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Achieving short-cut nitrification and denitrification in modified intermittently aerated constructed wetland

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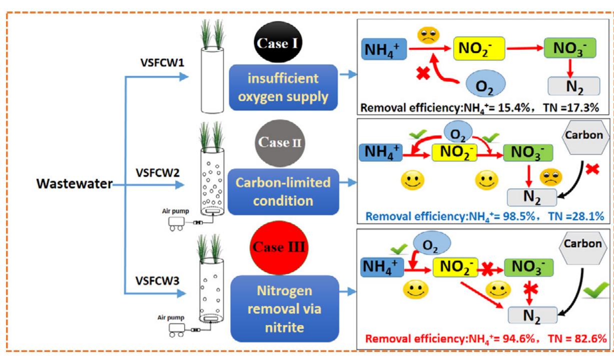
HIGHLIGHTS

- Nitrogen removal from nitrite pathway was achieved in CWs.
- Enhanced TN removal and reduced aeration costs were achieved.
- Appropriate alternating anoxic/aerobic condition results in nitritation.

GRAPHICAL ABSTRACT



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ABSTRACT

This study aim to enhance nitrogen removal performance via shifting nitrogen removal pathway from nitrate to nitrite pathway. It was demonstrated that nitrite pathway was successfully and stably achieved in CWs by using modified intermittent aeration control with aeration 20 min/non-aeration 100 min and reducing DO concentration during aeration, nitrite in the effluent could accumulate to over 70% of the total oxidized nitrogen. Q-PCR analysis showed that nitrifying microbial communities were optimized under the alternating anoxic and aerobic conditions, ammonia oxidizing bacteria increased from 7.15×10^6 to 8.99×10^6 copies/g, while nitrite oxidizing bacteria decreased approximately threefold after 234 days operation. Most importantly, high nitrogen removal efficiency with ammonium removal efficiency of 94.6%, and total nitrogen removal efficiency of 82.6% could be achieved via nitrite pathway even under carbon limiting conditions. In comparison to the nitrate pathway, the nitrite pathway could improve the TN removal by about 55%.

1. Introduction

Nitrogen must be removed from wastewater to protect lakes and other aquatic systems from eutrophication, which is a world-wide environmental problem (Chislock et al., 2013). Historically, the coupled nitrification-denitrification is a widely used process to remove nitrogen from wastewater. However, this combined process needs large amounts of oxygen and carbon sources. In recent years, new strategies in biological nitrogen removal shifted the focus from nitrogen removal via nitrate ($NH_4^+ \rightarrow NO_3^- \rightarrow N_2$, the nitrate pathway) to nitrogen removal via nitrite ($NH_4^+ \rightarrow NO_2^- \rightarrow N_2$, the nitrite pathway) (Gilbert et al., 2014). In the nitrite pathway,

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nitrification is stopped at nitrite (nitritation) and then the formed nitrite is reduced to dinitrogen gas via denitrification and/or anammox. Compared to conventional nitrate pathway, nitrite pathway presents significant advantages, as it can save at least 40% of the carbon sources demand, reduce 25% of the oxygen requirement and improve denitrification rate by 63% (Kornaros et al., 2010; Turk and Mavinic, 1987).

Nowadays, nitrogen removal via the nitrite pathway has been successfully achieved in diverse systems such as the biofilm systems (Chuang et al., 2007; Prá et al., 2013) and activated sludge system (Vlaeminck et al., 2009; Yang et al., 2007), and enhanced nitrogen removal efficiency can always be obtained via nitrite pathway in these systems. However, these wastewater treatment technologies are rather expensive and not feasible for treating widely distributed rural domestic wastewater (Maltais-Landry et al., 2009). Compared to conventional wastewater treatment systems, constructed wetlands (CWs) are efficient and environment-friendly wastewater treatment systems with low construction and operation cost (Li et al., 2014; Zhi et al., 2015). Such systems are especially suitable for treating decentralized domestic sewage from rural areas. Since low capacity and efficiency of nitrogen removal is one major problem that disturbs the application of CWs (Wu et al., 2014), shifting the nitrogen removal pathway from nitrate to nitrite in CWs seems to be a good choice. Nevertheless, nitrogen removal via nitrite pathway is usually limited in wetland systems as ammonium oxidizing bacteria (AOB) grows much slowly at moderate conditions than nitrite oxidizing bacteria (NOB). Although some recent studies have revealed that nitrogen removal via nitrite pathway (e.g. anammox, partial nitrification-denitrification process) may also exist in CWs (Hu et al., 2012a, b), the perception on N cycling in CWs remains still rooted on the classical view of nitrification and denitrification. Until now how to achieve a stably and high rate nitrite pathway in CWs remain uncertain.

To achieve a stable nitrite pathway process in CWs, a selectively inhibiting the activities of NOBs while maintaining the activities of AOBs should be obtained. Previously, several factors have been proposed for achieving nitritation in wastewater treatment plants, such as high temperature, high pH, appropriate dissolve oxygen (DO) level, high free nitrous acid (FNA) concentration and high free ammonia (FA) concentration (Abeling and Seyfried, 1992; Li et al., 2008; Park and Bae, 2009; Van Hulle et al., 2007). However, these factors are normally unavailable for CWs. For example, the water temperature in CWs is changed with seasons. It is hard to always keep the high temperature in CWs. High FA, FNA concentration can effectively inhibit the activity of NOB, while the FA and FNA concentrations are normally far below inhibition boundary conditions for NOB in typical ammonium-low wastewater treated by CWs.

Among the above factors, DO level seems to be the most possible factor which could be used to achieve nitrite accumulation in CWs. Theoretically, low DO concentration influences the activity of NOB more significantly than that of AOB due to the higher affinity of AOB with oxygen in comparison with NOB (Park and Noguera, 2004; Tokutomi, 2004). Additionally, many previous studies in active sludge system have shown that AOB recovered much more rapidly than NOB after anoxic disturbance, which give rise to nitrite accumulation when environment changed from anoxic to aerobic conditions (Ge et al., 2014; Gilbert et al., 2014). Therefore, it can be assumed that optimizing the oxygen levels (e.g. creating a shifting aerobic/anoxic conditions, limiting DO concentration during aerobic condition, providing appropriate aerobic/anoxic time ratios etc.) in CWs can selectively suppress/eliminate NOB, and thereby shifting nitrogen removal pathway from nitrate to nitrite. For ammonium-rich wastewater, nitritation has been achieved with the use of intermittent aeration strategy in CWs. However, for ammonium-low domestic wastewater, the effectiveness of using aerobic duration control for achieving nitritation in CWs has not been investigated yet. In fact, it is more difficult to repress NOB when treating ammonium-low wastewater because of lacking the inhibition factors such as high free ammonium (FA) and free nitrite (FNA).

Hence, the aim of the present study was (i) to investigate the feasibility of aerobic duration control in attaining sustained nitritation in CWs for ammonium-low wastewater treatment; (ii) to quantify whether satisfactory nitrogen removal could be obtained via nitrite pathway and to understand its mechanisms at the molecular level.

2. Materials and methods

2.1. Microcosm wetlands set up

Three microcosm vertical subsurface flow constructed wetlands (termed VSFCW1, VSFCW2 and VSFCW3) were constructed with PVC column (diameter 20 cm) in a greenhouse with temperature controlled at 23 ± 3 °C. The CW bed was made up of two functional layers (Fig. 1): a 10 cm depth water supporting layer (filled with gravel with diameter ranging from 20 to 30 mm) at the bottom and followed by a 60 cm depth treatment layer (filled with gravel with diameter ranging from 8 to 10 mm). The working volume of each CW was 8.79 L (initial porosity of 40%). Two vertical perforated PVC pipes (diameter 3 cm) were pre-buried in the middle of CW bed. One was used for monitoring various physical and chemical parameters (DO, pH, temperature) in situ. The other one filling with five columns (contained the same bed material of CWs) which wrapped up by highly permeable nylon mesh was used for microbial analysis using previously described methods (Zhi et al., 2015). Air was supplied via a diffuser placed on water distribution layer, and an air flow meter was used for controlling aeration rate. The top surface of each CW was planted with *Iris pseudacorus* at an initial density of 50 plants/m^2. The CWs were seeded with return sludge of a local municipal wastewater treatment plant (MWWTP) in Wuhan, China, and operated for about a month prior to the experiments.

2.2. Wastewater and operational conditions

Synthetic wastewater made from tap water was used in this study to minimize fluctuation in feed concentration. The composition of the wastewater were as follows: 282 mg/L (or 141 mg/L during phase 5) glucose, 236 mg/L $(NH_4)_2SO_4$, 13.2 mg/L KH_2PO_4, and 400 mg/L $NaHCO_3$, resulting in the following specific characteristics of the wastewater: COD of 300 mg/L (or 150 mg/L during phase 5), NH_4^+-N of 50 mg/L, PO_4^{3-}-P of 3.0 mg/L and pH of 7.2–7.5. Additionally, some supplement of mineral elements were also added to wastewater every two weeks using previously described formula (Li et al., 2011).

The experiment began on August 20, 2015 and lasted a total of 234 days. Based on different operation conditions, the experiments were divided into five phases (Table 1). In phase 1, in order to enhance the growth of nitrifiers (AOB and NOB), a high DO level was applied in VSFCW1–3 under continuous aeration with an aeration rate of 1 L/min. In phase 2, different aeration strategies were applied for VSFCW1–3. VSFCW1 was run as a control without aeration. VSFCW2 and VFSCW3 were operated with different intermittently aerated patterns. The respective operation conditions are summarized in Table 1. In phase 3, to inhibit the activity of NOB, 20 mg/L hydroxylamine was added into the wastewater. Also, in VSFCW2, to better inhibit the growth of NOB, the DO concentration during aeration was adjusted to 0.4–0.6 mg/L in this phase. In

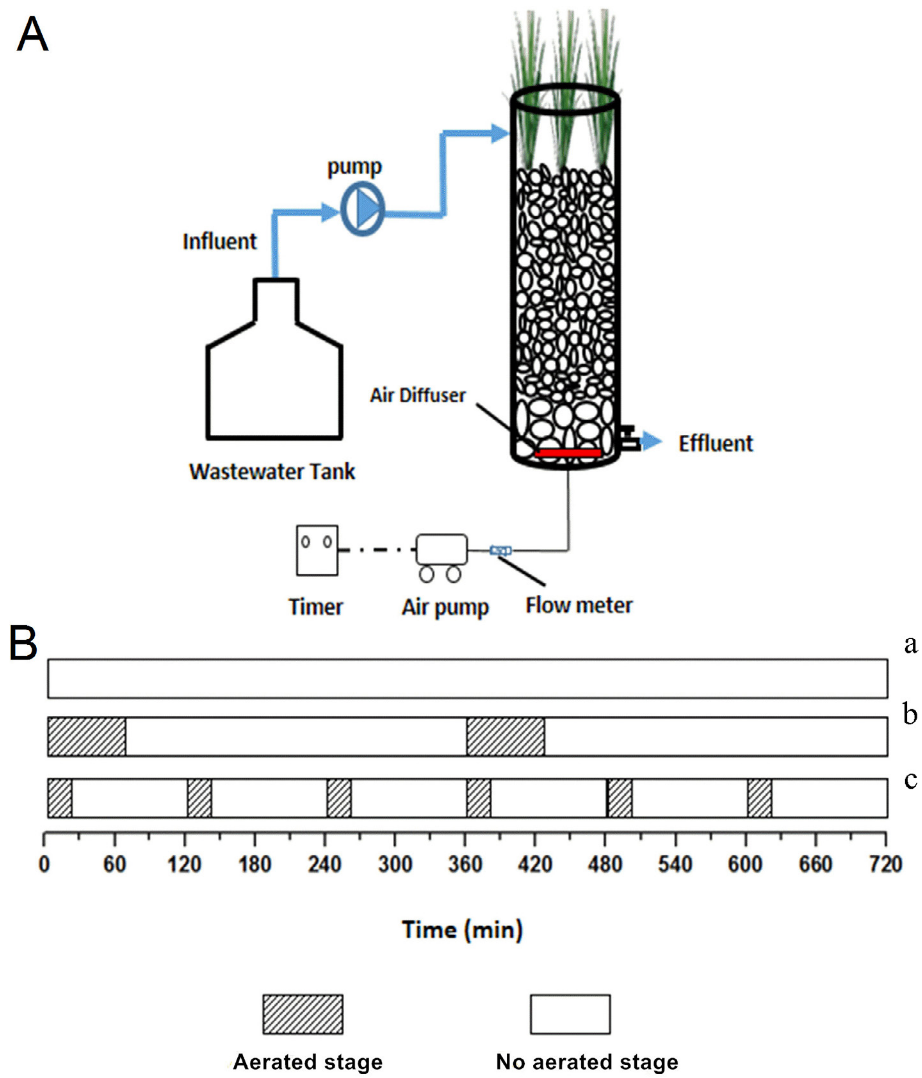

Fig. 1. Schematic of the experimental apparatus: (A) system setup, (B) aeration scheme in a typical phase, (a) VSFCW1; (b) VSFCW2; (c) VSFCW3.

Table 1
Experimental plan and operational conditions

Phase	Time (d)	Aeration mode No aeration (min): aeration (min)			Aeration rate (mL air min^{-1})			DO (mg/L)		
		VSFCW1	VSFCW2	VSFCW3	VSFCW1	VSFCW2	VSFCW3	VSFCW1[a]	VSFCW2[b]	VSFCW3[b]
1	1–27	Constant	Constant	Constant	1000	1000	1000	6.0–8.0	6.0–8.0	6.0–8.0
2	27–90	No aeration	300: 60	100:20	–	1000	30–50	0.2–0.25	6.0–8.0	0.4–0.6
3	90–111	No aeration	300: 60	100:20	–	30–50	30–50	0.2–0.25	0.4–0.6	0.4–0.6
4	111–171	No aeration	300: 60	100:20	–	1000	30–50 (90-156d) 50–70 (156-171d)	0.2–0.25	6.0–8.0	0.4–0.6 (90–156d) 0.8–1.2 (156–171d)
5	171–234	No aeration	300: 60	100:20	–	1000	50–70	0.2–0.25	6.0–8.0	0.8–1.2

[a] The effluent DO concentration.
[b] DO concentration during aerated stage.

phase 4, the hydroxylamine addition was stopped to verify whether the nitritation could be maintained by using intermittent aeration alone. Additionally, the DO concentration during aeration in VSFCW3 was increased to 0.8–1.2 mg/L to improve the ammonia oxidization performance at the end of phase 4 (from day 156). In phase 5, the COD/N ratio in influent was adjust to 3 to investigate how nitrogen removal performance responded to different influent C/N ratios. Throughout the study, the experiment cycle was 72 h for VSFCW1–3. At about 10:00 am on the first day of each cycle, the effluent was firstly drained from the outlets at the bottom of VSFCW1, VSFCW2 and VSFCW3, respectively, and then influent was supplied in batch mode into VSFCW1, VSFCW2 and VSFCW3 within 15 min by peristaltic pumps. Under such operation strategy, the hydraulic and nitrogen loading rates of VSFCW1-3 were maintained at 0.09 m^3/m^2 d and 4.5 g-N/m^2 d throughout the study, respectively, while the organic loading rate varied between 13.5 and 27 g-COD/m^2 d depending on different operation phase.

2.3. Sampling and analytical methods

Water samples from influent and effluent were collected every three days and were analyzed immediately in the laboratory. COD, NH_4^+-N, NO_2^--N, NO_3^--N and TN were measured according to Standard Methods (Apha, 1995). The temperature, pH, and DO were monitored in suit with an YSI Pro Professional Plus (USA). The nitrite accumulation percentage (NAP) was estimated using the equation as follows:

$$\mathrm{NAP} = \frac{\text{effluent } NO_2^- - N}{\text{effluent } NO_2^- - N + \text{effluent } NO_3^- - N} \times 100\%$$

Microbial samples were collected from pre-buried columns on days 27, 90, 111, 171 and 234. After sampling, the columns were firstly transferred to sterile plastic bags stored in an ice cooler and brought back to the laboratory immediately. Then the gravel in each columns were thoroughly mixed for biofilm collection. The attached biofilm was extracted according to previous method (Zhi and Ji, 2014). Briefly, 100 g of fresh gravel was weighed into 500 mL sterile Erlenmeyer flasks. Each flask contained 200 mL of phosphate buffer (1 mM, pH 7.4). The flask was firstly ultrasonic cleaned for 10 min, and then was shaken on an orbital shaker (250 rpm) at 20 °C for 1 h. The precipitate was collected in bottles after centrifuging twice at 4000 rpm for 20 min. Then the collected attached biofilm were stored at −80 °C for DNA extraction.

2.4. DNA extraction and qPCR

DNA was extracted from attached biofilms and seed sludge using UltraClean Soil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA) according to the manufacturer's instructions. The qPCR was performed to analyze the population dynamics of nitrifying bacteria. The absolute abundance of bacterial *amoA* gene (primer amoA1F/amoA2R), *Nitrospira* species 16S rRNA gene (primer 341f/NTSPAr) and *Nitrobacter* species 16S rRNA gene (primer FGPS872f/FGPS1269r) were quantified with an ABI STEPONE PLUS thermocycler (Applied Biosystems, Foster City, CA) using previously described protocols (Degrange and Bardin, 1995; Li et al., 2011; Nakamura et al., 2006). Standard curves were established with known quantities of cloned bacterial *amoA* gene, *Nitrospira* species 16S rRNA and nitrite *oxidoreductase* genes, respectively. The target gene copy numbers are presented per gram of dry weight (copies/g) of wetland media.

3. Results and discussions

3.1. Overall performance

In phase 1, a high DO concentration over 6 mg/L was maintained in three CWs at a continuously aeration rate of 1 L/min. Results showed that the removal efficiency of NH_4^+-N for VSFCW1–3 all exceeded 98%, with effluent NH_4^+-N concentration of 0.59 ± 0.12 mg/L (Figs. 2 and 3). While the influent NH_4^+-N was almost completely removed, the removal of TN was relatively low, the averaged TN removal effective was only about 22.3 ± 3.2%, with effluent TN concentration of 38.72 ± 0.94 mg/L. The major N component in the effluent was NO_3^--N, and the effluent NO_2^--N was neglectable, indicating that the nitrification was full-range nitrification in all three CWs as a result of continuously sufficient DO supplied.

In phase 2, different aeration strategies were applied for VSFCW1–3. For the non-aerated VSFCW1, oxygen was rapidly consumed during the first 2 h, and then the DO concentration was almost remained unchanged during the subsequence experiment period (Fig. S1). The average effluent DO concentration was merely 0.25 mg/L, indicating that the non-aerated VSFCW1 was under anoxic condition in most time of the cycle. As expected, incomplete nitrification became limiting step for TN removal in the VSFCW1. The effluent was dominated by high level of NH_4^+-N (37.38 ± 2.73 mg/L) (Fig. 2B), with average NH_4^+-N removal efficiency being only 25.0% (Fig. 3). The TN removal profile was similar to NH_4^+-N in VSFCW1. For traditional intermittent aerated VSFCW2, the DO concentration maintained at 6–8 mg/L during aeration and decreased below 0.30 mg/L when artificial aeration was terminated (Fig. S1). As shown in Fig. 3, the intermittent aeration did not have an obvious influence on NH_4^+-N removal in VSFCW2, the average NH_4^+-N removal efficiency exceeded 98%, which was equivalent to that in phase 1. However, with the intermittent aeration strategy, the TN removal efficiency was significantly improved in VSFCW2. The average TN removal efficiency increased from 22% in phase 1 to 60% in this phase. The major N component in the effluent was NO_3^--N (21.61 ± 1.48 mg/L), and the effluent NO_2^--N was neglectable (Fig. 2B). At the same, in order to achieve nitrite pathway, DO concentration was adjusted to 0.4–0.6 mg/L during aeration and the intermittent time was adjusted to aeration 20 min/non-aeration 100 min in VSFCW3 (Fig. S1). Results indicated that the period from day 30 to day 39 was a transition period, the NH_4^+-N removal efficiency recovered gradually during this period and then stabilized at about 83%. Although the effluent NH_4^+-N concentration was stable from day 39, the effluent nitrate concentration was quite unstable (Fig. 2D), which caused variation in the percent of TN removal. The maximal TN removal efficiency was near to 70%, while the minimum TN removal efficiency was only about 50% (Fig. 3).

In phase 3, 20 mg/L hydroxylamine was added into the wastewater to inhibit NOB activity. There was no significant change in effluent component for VSFCW1 (Fig. 2B). However, as to VSFCW2 and VSFCW3, the effluent nitrate decreased rapidly after hydroxylamine addition. At the end of phase 3, the NAP of VSFCW2 and VSFCW3 increased to 49.8% and 51.8% respectively (Fig. 2C and 2D).

During phase 4, after hydroxylamine addition stopped, stable nitritation process was maintained in VSFCW3 even at higher DO levels (0.8–1.2 mg/L) at the end of phase 4. As shown in Fig. 2 and Table S1, the NH_4^+-N conversion efficiency increased to 94.6% and NAP maintained at 55.6% at the end of phase 4 for VSFCW3. In contrast to the VSFCW3, the nitritation process only maintained 9 days in VSFCW2. Afterwards, the effluent was dominated by high concentration nitrate, and the effluent nitrite was neglectable (Fig. 2C).

From day 171 to day 234 (phase 5), wastewater with a much lower COD/N ratio of 3 was fed to VSFCW1–3. The change in COD/N ratio did not have obvious influences on NH_4^+-N conversion efficiency for three wetland systems. The NH_4^+-N conversion efficiencies were all equivalent to those at the end of phase 4 for three CWs (Fig. 3). However, denitrification became the limiting step for TN removal in VSFCW2 due to carbon deficiency. The effluent nitrate in VSFCW2 increased from 20.46 ± 2.11 in phase 4 to 36.90 ± 1.59 mg/L in phase 5. Consequently, the TN removal efficiency decreased from 63.6 ± 10.0% in phase 4 to 28.1 ± 3.2% in phase 5. For VSFCW3, the NAP increased gradually and reached 72.2% at the end of phase 5, indicating that the nitritation was successfully maintained and enhanced in VSFCW3. As the result of removing nitrogen via nitrite pathway, the TN removal efficiency in VSFCW3 was still maintained above 80%, even though the influent COD/N ratio was only 3.

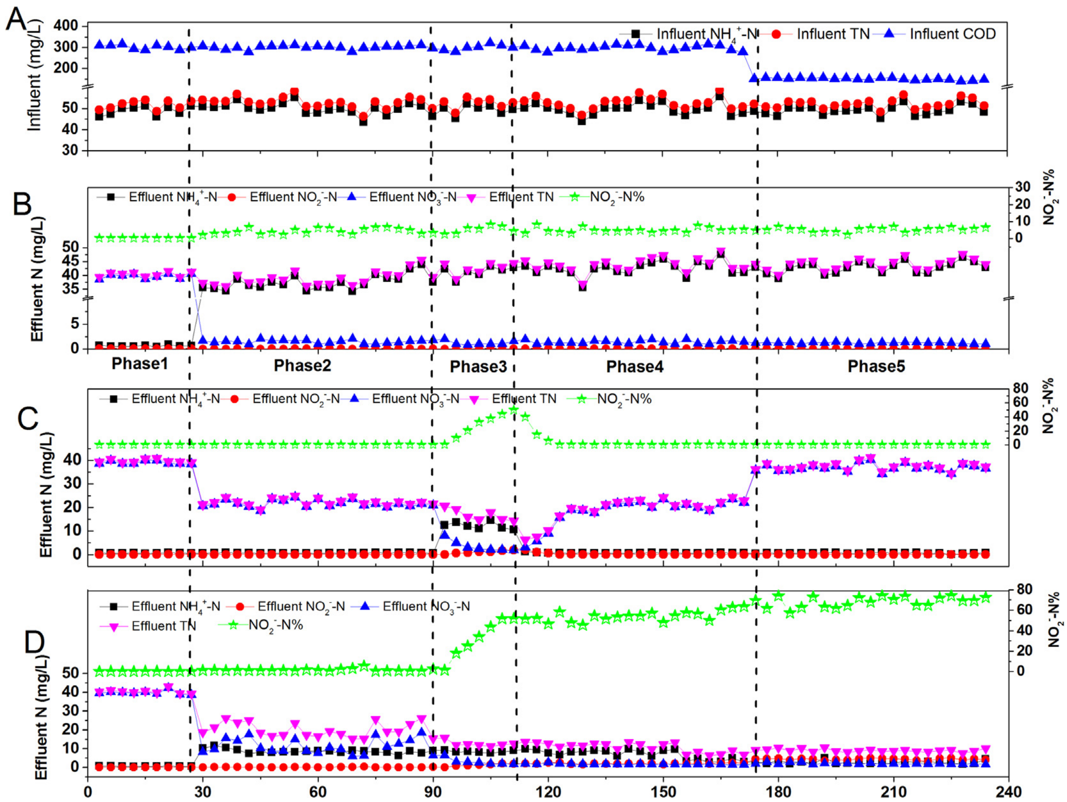

Fig. 2. VSFCWs performance: (A) the influent COD, NH_4^+-N and TN concentrations; the effluent NH_4^+-N, NO_2^--N, NO_3^--N, TN and NO_2^--N accumulation percent in VSFCW1 (B), VSFCW2 (C) and VSFCW3 (D).

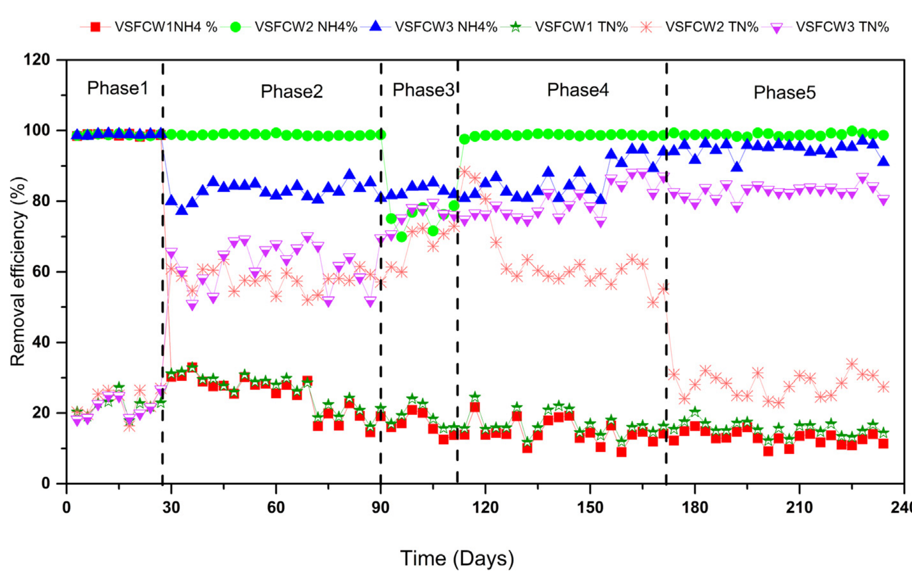

Fig. 3. Removal efficiency of NH_4^+-N and TN.

3.2. Quantification of AOB and NOB through qPCR

Quantification of the main nitrifiers was performed by qPCR. In the seed sludge, there was 4.22×10^9 copies/L of AOB and 2.94×10^9 copies/L of NOB. It was noteworthy that the *Nitrospira* spp. was the dominant NOB with abundance of 2.85×10^9 copies/L, while the abundance of *Nitrobacter* spp. was only 9.08×10^7 copies/L.

In VSFCW1, the abundance of both AOB and NOB decreased sharply since phase 2, and then stabilized at low levels throughout the subsequent experiment (Fig. 4). Compared to VSFCW1, the AOB population in VSFCW2 kept at a stable level from phase 1 to phase 4, while increased to 2.17×10^7 copies/g at the end of phase 5. On the other hand, the abundance of *Nitrospira* spp. and *Nitrobacter* spp. both decreased sharply at the end of phase 3 due to the addition of hydroxylamine, while the *Nitrospira* spp. number quickly recovered to 4.20×10^6 copies/g at the end of phase 4, which was similar to the amount during phase 2. In contrast, *Nitrobacter* spp. became undetectable during subsequent experiment.

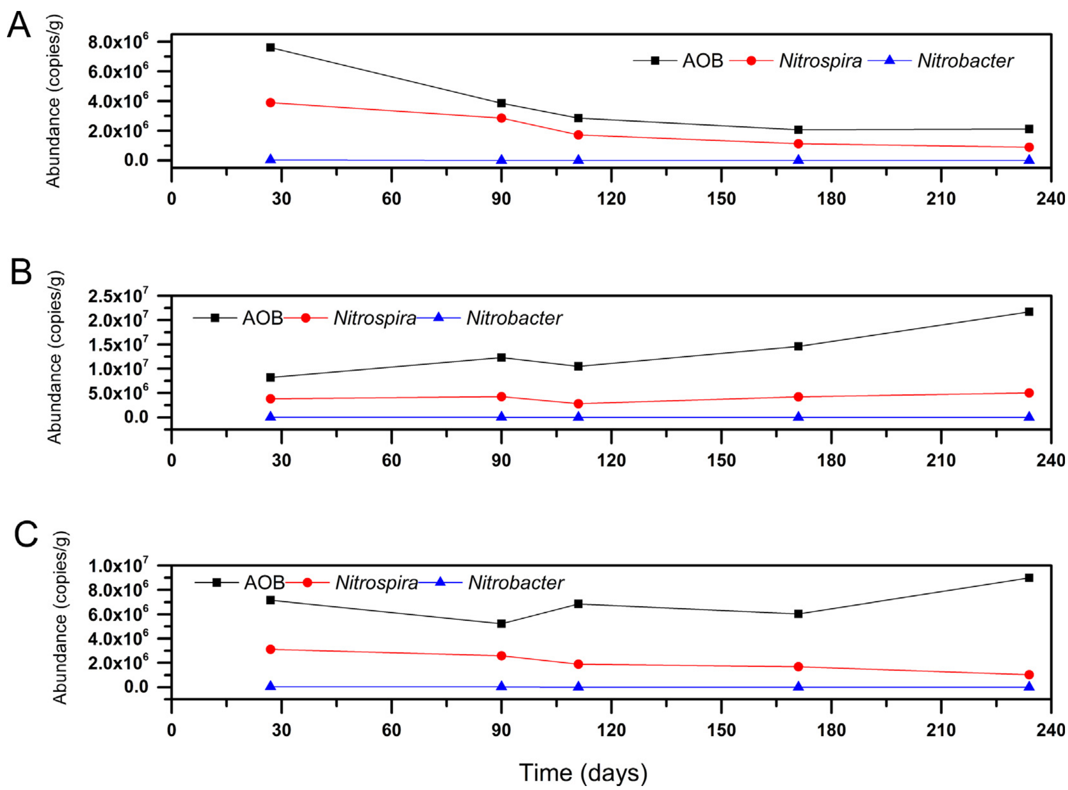

Fig. 4. Population variations of AOB and NOB in the VFCWs: (A) VSFCW1; (B) VSFCW2; (C) VSFCW3.

For VSFCW3, the population of both AOB and NOB showed a complex dynamics throughout the course of the study. In phase 1, 7.15×10^6 copies/g of AOB, 3.12×10^6 copies/g of *Nitrospira* spp and 3.99×10^4 copies/g of *Nitrobacter* spp were detected on day 27. In phase 2, due to the low DO (0.4–0.6 mg/L) level during aeration, both AOB and NOB decreased slightly compare to that in phase 1. In Phase 3, after adding hydroxylamine into system, NOB (both *Nitrospira* spp. and *Nitrobacter* spp.) decreased sharply while AOB increased slightly to 6.85×10^6 copies/g. In phase 4, the AOB abundance kept at a stable level which was almost the same as that at the end of Phase 3, while *Nitrobacter* spp. became undetectable and *Nitrospira* spp. decreased to 1.69×10^6 copies/g. In Phase 5, with a much lower COD/N ratio, the abundance of AOB increased to 8.99×10^6 copies/g, while the *Nitrospira* spp. decreased to 1.03×10^6 copies/g, which was about 1/3 of the number at the end of phase 1.

3.3. The mechanisms of achieving nitritation in CWs

In the literature, many parameters and conditions have been proposed for achieving nitritation through appropriate regulation factors that accumulate AOB while inhibit NOB. These factors include temperature, pH, FA, FNA, DO, as well as chemical inhibitors. In the present study, the temperature was controlled at 23 ± 3 °C, with pH maintaining at 7.0–7.5 in all CWs. Thus, the temperature and pH were unlikely contributing factors to nitritation in the present study. FA and FNA were widely accepted as common inhibitors on NOB activity. However, the maximum FA and FNA concentration in the present study was only 0.85 and 0.001 respectively, which was far below inhibition boundary conditions for NOB based on previous study (Anthonisen and Srinath, 1976; Turk and Mavinic, 1989). Control the DO at low concentration (below 1 mg/L) can favor the dominance of AOB due to the higher oxygen affinity of AOB compared with NOB (Park and Noguera, 2004; Tokutomi, 2004). However, the influence of low DO on NOB activity seemed to be limited in the present study. As shown in Fig. 2D, there was no significant nitrite accumulation in VSFCW3 when the average DO concentration during aeration was maintained at 0.4–0.6 mg/L in phase 2, while stable nitritation was achieved in phase 5 with a higher DO concentration (0.8–1.2 mg/L). These results indicated that low DO concentration was not the major selection factor for the successful start-up and maintenance of nitritation in this study.

In the present study, it was supposed that there were two main reasons for the successful achievement and stable maintenance of partial nitrification. The first one was a temporary addition of a selective inhibitor for NOB to initiate nitritation. In phase 3, free hydroxylamine was added to CWs with a concentration of 20 mg/L to inhibit the growth of NOB. As illustrated in Fig. 2, phase 3, a stable nitrite accumulation was attained in VSFCW2 and VSFCW3 during the hydroxylamine addition period. The corresponding NOB population (both *Nitrobacter* spp. and *Nitrospira* spp.) decreased sharply during the phase 3. The importance of the addition of hydroxylamine to begin attaining nitritation was confirmed by comparing with results obtained from VSFCW3 during phase 2, in which it was not successful to attain nitritation under the same intermittently-aeration mode. As shown in Fig 2D, phase 2, the effluent nitrate concentration in VSFCW3 ranged from 6.25 to 17.56 mg/L, while the effluent nitrite was neglectable. Additionally, after it was operated for 60 days of aeration duration control in phase 2, the *Nitrospira* spp. population in VSFCW3 still kept at 2.58×10^8 copies/g, which was almost the same as that at the end of Phase 1. The above results demonstrated that it was hard to start nitritation by only using the intermittent aeration strategy. Similarly, Li et al. (2011) found that in a sequencing batch reactor, if there was a high NOB population in reactor system, it was hard to achieve nitritation within a short time by using aeration duration control alone. One possible explanation for these phenomena is the fact that both NOBs and AOBs can attain growth opportunities during the aerated period of the cycle, the net loss of

NOBs per cycle may be small (Blackburne et al., 2008). Hence, it would take a long time to wash out the NOB if the initial NOB population is large. Additionally, the *Nitrospira* spp. was the dominant NOB in the present study. Compared to *Nitrobacter* spp., *Nitrospira* spp. has higher affinities for NO_2^- and DO. Consequently, *Nitrospira* spp. can effective compete oxygen with AOB when oxygen is insufficient, which limited the impact of low DO concentration (0.4–0.6 mg/L during aeration) on NOB suppression in phase 2. Therefore, it is beneficial to use an NOB inhibitor to reduce the time required to achieve nitritation.

The second factor was the appropriately intermittent aeration profile. The use of intermittent aeration had been found as an effective strategy to accomplish nitritation (Bournazou et al., 2013; Kornaros et al., 2010). In the present study, the nitritation was rapidly destroyed in VSFCW2 while successfully maintained in VSFCW3 after hydroxylamine addition stopped. Moreover, the nitrite accumulation percent was further enhanced in VSFCW3 in phase 5 without other inhibition factors. These observations proved that the nitrogen removal pathway (either nitrite pathway or nitrate pathway) could be selected in CWs by using aeration pattern control.

It was believed that the intermittent aeration can promote nitrite accumulation mainly through three ways: (1) NOB recovered more slowly than AOB when milieu changes from anoxic to aerobic conditions, which gave rise to nitrite accumulation when aeration was recovered (Kornaros et al., 2010); (2) timely consuming nitrite at anoxic stage by denitrifiers, which restricted the substrate for NOB in the aerobic conditions of next cycle (Hu et al., 2012a); (3) over long term operation of the alternating anoxic and aerobic condition, NOB was gradually washed out from the system as a result of limited substrate (nitrite) utilization by NOB and anoxic disturbance, which could lead to a high and stable nitritation (Ge et al., 2014). Based on the different function mechanisms, these three ways can be divided into two types. The way (1) and (2) could be classified as the first type, in which nitritation is achieved mainly through temporarily inhibiting the activities of NOBs, not by completely eliminating the NOBs. In this case, NOBs may still exist in the reactor systems, once the environment becomes suitable for NOB again, the activities of NOBs may recover at short notice and nitritation will be destroyed rapidly. Compared to the first type, nitritation is achieved through optimizing the nitrifying bacteria communities (washing out NOB) in the second type, which normally can result in a robust partial nitrification. In the present study, after hydroxylamine addition during phase 3, the *Nitrobacter* spp. was completely eliminated from VSFCW3, while there was still a small amount of *Nitrospira* spp. existed in the VSFCW3 at the end of phase 4. These results suggested that intermittent aeration might play its role mainly through the first type (inhibition of NOB activity) during the phase 4. After that, over many cycles of alternating anoxic and aerobic condition during phase 5, the *Nitrospira* spp. in VSFCW3 gradually reduced to 1.03×10^6 copies/g at the end of phase 5, which was only 1/3 of the number at the end of phase 1. Although the NOB was not completely washed out from VSFCW3, the greatly decreased NOB population seemed to be the primary factor in establishing the observed higher NAP in phase 5 than that in phase 4. Previous studies also showed that the optimized nitrifying microbial communities in the systems should be the most important reason to keep high and stable NO_2^--N accumulation after long term operation of the alternating anoxic and aerobic condition (Ge et al., 2014). However, contrary to the case of Ge et al. (2014), it was difficult to achieve more than 75% nitrite accumulation in this study. One possible explanation for these differences is that it was hard to accurately control DO concentration in microenvironments of CWs matrix. The plants in CWs can deliver oxygen to the wetland matrix through the rhizosphere, where is a suitable micro niche for NOBs. Hence, it was hard to completely wash out NOBs and counteract nitrate production in the present study.

As noted previously, both AOBs and NOBs can attain growth opportunities during the aerated period, an unsuitable anoxic and aerobic distribution may lead to NOB reactivate and destroy the nitritation. For example, the overlong aerobic time in VSFCW2 destroyed the nitritation rapidly after hydroxylamine addition was stopped. Additionally, DO concentration during aerated period is also important in the selection of AOB over NOB in intermittently aerated reactor systems (Gilbert et al., 2014). As reported (Mota, 2005), a high DO concentration will accelerate the recovery rate of NOBs, which made it hard to keep the expected AOB and NOB activities. Hence, although intermittent aeration strategy had been used widely in CWs nowadays, the primary nitrogen removal pathway was still the classical nitrate pathway in these studies due to the over long aeration time and over high DO concentration at aerated stage (Fan et al., 2013a,b; Uggetti et al., 2016). On the contrary, a modified intermittent aeration mode (suitable anoxic/aerobic distribution and limited DO concentration during aeration) was applied in VSFCW3, which ensure the inhibition of NOB activity before the following anoxic disturbance. This finally resulted in the changes of nitrogen pathway (from classical nitrate pathway to nitrite pathway) in CWs.

3.4. Environmental implications and significance

CWs have been intensively studied and proposed as feasible alternatives to conventional wastewater treatment plants. However, nitrogen removal efficiency reported for CWs was variable and often unsatisfactory. The poor performance of nitrogen removal might partly arise from the research directions over focused on how to improve nitrogen removal performance via enhancing nitrification-denitrification process. In the present study, the focus was shifted from nitrogen removal via nitrate to nitrogen removal via nitrite in CWs, and the results proved many benefits could be obtained through nitrite pathway.

As shown in Fig. 2A, the CWs had almost no nitrogen removal when there was no artificial aeration. This result was in accordance with many previous studies (Fan et al., 2013a; Maltais-Landry et al., 2009), which suggested that incomplete nitrification due to insufficient oxygen supply was the major limiting factor for TN removal in conventional CWs. Previously, many researches have proved greatly improved oxygen availability and NH_4^+-N removal efficiency can be obtained through artificial aeration (Wu et al., 2014). In the present study, the average NH_4^+-N removal efficiency of the VSFCW2 could exceed 98% when artificial aeration was applied. However, the TN removal efficiency was only 28.1 ± 3.2% with high effluent NO_3^--N (36.90 ± 1.59 mg/L) when influent COD/N ratio was low (phase 5). Similar results was observed in some other intermittent aerated CWs, in which the TN removal efficiency was only about 25%, with effluent NO_3^--N concentration of 30.89 ± 4.96 mg/L when the COD/N was 2.5 (Fan et al., 2013a). These results indicated that although intermittent aeration could provide the coexistence of aerobic and anoxic conditions, stimulating the simultaneous occurrence of nitrification and denitrification, the deficit of carbon supply for denitrification would become the limited for TN removal when influent COD/N ratio was low. Theoretically, 5–9 mg of BOD is needed for per 1 mg of NO_3^--N reduced by heterotrophic denitrifiers. Such high amount carbon sources are not always presenting in influent wastewater. Moreover, the rapid depletion of the influent carbon sources during aerobic stage will further reduce the carbon availability for denitrification during anoxic stage. Supplemental organic carbon, such as glucose, glycerol, starch and methanol, is not cost-effective for use in CWs. Furthermore, addition of external carbon sources may inhibit nitrification by increasing oxygen demand for the degradation of organic carbon, resulting in a null net effect on TN removal (Maltais-Landry et al., 2009).

Compared to classical nitrate pathway, a large number studies have demonstrated that enhanced nitrogen removal performance can always be achieved via nitrite pathway. The present study proved that it is possible to attain nitrogen removal via nitrite pathway in CWs by applying appropriately alternating anoxic/aerobic conditions, and the results again proved the advantages of the nitrite pathway for improving nitrogen removal. As shown in Fig. 3, phase 4 (COD/N = 6), better TN removal (79.9 ± 4.9%) was obtained in VSFCW3 via nitrite pathway than that (63.6 ± 10.0%) in VSFCW2 via classical nitrate pathway. More importantly, extraordinary TN removal performance (82.6 ± 1.9%) was maintained in VSFCW3 during phase 5, even the influent COD/N ratio was only 3. Under such a significantly carbon-limited condition, it would be expected that the TN removal would be far from satisfactory as seen in VSFCW2 (Fig. 3). However, in contrast to VSFCW2 in which the average effluent TN was 37.53 mg/L, the average effluent TN in VSFCW3 was only 8.96 mg/L, which could meet the class A discharge standard (GB18918-2002) for WWTPs in China. Overall, the above results clearly demonstrated the benefits of using the nitrite pathway in enhancing the nitrogen removal performance in CWs, especially when the wastewater is COD limited.

Another important benefit of the nitrite pathway is the reduced aeration costs. Compared with the VSFCW2 with complete nitrification, the average aeration rate was decreased from 1 L/min to 60 mL/min when the nitritation was established in VSFCW3, which saved over 90% aeration costs.

4. Conclusions

Firstly, this study proved that shifting nitrogen removal pathway from nitrate to nitrite can be realized in CWs. Secondly, intermittent aeration strategy was an effective method to accomplish nitrite pathway. Thirdly, an appropriate anoxic and aerobic distribution and limited DO concentration during aeration aggravated the difference between AOB and NOB growth rates, which was important for achieving nitrite pathway. Most important of all, in comparison to the classical nitrate pathway, nitrogen removal via nitrite can greatly improve the nitrogen removal efficiency of CWs especially while reduce the aeration cost.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2017.02.027.

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