR had little effect on NH$_4^+$-N removal in aerated SWISs within the scope of this study. DO concentrations were above 2.2 mg/L in the upper layers through artificial aeration in CA and IA SWISs, which favored the achievement of a high NH$_4^+$-N removal rate above 90% and effluent NH$_4^+$-N concentrations below 15 mg/L, even under the high HLR of 0.3 m$^3$/m$^2$ d).

Biological nitrification and denitrification is widely acknowledged to be the major nitrogen removal mechanisms (Wu et al. 2015). As shown in Figure 3, average TN removal rates were 25.6%–53.2%, 73.8%–82.2%, and 79.4%–85.8% under the HLR ranging from 0.06 to 0.3 m$^3$/m$^2$ d) for NA, CA, and IA SWISs, respectively. These results are consistent with the removal rates reported by Li et al. (2012) for conventional SWISs and by Pan et al. (2015) and Yang et al. (2016) for aerated SWISs. Average TN removal rates in IA and CA SWISs were significantly higher than that in NA ($P < 0.05$), which is in agreement with the studies of Maltais-Landry et al. (2009) and Liu et al. (2015). Under the HLR of 0.18 and 0.3 m$^3$/m$^2$ d), average effluent TN concentrations in NA SWIS were 21.85 and 29.56 mg/L, which were higher than the class I value (20 mg/L) according to the discharge standard of pollutant for municipal wastewater treatment plants in China (GB18918-2002). The effluents of NA SWIS were still dominated by high NH$_4^+$-N concentration due to limited nitrification. NA SWIS could not achieve high NH$_4^+$-N removal rate in anoxic or anaerobic conditions, which would greatly inhibit denitrification due to the insufficient supply of NO$_3^-$-N as electron acceptors. TN removal rates decreased with the increase of HLR in NA SWIS. The result was consistent with previous studies (Zou et al. 2013; Li et al. 2012). Effluent TN concentrations were between 10.70 and 7.31 mg/L for CA SWIS, and between 8.14 and 5.56 mg/L for IA SWIS. High-efficiency nitrification and denitrification were realized simultaneously in CA and IA SWISs. NH$_4^+$-N could be nitrified in aerobic zones and then processed via denitrification in anoxic or anaerobic zones. The needed carbon source was supplied by the continuous feeding, leading to high removal of TN. Average removal rate of TN was enhanced with increasing HLR in CA and IA SWISs, which was in accordance with a previous study (Yang et al. 2016). Under the same HLR, average COD removal rates of CA and IA SWISs were much higher than of NA SWIS. Decomposition of organic matter took place mainly in the upper matrix of the SWISs (Wang et al. 2010). In aerated SWISs, the aerobic conditions were created in the upper matrix by aeration and thus facilitated aerobic removal of organic matter.

![Figure 3](https://iwaponline.com/wst/article-pdf/76/1/210/451971/wst076010210.pdf)
Average COD removal rate decreased with the increase of HLR in NA, IA and CA SIWSS (Figure 3). Under 0.3 m³/(m² d), more carbon source in the lower layer was available than under 0.06 and 0.18 m³/(m² d), which led to higher denitification and resulted in lower effluent TN concentration in IA and CA SWISs under high HLR. Average TN removal rate of IA SWIS was higher than that of CA SWIS under the same HLR because of more carbon source obtained in IA SWIS.

COD, NH₄⁺-N and TN removal rates of aerated SWISs under the HLR of 0.3 m³/(m² d) were higher than previous studies (Li et al. 2011, 2012; Fan et al. 2013), though the HLR was nearly 5–10 times higher than in these studies. Taking TN removal performance, hydraulic efficiency and operation cost into consideration, IA mode and the HLR of 0.3 m³/(m² d) were recommended for SWISs.

Functional gene abundances involved in nitrogen removal

Figure 4 shows the abundances of nitrogen removal functional genes, which are involved in nitrification and denitrification processes. The amoA and nxrA genes are often regarded as the marker of oxidizing NH₄⁺-N to NO₂⁻ and oxidizing NO₂⁻ to NO₃⁻ (Poly et al. 2008), respectively. In NA, IA and CA SWISs, the abundances of amoA and nxrA decreased along the flow direction. This followed the same trend as DO, which showed a decrease...
along the flow direction. The metabolic activity, growth and enrichment of ammonia-oxidizing bacteria are related to DO (Wang et al. 2015a). DO concentrations (DO < 1.5 mg/L) were not in a suitable range for the growth of ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) (Fan et al. 2015). AOB perform NH$_4^+$-N to NO$_2^-$-N oxidation, providing substrate to NOB for the nitrite oxidation of NO$_2^-$-N to NO$_3^-$-N, which might explain the lower abundance of nxrA compared to amoA. With increasing HLR, the abundances of nxrA and amoA increased in IA and CA SWISs, and decreased in NA SWIS. When HLR increased by 0.12 m$^3/(m^2\text{d})$, the abundances of amoA and nxrA decreased by one order of magnitude at 50 cm depth in NA SWIS. Previous studies also found that high HLR reduced the growth of AOB and NOB in conventional infiltration systems (Rothrock et al. 2011). In IA and CA SWISs, the abundances of amoA and nxrA were significantly higher than those of NA SWIS under the same HLR (P < 0.05). Sufficient oxygen supply could greatly improve the number of nitrification bacteria and enzyme activities involved in NH$_4^+$-N removal under high HLR (Pan et al. 2015). DO concentrations of IA and CA SWISs (DO > 2.2 mg/L) were improved through aeration at 50 cm depth, which was favorable for the nitrification bacteria. Former studies also found that aeration obviously enhanced the growth of AOB and NOB by a fluorescence in situ hybridization analysis infiltration system (Pan et al. 2015).

NO$_3^-$-N to NO$_2^-$-N reduction, the first reaction process in denitrification, is catalyzed by the key gene narG and napA. As seen from Figure 4, the abundances of napA and narG declined with increasing HLR in NA SWIS similar to other study (Wang et al. 2015b). In IA and CA SWISs, the abundances of napA and narG increased with increasing HLR. Aeration enhanced DO concentrations in IA and CA SWISs, which was favorable for nitrification. More NO$_3^-$, as the substrate of anaerobic denitrification of NO$_2^-$ production, could be catalyzed, which improved the abundances of napA and narG. NO$_2^-$-N to NO reduction is the second reaction process in denitrification, which is catalyzed by nirS and nirK genes (Kandelers et al. 2006). With increasing HLR, the abundance of nirK gradually increased in IA and CA SWISs and decreased in NA SWIS. The abundance of nirS changed disorderly with HLR (Figure 4). It was reported that the composition of nirS genotypes varied greatly and nirK was more stable than nirS (Hallin et al. 2006). NO to N$_2$O reduction is the third process in denitrification, which is catalyzed by qnorB (Fujitwara & Fukumori 1996). With increasing HLR, the abundance of qnorB gradually increased in IA and CA SWISs, whereas it decreased in NA SWIS (Figure 4). N$_2$O to N$_2$ reduction catalyzed by nosZ is the last reaction in the denitrification process (Wang et al. 2015a). The abundance of nosZ increased along the flow direction and changed little with increasing HLR in NA, IA and CA SWISs, which was consistent with a former report (Wang et al. 2015b). Overall, the abundances of napA, narG, nirK and qnorB, which are intimately involved in the denitrification process, tended to gradually decline in NA and increase in IA and CA SWISs when HLR rose. In the HLR range of 0.06 and 0.3 m$^3/(m^2\text{d})$, more organic matter and nutrients provided good nutritional conditions for the growth and enrichment of anaerobic denitrifying bacteria with increasing HLR in IA and CA SWISs after nearly complete nitrification with aeration, in parallel to enhancing the enrichment of the four genes. Under the same HLR, the abundances of napA, narG, nirK and qnorB in IA SWIS were higher than those in CA SWIS at the same depth because IA SWIS provided more carbon source. This could further explain high removal of TN in IA SWIS.

The results suggested that a high HLR negatively affected the nitrifying and denitrifying community in conventional SWISs, and aeration improved the abundance of nitrogen functional genes, especially under high HLR, and favored nitrogen removal.

### CONCLUSIONS

Aeration strategy successfully created appropriate oxygen conditions for nitrification and denitrification, which increased COD, NH$_4^+$-N and TN removal rates, and enhanced the enrichment of nitrogen removal functional genes (amoA, nxrA, napA, narG, nirK and qnorB). Increasing HLR affected COD, NH$_4^+$-N and TN removal more in NA SWIS than in IA and CA SWISs. High TN removal rate (85.8%) was obtained in IA SWIS under the HLR of 0.3 m$^3/(m^2\text{d})$, a higher than that in CA SWIS (82.2%). Intermittent aeration is a reliable strategy to achieve low operation cost, and high HLR and TN removal for SWISs.

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