Nitrogen removal and \( \text{N}_2\text{O} \) emission by shunt distributing wastewater in aerated or non-aerated subsurface wastewater infiltration systems under different shunt ratios

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ABSTRACT

This study investigated matrix oxidation–reduction potential (ORP), nitrogen removal, \( \text{N}_2\text{O} \) emission and nitrogen removal functional gene abundance in three subsurface wastewater infiltration systems (SWISs), named SWIS A (without aeration or shunt distributing wastewater), SWIS B (with shunt distributing wastewater) and SWIS C (with intermittent aeration and shunt distributing wastewater) under different shunt ratios. Aerobic conditions were produced at a depth of 50 cm and anoxic or anaerobic conditions were not changed at depths of 80 and 110 cm by aeration in SWIS C. High average removal rates of chemical oxygen demand (COD) (83.1% for SWIS B, 90.9% for SWIS C), NH\(_3\)-N (74.3% for SWIS B, 90.8% for SWIS C) and total nitrogen (TN) (61.1% for SWIS B, 87.9% for SWIS C) were obtained under shunt ratios of 1:3 and 1:2 for SWIS B and C, respectively. The lowest \( \text{N}_2\text{O} \) emission rate (28.4 mg/(m\(^2\) d)) and highest nitrogen removal functional gene abundances were achieved in SWIS C under a 1:2 shunt ratio. The results suggested intermittent aeration and shunt distributing wastewater combined strategy would enhance nitrogen removal and reduce \( \text{N}_2\text{O} \) emission for SWISs.

Key words | intermittent aeration, \( \text{N}_2\text{O} \), shunt distributing wastewater, shunt ratio, subsurface wastewater infiltration system

INTRODUCTION

Subsurface wastewater infiltration systems (SWISs) have been applied to decentralized domestic wastewater treatment in small towns and rural areas due to its easy maintenance, and low investment and operational costs. However, nitrogen removal is around 50% for total nitrogen (TN) removal and generally 60–80% for ammonia nitrogen (NH\(_3\)-N) removal, which is still a major obstacle for the wide application of SWISs (Li et al. 2011; Pan et al. 2015; Sun et al. 2018).

Nitrification followed by denitrification is the main path for nitrogen removal in SWISs, but it can be influenced by various environmental parameters and operational conditions, of which dissolved oxygen (DO) and carbon source are vital (Pan et al. 2013, 2015). Previous studies have shown that nitrogen removal via nitrification and denitrification processes may be enhanced if the upper part of a SWIS has enough oxygen for complete nitrification; at the same time the lower part is anoxic and extra carbon is supplemented to improve the denitrifying process (Li et al. 2011; Song et al. 2016). Pan et al. (2016) concluded that intermittent artificial aeration combined with shunt distributing wastewater enhanced organic pollutant and nitrogen removal in SWISs. However, few studies have been carried out to investigate the effects of shunt ratios (hydraulic loading rate of shunt distributing wastewater to that of distributing wastewater) on pollutant removal performance, nitrous oxide (\( \text{N}_2\text{O} \)) emission and nitrogen removal functional gene abundance in SWISs that combine intermittent artificial aeration and shunt distributing wastewater. \( \text{N}_2\text{O} \), a byproduct of nitrification and denitrification processes, is an important greenhouse gas that accounts for approximately 5% of the total greenhouse effect and it has become a research focus in recent years. \( \text{N}_2\text{O} \) emissions from SWISs are affected by various parameters and environmental conditions such as DO, supply of available organic carbon, water temperature.

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and so on (Wang et al. 2014). Studies of NO₂ emission from SWISs are very limited (Li et al. 2017b). Ammonia monooxygenase (amoA), nitrite oxidoreductase (nxrA), periplasmic nitrate reductase (napA), membrane-bound nitrate reductase (narG), nitrite reductase (nirK/nirS), nitric oxide reductase (qnorB) and nitrous oxide reductase (nosZ) functional genes are all involved in nitrification and denitrification processes (Wang et al. 2015). The expression of nitrogen removal functional gene abundance in the matrix is relative to N₂O emission and nitrogen removal (Pan et al. 2017). However, most of studies have focused on individual functional genes.

Therefore, three batch-operated SWISs were used to treat domestic wastewater in the present study. The main objectives of this paper were: (1) to determine the characteristics of oxidation–reduction potential (ORP) profiles along aerated or non-aerated SWISs under different shunt ratios; (2) to assess the effects of shunt ratio on chemical oxygen demand (COD) removal, NH₃-N removal, TN removal, N₂O emission and nitrogen removal functional gene abundance in aerated or non-aerated SWISs; (3) to identify optimal operational schemes for high nitrogen removal and low N₂O emission in SWISs.

MATERIALS AND METHODS

SWISs description

Three pilot SWISs composed of a Plexiglass column (50 cm internal diameter and 120 cm in height) were operated under different conditions (Figure 1). There were three sampling holes in each, at depths of 50, 80 and 110 cm from the matrix surface, for nitrogen removal functional gene analysis. ORP detection probes were buried in the center of each SWIS at depths of 50, 80 and 110 cm from the matrix surface. A distributing pipe was installed at 50 cm below the surface in each infiltration system. A shunt distributing pipe was installed below the distributing pipe at a depth of 70 cm in SWIS B and C. The artificially aerated SWIS C was composed of an aerated unit which consisted of air compressor, air tube and micro-bubble diffuser at a depth of 40 cm. The micro-bubble diffuser and distributing pipe were surrounded by gravel (10–20 mm, diameter) to diffuse air and protect from clogging. Wastewater after treatment was collected from outlets in the

Figure 1 | Schematic diagram of three subsurface wastewater infiltration systems (SWIS). (1) Infiltration system body; (2) high-level tank; (3) liquid flow meter; (4) gas flow meter; (5) air compressor; (6) dissolved oxygen electrode; (7) distributing pipe; (8) shunt distributing pipe; (9) outlet; (10) sampling port.
lower part of three SWISs. Each system was filled with 10% coal slag, 10% biochar and 80% brown earth by weight ratio. The coal slag was from a furnace, 4–8 mm in diameter. The brown earth was collected from the top 20 cm of soil from Shenyang Ecological Station. Corn straw was carbonized under anaerobic conditions at 550 ± 10 °C for the biochar, to provide an environmentally friendly, low cost and renewable sample. All matrix components were mixed in a blender three times for 15 min each time to ensure uniformity. The mixed matrix had a hydraulic conductivity of (1.87 ± 0.5) × 10⁻³ cm/s, a surface area of 215.4 ± 3.8 m²/kg, total organics of 39.3 ± 1.6 g/kg, TN of 2.6 ± 0.4 g/kg and total phosphorus (TP) of 1.2 ± 0.2 g/kg.

### Operation of the system

Domestic wastewater pretreated in a septic tank was continuously fed into each SWIS. The total hydraulic loading rate was 0.12 m³/(m² d) for SWIS A, B and C. Experiments under different shunt ratios of 3:1, 2:1, 1:1, 1:2 and 1:3 were carried out in SWIS B and C. Each shunt ratio experiment lasted 60 days. SWIS C had four aerated/non-aerated cycles every day. In each cycle, SWIS C was firstly aerated for 1 hour with an airflow rate of 4.0 ± 0.2 L/min, and then had a 5 hour interval without aeration. The aeration start times were 1:00 am, 7:00 am, 1:00 pm and 10 pm.

### Analytical methods

Wastewater quality after pretreatment was: pH 7.1–7.4, COD 185.5–261.8 mg/L, TN 36.4–46.7 mg/L, TP 3.2–6.8 mg/L, NH₃-N 32.3–46.7 mg/L. Water samples were taken from the influent and the effluent every 5 days to analyze the COD, NH₃-N and TN according to Standard Methods for the Examination of Water and Wastewater (APHA 2005). The dichromate method was used for COD determination. The spectrophotometric method was used for NH₃-N and TN measurements. Pt detection probes were used to determine the ORP of the systems by depolarization ORP analytical methods. The signals were collected online by MDA-501 data acquisition system (Tuopu Co. Ltd; China) every 20 min. SPSS 12.0 was used to determine whether there was a significant difference of 0.05 in COD, NH₃-N, TN and N₂O emission from different SWISs (n = 12).

Gas samples were collected using a closed static chamber made of polymethyl methacrylate, with a total volume of 21 L. When sampling, the chambers were placed on the surface of systems. To ensure that the gas samples were well-mixed, the air inside the chambers was circulated with a battery-driven fan during the measurement. Five gas samples were collected at 0, 30, 60, 90 and 120 min after enclosure at the same time of day between 8:00 and 10:00 am every 5 days. The gases emitted from the system were trapped by the chamber, and then sampled from the air outlet at the middle part of each chamber into gas sampling bags by means of a mini gas pump. The N₂O concentration was analyzed by Agilent 6890N gas chromatography equipped with an electron capture detector and a Poropak Q column, and used 40 mL/min argon-containing 5% methane as the carrier gas. The temperature of the detector and oven was set at 500 °C and 120 °C, respectively. After determining the concentration of N₂O, the N₂O emission rate was calculated by the Equation (1) (Sun et al. 2013).

\[
\text{Flux} = \left( \frac{dC}{dt} \right) \times \left( \frac{1}{A} \right) \times (13.5M) \times [0.0225 \times 0.082 \\
\times (T + 273) \times 1000]^{-1}
\]

(1)

where Flux is the N₂O emission rate (mg/(m² h)); dC/dt is the slope of the best-fit line for the plot of gas concentration inside the chamber and time data points (mg/h); A is the section area of the gas chamber (m²); M is the molecular weight of N₂O; and T represents the air temperature inside the chamber (°C). The N₂O conversion ratio is the percentage of TN converted to N₂O.

Matrix samples were collected from sampling ports after each experiment. Soil DNA kits (Omega, D5625-01) were used to extract and purify the total genomic DNA from the matrix. Extracted genomic DNAs were detected by 1% agarose gel electrophoresis, and preserved at −20 °C in a freezer for further use. Functional gene abundance involved in nitrogen removal was quantified by quantitative polymerase chain reaction (qPCR) according to Wang et al. (2015). Quantitative analysis was performed on the target fragments of the following functional genes: amoA, nxrA, narG, napA, nirK, nirS, qnorB and nosZ. Each primer concentration was 10 pmol/μL. The protocol and parameters for each target gene were the same as in a previous study (Sun et al. 2017). 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. The standard samples were diluted to yield a series of 10-fold concentrations, and were subsequently used for qPCR standard curves. The real-time PCR standard curves of the functional genes were repeated three times and the coefficient of determination (R²) for each standard curve was greater than 0.99.

RESULTS AND DISCUSSION

ORP profiles in an aerated/non-aerated cycle

Soil redox conditions are easily distinguished using ORP. Therefore, it has been widely used instead of DO concentration to indicate soil aeration conditions. ORP over 100 mV indicates an aerobic environment, whereas less than −100 mV is commonly interpreted as being indicative of an anaerobic environment. ORP values between −100 mV and 100 mV indicate an anoxic environment (Ong et al. 2010). The ORP profiles of the SWISs with/without intermittent aeration in an aerated and non-aerated cycle are shown in Figure 2. The average ORP was −59.6, −176.5 and −262.1 mV for SWIS A at 50, 80 and 110 cm. The average ORP found at 50 cm was in the range of 46.6 to −44.2 mV. It was less than −176.4 and −285.4 mV at 80 and 110 cm in SWIS B under different shunt ratios, which indicated that the non-aerated systems were under anaerobic conditions at the depths of 80 and 110 cm, and under anoxic conditions at the depth of 50 cm. Furthermore, shunt distributing wastewater did not change the anoxic or anaerobic environment in SWIS B. This was consistent with previous studies, which found oxygen from air diffusing into the matrix was limited and the prevailing conditions in non-aerated SWISs were anoxic or anaerobic below the distributing pipe (Wang et al. 2010). As for the ORP changes in SWIS C, a similar tendency was observed under different shunt ratios, i.e. the matrix ORP increased in the aerated period, but decreased slowly in the non-aerated period at 50 cm. Under shunt ratios of 3:1, 2:1, 1:1, 1:2 and 1:3, the ORP at 50 cm was more than 286 mV during aeration and as high as 108 mV when supplementary aeration were switched off. At 80 and 110 cm, the ORP was below −109.1 and −268.6 mV during aeration, and was below −150.5 and −271.5 mV without aeration under the experimental shunt ratios, respectively. It could be concluded that aerobic conditions were effectively developed at 50 cm and anoxic or anaerobic conditions were not changed at 80 and 110 cm within SWIS C, that is to say, sequential aerobic and anaerobic conditions were well developed by intermittent aeration. The high aeration-induced ORP was reduced by the decomposition of nutrients and organic matter, which may explain the decreasing tendency in the non-aerated period. ORP decreased with the decrease of shunt ratios at 50 cm in SWIS B and C which could be explained by the fact that more oxygen was consumed for the degradation of organic matter and nutrients under lower shunt ratios.

COD, NH₃-N and TN removal

Figure 3 presents the average effluent concentrations and removal rates of COD, NH₃-N and TN. The average COD removal rate was above 83.4% in SWIS A, which reflected the effectiveness of conventional SWISs in removing organic matter. Similar results were detected in other studies (Li et al. 2011; Song et al. 2016). However, COD removal rates increased as shunt ratios decreased in shunt distributing wastewater SWISs. Average COD removal rates increased from 46.1% and 53.4% under the shunt ratio of 3:1 to 83.1% and 94.2% under the shunt ratio of 1:3 for SWIS B and C, respectively. A shorter hydraulic retention time was achieved under the higher shunt ratio leading to organic matter in the shunt wastewater not being completely oxidized by microbial organisms in anoxic or anaerobic conditions. Average effluent COD concentrations in SWIS B and C were above Chinese criterion for water discharge from municipal wastewater treatment plants (GB18921-2002) under shunt ratios of 3:1 and 2:1, which was in accord with Wang et al. (2010) and Pan et al. (2013). The average COD removal rate of SWIS C was significantly higher than that of SWIS B under shunt ratios of 1:2 and 1:3 (P < 0.05). The intermittent aeration strategy obviously improved COD removal. Organic pollutants in the influent are mainly oxidized around the distributing pipe with sufficient oxygen (Li et al. 2011). Oxygen concentration decreases with the increase of soil depth and the surroundings become either anoxic or anaerobic. Disadvantageous aerobic and anaerobic environment always limit organic pollutant degradation (Wang et al. 2010). Although organic matter can be degraded both aerobically and anaerobically by heterotrophic bacteria, aerobic degradation is usually more important (Wu et al. 2015). Yang et al. (2016) reported that sufficient oxygen would greatly elevate the performance of aerobic biochemical oxidation. In SWIS C, aerobic
conditions in the upper matrix were improved by intermittent aeration and thus facilitated aerobic removal of organic pollutants.

Nitrogen transformations in the SWIS include nitrification, denitrification, ammonia volatilization, adsorption and cation exchange for ammonia (Wang et al. 2010). Among these, nitrification coupled with denitrification was the major removal process (Sun et al. 2017). TN removal firstly depends on complete nitrification, which is an aerobic chemo-autotrophic microbial process and is usually the limiting step for nitrogen removal in conventional SWISs because of the insufficient oxygen supply (Fei et al. 2017).

As shown in Figure 3, SWIS A achieved an average effluent NH$_3$-N concentration of 10.21 mg/L, with an NH$_3$-N removal rate of 73.7%, which was consistent with many conventional SWISs studies (Li et al. 2011; Pan et al. 2015). Most conventional SWISs fail to achieve efficient nitrification due to an insufficient oxygen supply (Pan et al. 2015). In shunt SWISs, NH$_3$-N removal rates decreased as the shunt ratio increased in SWIS B and C. The average effluent NH$_3$-N concentration in SWIS B under shunt ratios of 3:1, 2:1, 1:1 and 1:2 was higher that of SWIS A, while for SWIS C, the shunt ratios were 3:1, 2:1 and 1:1. The reason was that NH$_3$-N in the shunt wastewater was not oxidized completely
due to shorter retention time of a larger quantity of shunt wastewater and adverse anoxic or anaerobic conditions in the lower matrix. In SWIS B, the average NH$_3$-N concentration in the effluent under the shunt ratio of 1:3 was lower than that of SWIS A. A small amount of wastewater was introduced to the lower matrix by shunt wastewater which increased nitrification because of ORP improvement. Nevertheless, the average NH$_3$-N removal rate of SWIS C was above 90% with intermittent aeration under shunt ratios of 1:2 and 1:3, which was significantly higher than that of SWIS B ($P < 0.05$). Additional artificial aeration appeared to be the most effective alternative to substantially improve the oxidation of the matrix (Wu et al. 2015; Fei et al. 2017). ORP results showed that the oxidative conditions of the upper matrix were enhanced through intermittent aeration in SWIS C, which was favorable for the nitrification process. Previous studies also found that more nitrifying bacteria, other viable bacteria and enzyme activities involved in nitrogen removal were detected in intermittently aerated SWISs than in non-aerated SWISs (Pan et al. 2018; Fei et al. 2011). These results indicated that artificial aeration was an effective strategy for shunt distributing wastewater SWISs to enhance NH$_3$-N removal.

The nitrified nitrogen must be processed by anaerobic microbial denitrification to be removed from wastewater permanently. Various factors such as an insufficient organic carbon and excess oxygen could limit its completion (Yang et al. 2016). Organic matter was adsorbed and degraded by aerobic microorganisms in the upper matrix, which led to a lack of carbon in the lower part and low denitrification (Wang et al. 2010; Li et al. 2011). As shown in Figure 3, the average TN removal rate was 51.2% for SWIS A, with an average effluent TN concentration of 20.39 mg/L, which was mainly attributed to poor nitrification. SWIS A could not attain high NH$_3$-N removal in insufficient DO conditions, which greatly restrict denitrification. Average TN removal rates were increased from 51.2% in SWIS A to 61.1% under the shunt ratio of 1:3 for SWIS B, to 87.9% and 78.7% under the shunt ratios of 1:2 and 1:3 for SWIS C. TN removal rates of SWIS B and C under the shunt ratios of 2:1, 3:1 and 1:1 were lower than that of SWIS A due to the unbalanced ratio of carbon to nitrogen and low nitrification. The average TN removal rate of SWIS C was significantly higher than that of SWIS B under the shunt ratios of 1:2 and 1:3 ($P < 0.05$). After effective nitrification under aerobic conditions, the NO$_3$-N as electron acceptor could not be eliminated permanently unless sufficient organic carbon was supplied as the electron donor (Song et al. 2016). In SWIS C, intermittent aeration achieved good nitrification and well developed aerobic conditions in the upper matrix and anoxic or anaerobic conditions in the subsequent matrix simultaneously in one cycle (Figure 2). Therefore, extra carbon from shunt wastewater greatly enhanced denitrification in SWIS C. The best TN removal performance was achieved in SWIS C under the shunt ratio of 1:2, which was a substantial enhancement compared to TN removal (40–60%) in conventional SWISs reported by Li et al. (2011) and Wang et al. (2010).
Taking COD, NH$_3$-N and TN removal performance into consideration, the shunt ratios of 1:2 for the intermittent artificial aeration and shunt distributing wastewater combined SWIS and 1:3 for the shunt distributing wastewater SWIS are recommended. The performances of intermittent artificial aeration and shunt distributing wastewater combined SWIS under the optimal shunt ratio (1:2) were substantially enhanced compared to COD, NH$_3$-N and TN removal performances in other enhanced SWISs (Zou et al. 2009; Wang et al. 2010; Li et al. 2015). Zou et al. (2009) mixed microbial inoculums into soil to enhance the ammonifying, nitrifying and denitrifying biomass for nitrification and denitrification in SWISs which achieved COD, NH$_3$-N and TN removal rates of 88.6%, 87.0% and 60.6%, respectively. Wang et al. (2010) adopted shunt distributing wastewater in a SWIS to enhance the carbon supply for denitrification, and the average removal efficiencies for COD, NH$_3$-N and TN were 81.6%, 78.4% and 65.1%, respectively. Li et al. (2015) used intermittent operation to improve the oxidative conditions for nitrification in SWISs and obtained average COD, NH$_3$-N and TN removal rate of 90.5%, 86.2% and 70.8%, respectively.

N$_2$O emission from SWISs

Both nitrification and denitrification lead to N$_2$O emission in SWISs (Jiang et al. 2017; Li et al. 2017b). N$_2$O emission depends on various operational and ambient conditions, of which DO and influent C/N ratio are vital (Wang et al. 2014). Figure 4 shows the N$_2$O emission rate and conversion ratio of each SWIS. The N$_2$O emission rates of SWIS B and C were as follows: 22.7 and 23.6 mg/(m$^2$ d) under the shunt ratio of 3:1, 26.3 and 40.9 mg/(m$^2$ d) under the shunt ratio of 2:1, 44.6 and 45.5 mg/(m$^2$ d) under the shunt ratio of 1:1, 50.2 and 28.4 mg/(m$^2$ d) under the shunt ratio of 1:2, 44.1 and 36.8 mg/(m$^2$ d) under the shunt ratio of 1:3, respectively. N$_2$O conversion ratios of the three SWISs were in the range of 0.45% to 1.10%, which were similar to previous studies (Li et al. 2017a). Denitrification is an anaerobic heterotrophic process, which is generally regarded as the dominant process responsible for N$_2$O emission in SWISs (Kong et al. 2014). However, denitrification is highly dependent on nitrification, an aerobic chemo-autotrophic process, which produces nitrate from ammonium. The N$_2$O emission rate increased with shunt ratio decreasing from 3:1 to 1:2 in SWIS B and with shunt ratio decreasing from 3:1 to 1:1 in SWIS C because denitrification was hindered by low nitrification. The N$_2$O emission rate of SWIS B under the shunt ratio of 1:5 was higher than that of SWIS C under the shunt ratios of 1:2 and 1:3, which was a little lower than that of SWIS A (49.5 mg/(m$^2$ d)). Nitrification was poor due to an insufficient oxygen supply in SWIS B. Although shunt distributing wastewater supplied organic carbon as the electron donor, the enhancement of denitrification was limited under the shunt ratio of 1:3. The lowest N$_2$O emission rate was achieved in SWIS C under the shunt ratio of 1:2, which was significantly lower than that of SWIS A ($P < 0.05$). Intermittent aeration achieved good nitrification and well developed aerobic conditions in the upper matrix and anoxic or anaerobic conditions in the subsequent matrix in SWIS C. Simultaneously, an optimal quantity of carbon from shunt wastewater enabled N$_2$O transformation into N$_2$ under the shunt ratio of 1:2. Previous studies reported that sufficient carbon supplied after efficient nitrification could greatly reduce N$_2$O emission (Wang et al. 2014; Jiang et al. 2017). The combination of artificial aeration and shunt distributing wastewater was an effective strategy to reduce N$_2$O emission in SWISs.

**Functional gene abundance involved in nitrogen removal**

The use of environmental data from functional genomics plays an important role in determining the genetic relationships of microbes and their ecological processes (Ji et al. 2012). Nitrogen removal functional gene abundances are shown in Figure 5. The amoA and nxaA genes oxidize NH$_4^+$-N to NO$_2^-$-N and NO$_2^-$-N to NO$_3^-$-N (Wang et al. 2015), respectively. At 50 cm in SWIS C under shunt ratios of 1:2 and 1:3, the abundances of amoA and nxaA were significantly higher than in SWIS A and B ($P < 0.05$). Sufficient oxygen supplied by aeration in SWIS C greatly enhanced the

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Figure 4 | N$_2$O emission rate and conversion ratio in SWIS A (without shunt distributing wastewater or aeration), SWIS B (without aeration) and SWIS C (with intermittent aeration) under shunt ratios of 3:1, 2:1, 1:1, 1:2 and 1:3.
abundances of amoA and nxrA involved in NH3-N transformation. The result was consistent with a previous study (Jiang et al. 2014). The abundances of amoA and nxrA were the highest under the shunt ratio of 1:3 in SWIS C, which could further explain the highest removal rate of NH3-N. In the experimental SWISs, the abundances of amoA and nxrA decreased along the direction of flow. ORP also showed a decrease along the direction of flow. The metabolic activity, growth and enrichment of ammonia-oxidizing bacteria are related to aerobic or anaerobic conditions (Wang et al. 2018).

NarG, napA, nirS, nirK, qnorB and nosZ genes are the six functional genes involved in denitrification (Ji et al. 2012; Wang et al. 2015). The NarG and napA convert NO3-N into NO2-N, which is the first step of denitrification. The second process is NO2-N to NO reduction catalyzed by nirS and nirK. NO to N2O reduction is the third process, which is catalyzed by qnorB. The last reaction is N2O to N2 reduction catalyzed by nosZ, which acts as a marker for complete denitrification. As can be seen in Figure 5, the abundances of narG, napA, nirS, nirK, qnorB and nosZ in SWIS C under shunt ratios of 1:2 and 1:3 were significantly higher than in SWIS A and B at the same depths of 80 cm and 110 cm (P < 0.05). Aeration enhanced the DO supply in SWIS C, which was favorable for nitrification. More NO3 as the substrate of anaerobic denitrification and

Figure 5 | Abundances of nitrogen removal functional genes in SWIS A (without shunt distributing wastewater or aeration), SWIS B (without aeration) and SWIS C (with intermittent aeration) under shunt ratios of 3:1, 2:1, 1:1, 1:2 and 1:3.
carbon from shunt wastewater enhanced the abundances of the six genes. The nosZ is a key factor determining whether N₂O is converted to N₂ or released into the atmosphere as a greenhouse gas (Thomson et al. 2012). The abundance of nosZ in SWIS C under the shunt ratio of 1:2 was the highest, which could further explain the lowest N₂O emission. Optimal quantities of organic matter and nutrients provided good nutritional conditions for the growth and enrichment of anaerobic denitrifying bacteria with shunt distributing wastewater after efficient nitrification with aeration under the shunt ratio of 1:2, in parallel to improving N₂O to N₂ conversion.

CONCLUSIONS

Aerobic conditions were effectively created at a depth of 50 cm, and anoxic or anaerobic conditions were not changed at depths of 80 and 110 cm by intermittent aeration. The combined strategy of intermittent aeration and shunt distributing wastewater achieved efficient nitrification and denitrification with a low N₂O emission rate (28.4 mg/ (m² d)), high COD (90.9%), NH₃-N (90.8%) and TN removal (87.9%) under the shunt ratio of 1:2, which enhanced the abundances of nitrogen removal functional genes (amoA, nxrA, narG, napA, nirS, nirK, qnorB and nosZ) simultaneously. This study will help to improve the design, operation and nitrogen removal performance, and reduce the N₂O emissions of SWISs.

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