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RESEARCH ARTICLE

Enhanced nitrogen removal and microbial analysis in partially saturated constructed wetland for treating anaerobically digested swine wastewater

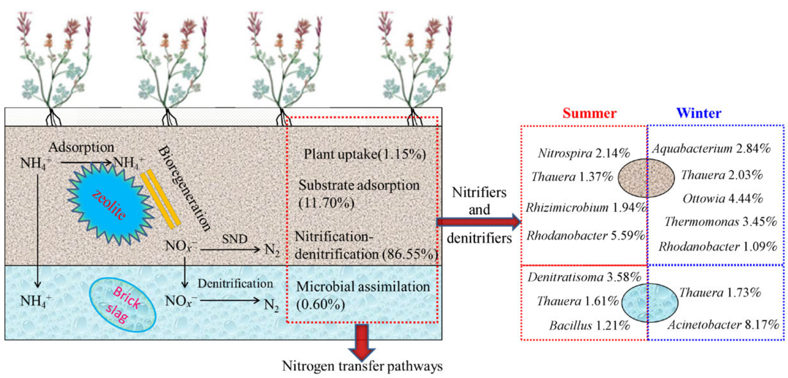
Zhenfeng Han[1,2], Ying Miao[3], Jing Dong[4], Zhiqiang Shen (✉)[1], Yuexi Zhou (✉)[1], Shan Liu[3], Chunping Yang[2]

1 State Key Laboratory of Environmental Criteria and Risk Assessment, Chinese Research Academy of Environmental Sciences, Beijing 100012, China
2 College of Environmental Science and Engineering, Hunan University and Key Laboratory of Environmental Biology and Pollution Control (Hunan University), Ministry of Education, Changsha 410082, China
3 College of Environmental Science and Engineering, Chang'an University, Xi'an 710061, China
4 Beijing Municipal Research Institute of Environmental Protection, Beijing 100037, China

HIGHLIGHTS

- Anaerobically digested swine wastewater was treated by a novel constructed wetland.
- Tidal operation was better for total nitrogen removal than intermittent flow.
- Mechanism of nitrogen removal by biozeolite-based constructed wetland was discussed.
- Simultaneous nitrification and denitrification were determined in zeolite layer.

GRAPHIC ABSTRACT



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ABSTRACT

Nitrogen removal of wastewater containing high-strength ammonium by the constructed wetlands (CWs) has been paid much attention. In this study, the ability of a partially saturated CW to treat anaerobically-digested decentralized swine wastewater under varying operating parameters from summer to winter was investigated. The partially saturated CW achieved better NH_4^+-N and TN removal by tidal flow than intermittent flow. With surface loading rates of 0.108, 0.027, and 0.029 kg/(m^2·d) for COD, NH_4^+-N, and TN, the partially saturated CW by tidal operation achieved corresponding removal efficiencies of 85.94%, 61.20%, and 57.41%, respectively, even at 10°C. When the rapid-adsorption of NH_4^+-N and the bioregeneration of zeolites reached dynamically stable, the simultaneous nitrification and denitrification in the aerobic zeolite layer was observed and accounted for 58.82% of the total denitrification of CW. The results of Illumina high-throughput sequencing also indicated that nitrifiers (*Nitrospira* and *Rhizomicrobium*) and denitrifiers (*Rhodanobacter* and *Thauera*) simultaneously existed in the zeolite layer. The dominant existence of versatile organic degraders and nitrifiers/denitrifiers in the zeolite layer was related to the removal of most COD and nitrogen in this zone. The contribution of the possible nitrogen removal pathways in the CW was as follows: nitrification-denitrification (86.55%)>substrate adsorption (11.70%)>plant uptake (1.15%) >microbial assimilation (0.60%).

1 Introduction

Decentralized swine wastewater has greatly aggravated the pollution of water bodies in China due to the large demand

✉ Corresponding authors
E-mail: shenzq@craes.org.cn (Shen Z Q); zhouyuexi@263.net (Zhou Y X)

for pork and the expansion of swine production (Luo et al., 2016). Although intensive pig farms (with over 500 pigs) are now recommended in the swine industry to replace decentralized pigsties, the number of the feeding pig population from the intensive pig farms only accounted for 42% of the total pig population in 2014, and this is estimated to merely increase to 52% by 2020, based on the National Development Plan on the Swine Industry (2016–2020) by the Ministry of Agriculture of China. Due to the shortage of suitable technology and economic support in small villages or communities, decentralized swine wastewater is commonly discharged directly or after simple anaerobic treatment, which could result in excessive nutrient discharge. Decentralized swine wastewater contains high-strength nitrogen and the ratio of organics to nitrogen (C/N) is low. In addition, its water quantity and quality fluctuates heavily as decentralized pigsties are washed artificially and randomly by their owners. This also challenges the stability of treatment processes. As a result, processes with low cost and low demand for skilled labor and management are required for treating decentralized swine wastewater.

Anaerobic digestion and constructed wetlands (CWs) are both biological treatment processes with easy maintenance and low cost. A combination of these processes is appealing for treating decentralized swine wastewater (Liu et al., 2015). Over the past several decades, CWs have been singly or jointly used to treat livestock wastewater (Lee et al., 2014; Huang et al., 2017b). However, anaerobically digested swine wastewater (ADSW) still has a high nitrogen content, especially ammonium, and poor total nitrogen (TN) removal by CWs has been reported frequently (Knight et al., 2000; Lee et al., 2014). Thus, the efficient elimination of nitrogen is a key factor determining the application of CWs for treating ADSW.

The nitrogen removal pathways in CW are complicated, but the main pathway is still nitrification followed by denitrification (Yu et al., 2019). Aerobic and anaerobic zones were unable to exist simultaneously in one-stage CW; thus, single CWs were unable to achieve good TN removal (Vymazal, 2007). For instance, vertical flow CWs could facilitate nitrification instead of denitrification, while horizontal flow CW could be beneficial for denitrification (Zhi and Ji, 2014). Besides, the properties of wastewater could also influence the nitrification-denitrification process. High concentrations of contaminants, such as COD, in ADSW could be conducive for competition between aerobic heterotrophic microbes and nitrifiers, resulting in the shortage of oxygen. In addition, owing to the low C/N of ADSW, the deficiency of available organics for denitrification could also suppress nitrogen removal. Therefore, it is of great significance to utilize the organics in ADSW to improve nitrogen removal.

A newly designed one-stage CW, called the partially saturated CW with a bottom saturated zone, allowed the simultaneous existence of aerobic/anoxic zones (Prigent et al., 2013). Some reports about the use of a partially saturated CW for wastewater treatment were published recently (Prigent et al., 2013; Silveira et al., 2015; Saeed and Sun, 2017; Pelissari et al., 2018). However, these reports employed the partially saturated vertical flow CWs (VFCWs) to purify domestic/municipal/urban wastewater. The performance of partially saturated CWs for treating swine wastewater remains unknown. Saeed and Sun (2017) reported that nitrification in partially saturated VFCWs was the limiting step for nitrogen removal. The enhancement of nitrification involves improving oxygen regeneration or decreasing competition from aerobic microbes.

In this study, a partially saturated tidal flow CW (TFCW) packed with biozeolites was constructed. Wastewater under tidal flow operation could serve as a passive pump to expel and draw air into CW (Zhi and Ji, 2014), thus ensuring oxygen regeneration in the upper free-drainage zone. As a result, this could enhance nitrification. Zeolites have been widely used for the treatment of wastewater because of the features of high ion exchange capacity, advanced porous structure (molecular sieve) and good ion exchange selectivity for the NH_4^+-N (Luo et al., 2014). With microbes attached into the pores and onto the surface of zeolites (referred as biozeolites), the ammonium adsorbed onto the biozeolites could be transformed and removed by the following microbial processes. Zhang et al., (2015) constructed biozeolite-based reactor to treat rural wastewater and the TN and NH_4^+-N were removed by 92.8% and 96.1%, respectively. Liu et al., (2014) indicated that zeolite-based TFCW outperformed the quartz sand, ceramsite, and volcanic-based TFCWs in treating high ammonium wastewater.

The objectives of this study were to (1) evaluate the performance of the partially saturated CW for the treating real ADSW under varying operational modes, hydraulic loading rates, and pollutant loads from summer to winter; (2) discuss the mechanism of nitrogen removal based on bio-zeolites, (3) assess the contribution of different nitrogen removal pathways (such as denitrification, substrate adsorption, plant uptake, and microbial assimilation), and (4) explore the microbial composition of the different zones in the CW.

2 Materials and methods

2.1 Experimental apparatus and materials

The schematic configuration of the partially saturated CW is presented in Fig. 1. A CW (1000 mm in length × 500 mm in width × 1200 mm in height) was constructed from polyvinyl chloride (PVC) plates, and it was mainly packed with a brick slag layer and mixed layer of zeolite and limestone with a dry weight ratio of 5:1. The brick slag and mixed layer are denoted as “BL” and “ZL,” respectively, in the following sections. There was a further 50 mm of extra height above the soil layer. *Cannas* were vegetated in the red soil layer with a density of 16 plants/m^2. Four similar PVC pipes in parallel were buried in the upper gravel layer as the water distributing system. The influent pipes were composed of perforated PVC (30 mm in diameter) with circular apertures (5 mm in diameter) every 100 mm. An evenly perforated collecting pipe (50 mm in diameter) was buried in the bottom gravel layer, and was connected to two vertical PVC pipes (50 mm in diameter, 120 cm in length), which were also perforated at a height of 75–105 cm (exactly corresponding to the ZL) to facilitate oxygen regeneration in the ZL. The BL, serving as the anaerobic zone, was saturated with water throughout the experiment. In addition to the bottom outlet, another outlet was located at the bottom of the ZL to assay the parameters of its effluent. In addition, there were two circular sampling sites (60 mm in diameter) with matching plugs in the middle of the ZL and BL on the four plates of the CW. When needed, substrates were retrieved from these two sampling sites.

Natural zeolites were purchased from Jinyun, Zhejiang Province, China. All zeolites (7–10 mm in diameter), gravels (20–30 mm in diameter), brick slags (5–10 mm in diameter), and limestones (3–5 mm in diameter) were washed with tap water and air-dried before packing. The activated sludge utilized to seed the wetland was obtained from the Huanghua Wastewater Treatment Plant, Changsha City, Hunan Province, China.

2.2 Wastewater for treatment and operational conditions

The wastewater used in the experiment was the effluent from an anaerobic baffled reactor (ABR) in a decentralized piggery. Before anaerobic treatment, the swine wastewater was first treated by solid-liquid separation to separate manure and liquid-liquid separation to separate swine urine.

The CW was mainly operated for four phases (259 d in total, from May to January), during which the effects of operational pattern, hydraulic loading rate, and pollutant loads were studied. To seed the CW prior to the formal experiment, digested swine wastewater mixed with activated sludge at a concentration of 1000 mgSS/L was pumped into the system for the initial 7 days. Then, in the following 3 days, only the ADSW was fed and drained to prevent the remaining sludge from influencing the results. Afterwards, the formal experiment was started.

Following the successful startup of the CW, an inflow of 90 L/d was employed for phases 1 and 2, whose operating patterns were intermittent flow with a constant water saturation level, and tidal flow with a varying water saturation level, respectively. The influent was supplied from 09:00 and for 1 h. Under intermittent flow, the valve indicated in Fig. 1 was open, ensuring a constant water saturation level of 750 mm, and the final outlet 1 was used. After 1 h, the CW rested for 23 h. Under tidal operation, the valve was closed and the final outlet 2 was used. The water saturation level was 1050 mm in the flooding stage. After 1 h, the water saturation level was quickly varied from 1050 to 750 mm by the siphon, and the CW rested for 23 h until the next operational cycle. Subsequently, phases 3 and 4 were operated under the tidal flow pattern with the same inflow of 50 L/d, but different pollutant loads. Denitrification in phase 3 was insufficient, while the COD and

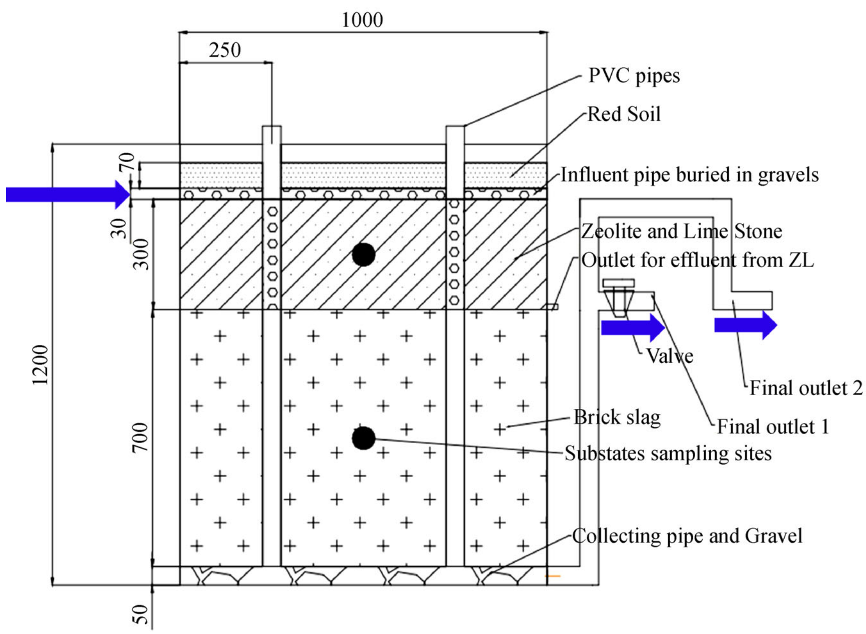

Fig. 1 Schematic configuration of partially saturated CW (all the values in mm).

ammonium loads in phase 4 were increased by the use of less water when washing the pigsties. In phase 4, the COD and NH_4^+-N were increased from approximately 500 to 1000 mg/L and 150 to 250 mg/L, respectively. The average ambient temperature in phases 1 and 2 was 24°C–28°C, but that of phases 3 and 4 was 17°C and 10°C, respectively.

2.3 Water sampling and analytical methods

The determination of pollutant concentrations in zeolite layer (ZL) was special. To make sure that the results of pollutant concentrations in ZL was accurate, all the wastewater in ZL was drained and mixed evenly before measurement. Thus, when the wastewater in ZL was drained, the concentrations of pollutants in brick slag layer (BL) weren't able to be determined. Besides, the determination of pollutant concentrations in ZL was conducted every two days so as to ensure the activity of microorganisms in BL and the stable performance of the whole CW. All water samples were filtered through a 0.45-μm cellulose acetate membrane before analysis. The methods for determining water parameters were as follows: potassium dichromate method for COD_{cr}, Nessler's reagent colorimetric method for NH_4^+-N, potassium persulfate oxidation-ultraviolet spectrophotometry for TN, ultraviolet spectrophotometry for NO_3^--N, and the N-(1-naphthyl)-ethylenediamine spectrophotometric method for NO_2^--N (State Environmental Protection Administration of China (SEPA) (2002)). The pH was determined using a pH meter (METTLER TOLEDO). The oxidation-reduction potential (ORP) in the zeolite during the drainage stage was determined using an ORP instrument (FJA-6). Three probes were buried at heights of 0, 15, and 30 cm in the ZL, respectively.

2.4 Determination of the nitrogen content of the substrate, plant, and biofilms

After the experiment was terminated on day 259, zeolites and brick slags at the middle height of the ZL and BL were collected from the sampling sites. The substrates were submerged into 2-mol/L KCl solution, and then the NH_4^+-N in the supernatant were determined by the Nessler's reagent colorimetric method. The *Cannas* were harvested and air-dried. Afterwards, they were digested using H_2SO_4-H_2O_2. The TN in the digestion solution was then determined by potassium persulfate oxidation-ultraviolet spectrophotometry. The nitrogen content in the biofilm was assayed by fumigation-extraction (Wu et al., 1990).

2.5 16S rRNA-based Illumina high-throughput sequencing

Substrates were sampled from the sampling sites on days 90 (August) and 259 (January). Around 100 g of substrates were sampled from each sampling sites on four plates of CW, and the substrates from the same layer were mixed evenly. The samples were stored at −80°C. The 16s rRNA-based Illumina high-throughput sequencing was performed in Majorbio Bio-pharm Biotechnology Co., Ltd. (Shanghai, China). The procedures included DNA extraction performed by the E.Z.N.A.® Soil DNA Kit (Omega Bio-tek, Norcross, GA, US) and polymerase chain reaction (PCR) amplification of V3-V4 regions using the primers 338F 5′-ACTCCTACGGGAGGCAGCAG-3′ and 806R 5′-GGACTACHVGGGTWTCTAAT-3′. Afterwards, the high-throughput sequencing of PCR amplicons was performed using an Illumina Miseq PE300 platform (Illimina, USA). The detailed description of procedures could be referred to Wang et al. (2019).

3 Results and discussion

3.1 The COD and N removal of the CW

The performances of COD and N removal by the CW during the four operating phases are presented in Fig. 2. The effluent from the ZL and BL is denoted with subscripts of "zeo" and "bri," respectively. For example, COD_{zeo} is the COD concentration of the effluent from the ZL, and COD_{bri} denotes the COD concentration of the final effluent.

According to Fig. 2, despite the fluctuant water quality of the influent, the pollutant concentrations of the effluent remained relatively stable. The CW exhibited excellent COD removal throughout the experiment, with an average COD removal rate of 71.65%–85.94% under a surface loading rate ranging from 0.055 to 0.108 kg/($m^2 \cdot d$). This efficiency was comparable to that reported by previous studies on CWs (Huang et al., 2017b; Li et al., 2017). Even in the winter (phase 4), with an average temperature of approximately 10°C and influent COD of approximately 1000 mg/L, the average COD removal rate was still 85.94%. The NH_4^+-N removal rate was 34.65% in phase 1, and increased from 45.70% to 61.20% in the following three phases. Most of the COD and NH_4^+-N were removed in the ZL. The NH_4^+-N concentration of the effluents from the ZL and BL exhibited slight change, indicating that brick slags could only slightly adsorb NH_4^+-N. No NO_2^--N and NO_3^--N were detected in the influent; therefore, only the concentrations of NO_2^--N and NO_3^--N in the effluents from the ZL and BL are plotted in Fig. 2(c). NO_2^--N_{zeo} and NO_3^--N_{zeo} significantly increased during phases 2 and 3, which was due to the enhanced oxygen regeneration by tidal flow and decreased inflow, respectively. Comparing NO_x^--N_{zeo} (NO_2^--N_{zeo} and NO_3^--N_{zeo}) between phases 1 and 2, it was concluded that nitrification was better under tidal flow than that under intermittent flow. With the increase in NO_x^--N_{zeo} during phase 2, increased TN removal of 40.58% was observed. NO_2^--N_{bri} remained relatively stable during four phases, while NO_3^--N_{bri} increased visibly during phase 3 and declined again in

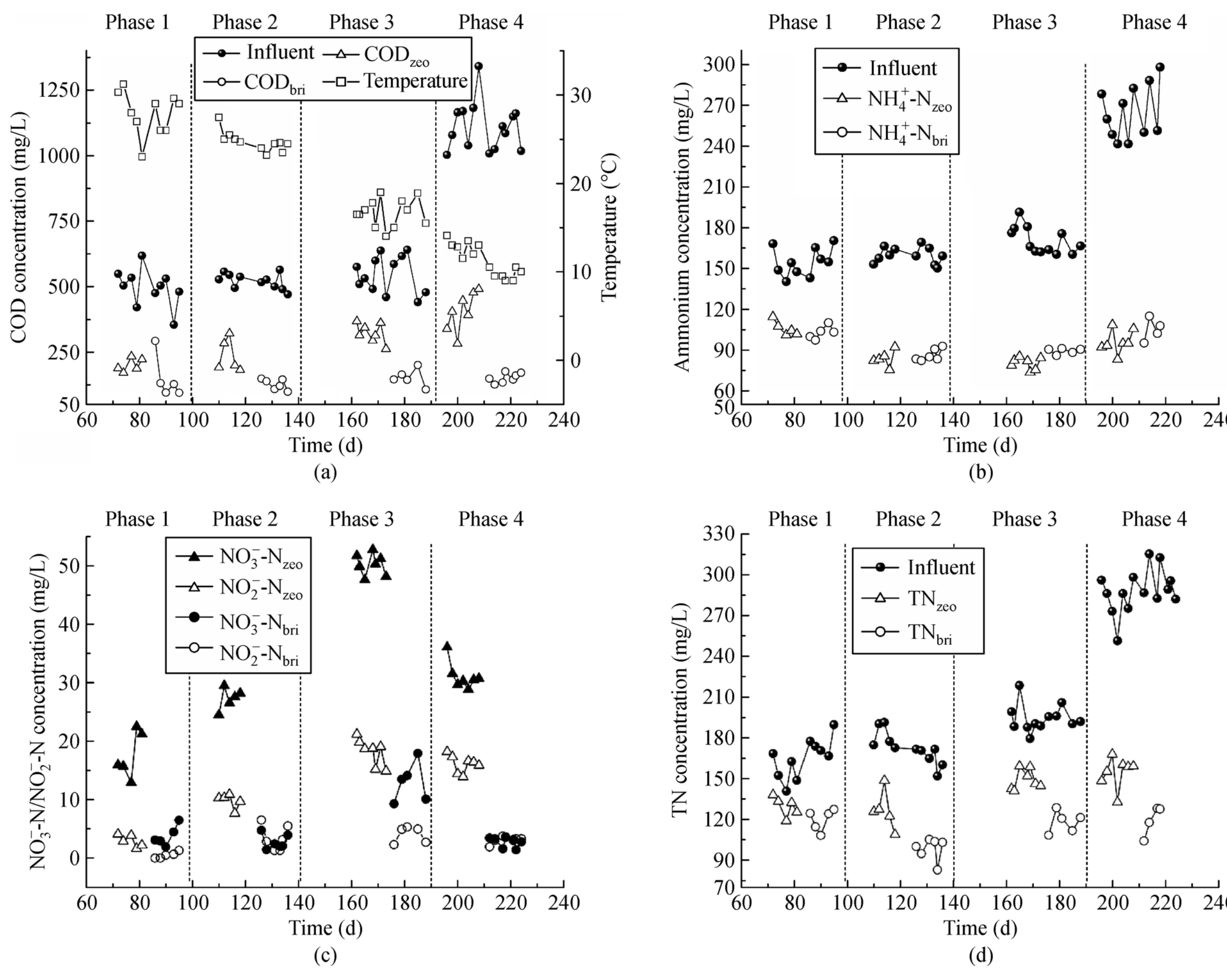

Fig. 2 Performances of the CW (a) COD, (b) ammonium, (c) oxidized nitrogen in effluent, (d) TN.

phase 4 with the increase in the COD load. As a result, the TN removal rate increased to 57.41% during phase 4. Overall, CW achieved better pollutants removal by tidal flow than intermittent flow.

Nitrogen transformation under tidal operation was alternate [9]. In the flooding stage, the NH_4^+-N in swine wastewater rapidly adsorbed onto the zeolite, and the autotrophic nitrifiers attached to the zeolites could oxidize NH_4^+-N into NO_2^--N and further into NO_3^--N as oxygen regenerated during the drainage stage. In the flooding stage, the ZL was flooded with wastewater and became anaerobic. After 1 h, the wastewater was quickly drained with a siphon. This allowed air to be drawn into the zeolites. The average ORPs (Fig. 3) at heights of 0, 10, and 30 cm in the zeolite layer during the drainage stage were 506.14, 420.29, and 402.57 mV, respectively. The high ORP values in the ZL indicted that the air was quickly drawn into the ZL by tidal operation, and the ZL became aerobic, which was beneficial for the bioregeneration of zeolites by the nitrifiers. The bioregeneration of zeolites involves two processes. According to Jung et al., (2004), the adsorbed NH_4^+-N was first desorbed and then nitrified by nitrifiers. Zhang et al. (2017) also indicated that the adsorption of NH_4^+-N onto zeolites involved rapid surface adsorption and slow intra-particle diffusion stage, and the desorption of NH_4^+-N from the surface was rapid, which would not restrain the following nitrification. Therefore, based on the rapid adsorption of zeolites, a theory of dynamic "rapid-adsorption bioregeneration" (rapid-adsorption of NH_4^+-N and the bioregeneration of zeolites) for nitrogen removal was proposed for biozeolite-based CWs. With a mature biofilm under appropriate conditions for microbes in a CW, the dynamic process could reach the equilibrium, which meant that NH_4^+-N rapidly adsorbed during the flooding stage amounted to NO_x^--N produced by bioregeneration during the drainage stage. During the long-term operation of CW, this dynamic "rapid-adsorption bioregeneration" process ensured the persistent removal of nitrogen in the ZL of the CW. The NO_x^--N that regenerated during the drainage stage would be removed by denitrification when it was washed out by the influent in the next flooding stage. Most pollutants were removed in the ZL. After flooding stage, the wastewater in ZL entered the BL, and the remaining

NO_x^--N was further denitrified. As shown in Fig. 2(c), denitrification also occurred in the BL. Therefore, the NO_x^--N content continued to decrease in the BL. In phase 3, the NO_3^--N_{bri} content increased, which indicated the insufficient denitrification. As the influent COD increased from 500 to 1000 mg/L in phase 4, the NO_3^--N_{bri} decreased accordingly.

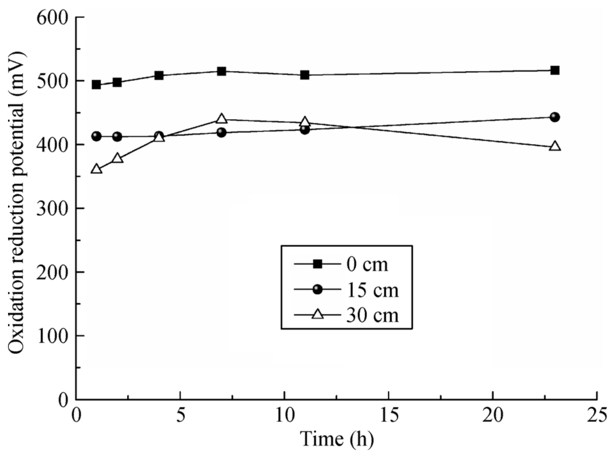

Fig. 3 ORPs at different depths in the zeolite layer

The dynamic process in the ZL could be spontaneously adjusted, thereby making the CW tolerable to pollutant shock loads to some extent. This would ensure the applicability of biozeolite-based CW for treating ADSW. When the adsorbed NH_4^+-N was beyond what the nitrifiers could regenerate, NH_4^+-N would accumulate in the zeolites. The gradual increase of NH_4^+-N in the zeolites affected the dynamic process, reducing the adsorption of NH_4^+-N in the following operational cycle and also the TN removal. However, the lower adsorption of NH_4^+-N in zeolites caused the higher adsorption of NH_4^+-N in the subsequent phases. This might be the reason for the unsatisfactory NH_4^+-N removal during phase 1, which was operated following the startup phase. The adsorption of zeolites dominated during the startup phase due to the immature biofilm and weak microbial regeneration. Excessive NH_4^+-N was adsorbed in the startup phase, which cause less NH_4^+-N adsorption in phase 1 and resulted in unsatisfactory NH_4^+-N removal efficiency. With the release of adsorption sites during phase 1 and a suitable temperature for microbial nitrification in phase 2, the dynamic "rapid-adsorption bioregeneration" process reached its equilibrium. During equilibrium phase 2, NH_4^+-N removal was enhanced. In phase 3, with a decrease in the hydraulic loading rate, NH_4^+-N removal should have been enhanced; however, microbial regeneration was inhibited due to the decreased temperature, which could decrease NH_4^+-N removal. Therefore, NH_4^+-N removal exhibited almost no change in phase 3. In phase 4, owing to the further decrease in temperature, adsorption dominated as it did in the startup phase. After 250 d operation (including 62 days under a temperature of approximately 10°C), the NH_4^{++}-N adsorbed onto the zeolites was 1.75 mg/g, which was below the maximum adsorption capacity of 10.99 mg/g. Therefore, it could be concluded that the CW held low-temperature recalcitrance. With the increase of the temperature during the following spring and summer, biological regeneration would be regained.

The amount of NH_4^+-N removed in the ZL was always higher than that of NO_x^--N_{zeo}. This significant nitrogen loss could be due to several explanations, including anaerobic ammonia oxidation (ANAMMOX) and simultaneous nitrification and denitrification (SND) (Hu et al., 2014). However, ANAMMOX has not been considered in tidal flow CW because it can only occur under strict anaerobic or low-organic matter conditions. Besides, according to the microbial results in the following part, no ANAMMOX genera were identified. In this study, the NO_x^--N from the previous drainage stage in the ZL was washed out with the influent during the flooding stage, and it could be denitrified with a sufficient amount of organics. Therefore, the significant SND in the ZL might be responsible for the nitrogen loss.

SND in the zeolite layer has been corroborated by many previous studies (Zhang et al., 2015). To ensure the occurrence of SND in ZL, the nitrogen mass balance in equilibrium phase 2 was calculated. Theoretically, the NH_4^+-N removed in ZL was identical to the total NO_x^--N produced in the ZL during phase 2. The NO_x^--N produced was identical to the nitrogen removed by SND plus NO_x^--N_{zeo}. The amount of different nitrogen species in phase 2 was calculated and provided in Table 1. During phase 2, the amount of NH_4^+-N removed in the CW was 137.25 g and the nitrogen removed by SND in the ZL was calculated as 70.60 g, which was more than the nitrogen denitrified in the BL during phase 2. The amount of nitrogen denitrified in the ZL accounted for 58.82% of the total denitrification in the CW.

Table 1 The amount of nitrogen in the CW in phase 2

Nitrogen species	Amount (g)
NH_4^+-N removed in the CW	137.25
Effluent NO_x^--N from the ZL	66.65
Nitrogen denitrified in ZL	70.60
Nitrogen denitrified in the whole CW	120.02

However, instead of only one unit, some researchers investigated the CW in parallel units, which can get more directly comparable results (Martínez et al., 2018). Thus, this study could provide only preliminary instructions for the application of this novel CW.

3.2 Nitrogen removal pathway

The nitrogen in CWs could be removed by microbial nitrification-denitrification, plant uptake, microbe assimilation, plant uptake, ammonium volatilization, substrate adsorption and ANAMMOX (Vymazal, 2007). Generally, the mass balance calculation of nitrogen could be conducted by two methods: direct measurements (N_2 production, stable isotopes and, acetylene block) and indirect measurements. The direct measurement could result in more conservative estimates of the N removal by denitrification. Indirect measurement was employed in this study. The N loss by ammonium volatilization was negligible due to the neutral pH in the effluent (7.59–7.72) (Chen et al., 2014). As aforementioned in Section 3.1, the ANAMMOX was not considered. The amount of TN removed after all operational phases was 2046.47 g. The amount of TN removed by substrate adsorption, plant uptake, and microbe assimilation was presented in Table 2.

In conclusion, the fractions of the possible nitrogen removal pathways were as follows: nitrification-denitrification (86.55%), substrate adsorption (11.70%), plant uptake (1.15%) and microbial assimilation (0.60%). Similar results were also obtained by Zhang et al. (2016), who also concluded that microbial nitrification-denitrification up to 79.9% was the main nitrogen removal pathway. Wen et al., (2012) also described that biological nitrogen removal accounted for over 79% of TN removal in their integrated CWs.

3.3 Microbiological analysis

In this part, A and B represent the samples collected from the ZL and BL in summer, and C and D represent the samples collected in winter, respectively. The high-quality sequences generated for samples A, B, C, and D were 35350, 39929, 28451, and 32121, respectively. The operational taxonomic units (OTUs) of the four samples clustered at the 97% similarity level were 382, 507, 304, and 441. The Shannon index values for the four samples were 4.04, 4.36, 3.13, and 3.61, respectively. It was found that the microbial richness and diversity of the CW were higher in summer than winter, and the microbial community was more abundant and diverse in the BL than in the ZL. The Excellent Good's coverage (all above 0.99) indicated that samples were sequenced at high depth.

The microbial composition of the four samples at the phylum level is presented in Fig. 4. The dominant phyla in samples 8 and 9 were similar, but their contents differed. In the summer, *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Chloroflexi*, *Actinobacteria Acidobacteria*, and *Ignavibacteriae* dominated in both the ZL and BL, which was consistent with many previous papers (Zhong et al., 2016); however, *Saccharibacteria* was predominant in both layers

Table 2 Fractions of possible nitrogen removal pathways

Nitrogen removal pathways	The amount of TN removed (g)	Fraction (%)
Substrate adsorption	239.51	11.70
Plant uptake	23.54	1.15
Microbial assimilation	12.25	0.60
Nitrification-denitrification	1771.17	86.55

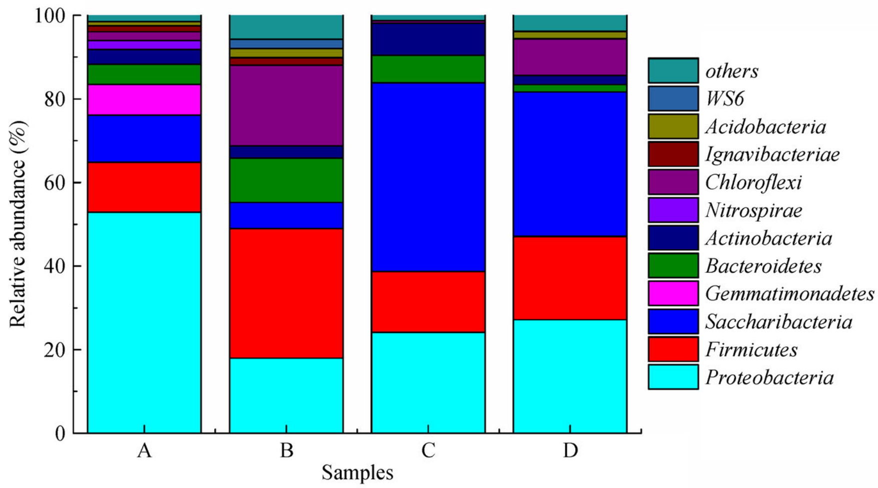

Fig. 4 Microbial composition at the phylum level.

in our study. *Gemmatimonadetes* (7.38%) and *Nitrospirae* (2.14%) dominated uniquely in the ZL, while *WS6* (2.20%) was only found in the BL. *Proteobacteria* was detected frequently in natural or constructed wetlands, and they were integral to nitrogen removal (Arroyo et al., 2015). *Gamma*- and *beta-Proteobacteria* were possibly associated with nitrate abatement (Cheng et al., 2016). Cardinali-Rezende et al. (2012) revealed the abundance of *Bacteroidetes* and *Firmicutes* in swine wastewater. It has been speculated that *Bacteroidetes* can proliferate well in swine wastewater due to the high organic matter content (Zhong et al., 2016), and it was also regarded as a representative denitrifier (Cheng et al., 2016). *Firmicutes* is able to heterotrophically denitrify (Chen et al., 2017). *Saccharibacteria* (former TM7) is ubiquitous in nature, but only a few previous reports have detected it as a dominant phylum, and little information is available about its role in wastewater treatment. Kindaichi et al. (2016) established that *Saccharibacteria* is a phylogenetically diverse group that is involved in the degradation of various organic compounds (versatile organic degrader). Remmas et al. (2017b) also suggested that the *Saccharibacteria* phylum prefer ammonium-rich environments. *Nitrospirae* is well-known for nitrification.

In the winter, the presence of *Saccharibacteria* significantly increased in both the ZL and BL. COD was increased during winter, and thus the increase in *Saccharibacteria* was reasonable. The relative abundance of *Proteobacter* increased in the BL. Based on Fig. 2(c), more NO_x^--N was removed in the BL during the winter than the summer. As many microbes belonging to the *Proteobacter* are denitrifiers, the increase of this group during the winter might be related to the increased removal of NO_x^--N in the BL. *Firmicutes* is resistant to adverse environments, which may explain why it increased in the ZL during winter. The ZL was drained for 23 h in every operational cycle; therefore, the microbes in the ZL could directly receive cold air.

The relative abundance of microorganisms at the genus level is presented in Fig. 5. The main dominant genera in the ZL during the summer were *Mizugakiibacter* (21.99%), *norank_p_saccharibacteria* (10.80%), *norank_f_Gemmatimonadaceae* (7.13%), *Rhodanobacter* (5.59%), *Clostridium_sensu_stricto_1*(4.61%), *Arenimonas* (2.83%), *Alkanibacter* (2.14%), *Nitrospira* (2.14%), *Lactococcus* (2.10%), *Rhizomicrobium* (1.93%), and *Thauera* (1.37%). *Nitrosomonas* and *Nitrospira*, which are typical ammonium-oxidizing bacteria (AOB) and nitrate-oxidizing

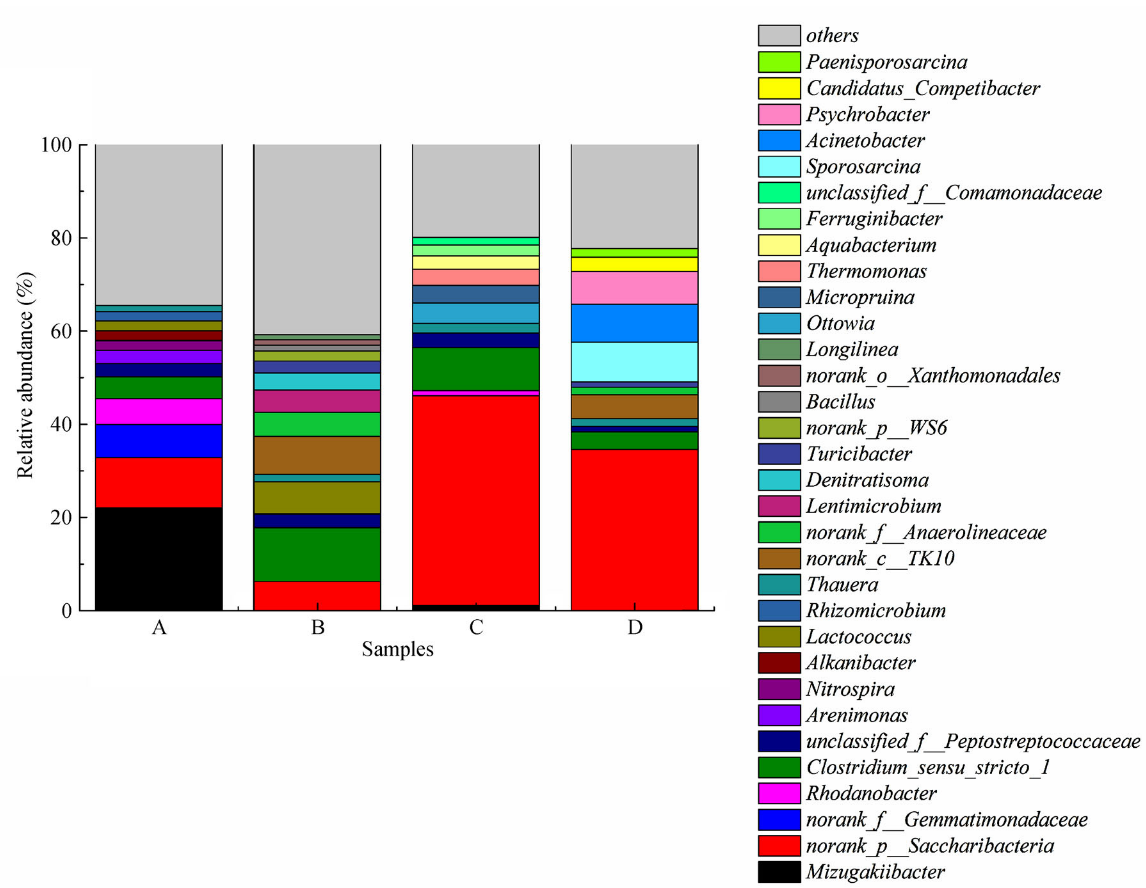

Fig. 5 Microbial composition at the genus level.

bacteria (NOB) have been extensively reported to take part in ammonium oxidization and nitrification in CWs, respectively (Arroyo et al., 2015). In this mesocosm CW, 2.14% of the sequences were assigned to *Nitrospira* in the ZL, while *Nitrosomonas* was not shown on the bar chart as its relative abundance was below 0.5%. Therefore, according to the hypothesis of Zhong et al. (2016), AOB were likely not resistant to external changes. The fluctuating influent quality in this study may have exerted an adverse effect on the activities or survival of AOB. In addition, *Rhizomicrobium* may participate in soil nitrification (Cheng et al., 2017). It was observed that *Rhodanobacteria* and *Thauera*, both denitrifiers, were predominant in the ZL during summer, which verified the existence of simultaneous denitrification in the aerobic zone from the microbial level. In the flooding stage, the ZL was flooded by wastewater for 1 h and became anaerobic. During this stage, the NO_x^--N from the previous drainage stage were mixed with the influent containing high amounts of organics; therefore, denitrification could occur, as discussed above. The imbalance in the amounts of NH_4^+-N removed and NO_x^--N_{zeo}, as well as the COD degraders and denitrifiers that were detected dominantly in the ZL, also demonstrated this. In the drainage stage, air was quickly drawn into the ZL due to the tidal operation. The high ORPs exceeding 400 mV in the ZL also demonstrated the aerobic atmosphere of the ZL during draining stage, which was beneficial for nitrification. This could explain the predominance of nitrifiers in the ZL. The predominant *norank_p_saccharibacteria* (a newly verified versatile organics degrader), other organic matter degraders, and the nitrifiers/denitrifiers could explain the removal of COD, NH_4^+-N, and TN in the ZL.

For the ZL during winter, the relative abundance of *Nitrospira* was decreased and it was not dominant (0.1%). The *norank_p_Saccharibacteria* (15.11%) and *Thauera* (2.03%) were increased. *Ottowia* (4.44%), *Thermomonas* (3.45%), *Aquabacterium* (2.83%), and *Ferruginibacter* (2.36%) were emerging genera in the ZL during the winter. *Ottowia*, *Aquabacterium*, and *Thauera* are all denitrifiers (Huang et al., 2017a). In addition, *Thermomonas* could potentially aid in denitrification (Zhang et al., 2012). *Ferruginibacter* is a versatile heterotrophic degrader for organics (Meng et al., 2016). The increase of *Ferruginibacter* and *norank_p_Saccharibacteria* was due to the increase of COD during winter. The microbial composition at the genus level indicated that the low temperature had a more adverse effect on nitrifiers than it did on denitrifiers and organic degraders.

For the BL during summer, *Denitratisoma* (3.58%), *Thauera* (1.61%), and *Bacillus* (1.21%) were the dominant denitrifiers in the anaerobic zone (Cheng et al., 2016). In addition, *norank_o_Xathomonadales* (1.20%), belonging to the *Xanthomonadales* order, was also predominant. A previous report by Kyongmi et al. (2015) mentioned that the *Xanthomonadales* bacteria in their CW were heterotrophic denitrifiers. *Norank_p_Saccharibacteria* (6.21%) and *norank_f_Anaerolineaceae* (5.14%) were also detected. *Saccharibacteria* was responsible for the degradation of COD. *Anaerolineae* could degrade carbohydrate (Yang et al., 2015), which might also be related to COD removal. The BL was saturated with wastewater during the whole experiment, and contained large amounts of anaerobic denitrifiers and organic matter-degraders. However, for the BL during winter, the relative abundance of *norank_p_Saccharibacteria* significantly increased to 34.5% due to the increased influent COD during winter, and higher COD removal was also observed in the BL in phase 4 than that in phase 1 (Fig. 2(a)). The dominant denitrifiers in the BL during winter were *Acinetobacter* (8.17%) and *Thauera* (1.73%). Although the variety of denitrifiers decreased in the BL during winter, the total relative abundance of the dominant denitrifiers in the winter (9.9%) was higher than that in the summer (7.60%), and NO_x^--N removal in the BL was also higher in phase 4 than in phase 1. Additionally, based on the current literature, the anammox genera included *Brocadia*, *Kuenenia*, *Anammoxoglobus*, *Jettenia* (all fresh water species), and *Scalindua* (marine species) (Jetten et al., 2009). However, according to the microbial results, no anammox genera were identified in the CW.

4 Conclusions

Due to the “rapid-adsorption bioregeneration” process that could reach dynamically stable, the biozeolite-based CW could achieve persistent ammonium removal on treating decentralized swine wastewater. Meanwhile, this process also ensured that the adsorption sites of zeolites were not all occupied. Thus, the CW could operate with the fluctuant influent water quality and under low temperatures to some extent. Tidal flow was better for TN removal than intermittent flow in the partially saturated CW. With surface loading rates of 0.108, 0.027, and 0.029 kg/($m^2 \cdot d$) for COD, NH_4^+-N, and TN, the CW could achieve corresponding removal efficiencies of 85.94%, 61.20%, and 57.41%, respectively, even at 10°C. When the “rapid-adsorption bioregeneration” process reached dynamically stable, the SND was observed in the zeolite layer, and it accounted for 58.82% of the total denitrification in the CW. Illumina high-throughput sequencing also validated that *Nitrospira*, *Rhodanobacter*, and *Thauera* were the dominant genera in the zeolite layer. In the winter, more reported or potential denitrifiers, including *Ottowia*, *Aquabacterium*, *Thauera*, and *Thermomonas* were predominant in the zeolite layer. The contribution of the possible nitrogen removal pathways in the CW was as follows: nitrification-denitrification (86.55%) > substrate adsorption (11.70%) > plant uptake (1.15%) > microbial assimilation (0.60%).

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