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# Achieving nitrification at low temperatures using free ammonia inhibition on *Nitrobacter* and real-time control in an SBR treating landfill leachate

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## ABSTRACT

Free ammonia (FA) inhibition on nitrite-oxidizing bacteria (NOB) and real-time control are used to achieve nitrogen removal from landfill leachate via nitrite pathway at low temperatures in sequencing batch reactor. The inhibition of FA on NOB activity during the aerobic period was prolonged using real-time control. The degree of nitrite accumulation was monitored along with variations of the ammonia-oxidizing bacteria and NOB population using fluorescence *in situ* hybridization techniques. It is demonstrated that the end-point of ammonia oxidation is detected from the on-line measured dissolved oxygen, oxidation-reduction potential, and pH signals, which could avoid the loss the FA inhibition on NOB caused by excess aeration. At low temperature (13.0–17.6°C), the level of nitrite pathway rapidly increased from 19.8% to 90%, suggesting that nitrification was successfully started up at low temperature by applying syntrophic association of the FA inhibition and real-time control, and then this high level of nitrite pathway was stably maintained for as long as 233 days. Mechanism analysis shows that the establishment of nitrification was primarily the result of predominant ammonia-oxidizing bacteria developed in the nitrifying bacteria population compared to NOB. This was mainly due to a gradual reduction of nitrite amount that is available to provide energy for the growth of NOB, eventually leading to the elimination of NOB from the bacterial clusters in sequencing batch reactor sludge system.

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## Introduction

From an environmental and economic point of view, the biological nitrogen removal (BNR) could be an interesting methodology for treating ammonium-rich landfill leachate (Tchobanoglous et al., 2003; Renou et al., 2008). Compared to the chemical methods, BNR reduces chemical consumption and cost, reduces the production of waste solids, and has lower energy requirement (Zhou et al., 2011). The BNR process includes two steps: oxidation of ammonia to

nitrate or nitrification and reduction of nitrate to nitrogen gas or denitrification. Nitrification is a two-step reaction. Firstly, ammonia is oxidized to nitrite by ammonia oxidizing bacteria (AOB), which is called nitrification. Secondly, nitrite is oxidized to nitrate by nitrite oxidizing bacteria (NOB), which is called nitrification (Torà et al., 2010).

Typically, AOB and NOB co-exist within bacteria clusters in the BNR systems with a synergetic interaction to accomplish nitrification. AOB oxidizes ammonia to nitrite, which serves as energy

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source for NOB, and NOB oxidizes nitrite (Lemaire et al., 2008). However, the achievement of nitrification process requires the decoupling of AOB and NOB activities so that nitrogen is removed via nitrite, bringing about significant reductions in both oxygen and COD demand (Turk and Mavinic, 1986; Vadivelu et al., 2006; Wang et al., 2013).

Temperature, dissolved oxygen (DO), free ammonia (FA), and free nitrous acid are useful to start-up nitrification in many studies. It has been widely reported that a high concentration of FA can promote nitrification by selectively inhibiting the activity of NOB over AOB (Zhang et al., 2012; Peng et al., 2008; Kim et al., 2006; Park et al., 2010). Moreover, it is believed that high temperature (30–35°C) is an optimal condition for achieving nitrification (Hellinga et al., 1998). Furthermore, nitrification at mild temperatures (20–25°C) has been proved (Guo et al., 2010; Qiao et al., 2010). Nitrification in a pilot-scale sequencing batch reactor (SBR) was achieved at mild water temperatures (26°C) and then at low water temperatures (11.8°C) in a long-term operation by Yang et al. (2007). To the best of our knowledge, however, only Gu et al. (2012) successfully started up nitrification at low temperatures (11–16°C) with a real-time control strategy based on blower frequency and pH in an SBR treating municipal wastewater.

In this study, a lab-scale SBR, operated an alternating aerobic/anoxic strategy, was employed to eliminate nitrogen via nitrite from upflow anaerobic sludge bed (UASB)-treated landfill leachate. We investigate the possibility of establishing a nitrification process at low temperatures by integrating FA inhibition on NOB activity with a real-time control. The mechanism responsible for the start-up of the nitrification is explained using fluorescence in situ hybridization (FISH) analysis.

## 1. Materials and methods

### 1.1. Reactor and operation

A lab-scale SBR with a working volume of 8 L was used. The SBR cycle operation consisted of 2 min feeding; aerobic reaction, anoxic reaction, 30 min settling, 15 min decanting, and idling. The durations of aerobic and anoxic reactions were controlled by using a real-time control.

In each cycle, 4 L of landfill leachate was pumped into the reactor during the filling period. Each filling period was followed by an aerobic period. During aerobic period, enough oxygen was provided by an air blower to keep the DO level between 0.5 and 2.0 mg/L. The DO, pH, and oxidation–reduction potential (ORP) signals were used to indicate the completion of ammonia oxidation. During the followed anoxic period, an additional quantity of 0.3 mL of methanol was added into the reactor as an external carbon source for denitrification. At the same time, the SBR was mixed with an overhead mixer to ensure complete mixing. The pH and ORP signals were also recorded to give indication of the denitrification finish. Sludge settlement and supernatant discharge then followed.

### 1.2. Wastewater and inoculated sludge

The raw leachate was collected from a municipal landfill site (located at Beijing, China) and the leachate characteristics are as following (range, average): COD (460–850, 665 mg/L), TN (172–225, 199 mg/L),  $\text{NH}_4^+\text{-N}$  (131–180, 155 mg/L),  $\text{NO}_3^-\text{-N}$  (0.02–2.3, 0.56 mg/L),  $\text{NO}_2^-\text{-N}$  (0.01–0.66, 0.04 mg/L), pH (7.5–8.8, 8.5) and alkalinity (5000–8500, 6500 mg  $\text{CaCO}_3/\text{L}$ ).

The seed sludge was taken from the municipal wastewater treatment plant in Beijing, China. The seeding sludge was a mixture of heterotrophic and autotrophic microorganism. The average levels of mixed liquor suspended solids (MLSS) and volatile MLSS (MLVSS) in the SBR were 1500–2500 and 1200–2100 mg/L, respectively.

### 1.3. Analytical methods

The chemical oxygen demand (COD), ammonia nitrogen ( $\text{NH}_4^+\text{-N}$ ), nitrate nitrogen ( $\text{NO}_3^-\text{-N}$ ), nitrite nitrogen ( $\text{NO}_2^-\text{-N}$ ), MLSS, and MLVSS were analyzed according to the standard methods (APHA, 1998). The total nitrogen (TN) was analyzed using a total organic carbon analyzer (Multi N/C 3000, Analytik Jena AG, Jena, Germany). DO, pH and ORP were continuously detected using a pH/oxi 340 analyzer (WTW Company, Munich, Germany).

FISH was performed as specified in Amann (1995). Specific oligonucleotide probes used in this study were EUBmix (Daims et al., 2001) for the detection of all bacteria, Nso1225 for ammonia-oxidizing  $\beta$ -proteobacteria (Mobarry et al., 1996), NIT3 for *Nitrobacter* and Ntspa662 for *Nitrospira*. FISH images were captured using an OLYMPUS-BX52 fluorescence microscope (Olympus Corporation, Tokyo, Japan). Fish quantification was performed, where the relative abundance of the each group was determined in triplicate as mean percentage of all bacteria.

### 1.4. Calculations

The FA concentration (mg/L) was calculated as a function of pH, temperature (T), and total ammonium as nitrogen (TAN); the calculation was modified from Anthonisen et al. (1976):

$$\text{FA}(\text{mg/L}) = \frac{17}{14} \frac{\text{TAN} \times 10^{\text{pH}}}{\exp\left(\frac{6334}{273 + T}\right) + 10^{\text{pH}}} \quad (1)$$

The nitrite accumulation ratio (NAR, %) was calculated using Eq. (2):

$$\text{NAR} = \frac{\text{NO}_2^-\text{-N}}{\text{NO}_2^-\text{-N} + \text{NO}_3^-\text{-N}} \times 100\% \quad (2)$$

## 2. Result and discussion

### 2.1. FA inhibition and real-time control to promote nitrite pathway

#### 2.1.1. NOB inhibition by FA to optimize nitrifying bacteria population

The inhibitory effects of FA on AOB and NOB have been widely reported, NOB has been described to be much more sensitive to FA than AOB. The difference in the inhibitory effects of FA on the metabolisms of AOB and NOB indeed have been suggested as a major factor leading to elimination of NOB, which is critical for achieving nitrification.

Fig. 1 presents typical profiles of the FA, nitrogen, DO, pH, and ORP during each SBR cycle. FA levels were plotted during the nitrification process, which clearly show that the FA level

gradually decreased from 18.8 mg/L at the beginning of nitrification to 0.21 mg/L at the end of nitrification because both the ammonium concentration and the pH decreased (there were only slight changes in ambient temperature, which was of  $26.5 \pm 0.7^\circ\text{C}$ ) throughout the SBR cycle. This decreasing FA concentration implies that the inhibitory effect that FA placed on NOB activity gradually weakened as nitrification proceeded.

If the aeration is continued after complete oxidation of ammonia, the extremely low level of FA would lose its inhibition on NOB activity, resulting in the conversion of nitrite to nitrate by NOB in the system. Therefore, how to avoid the loss of NOB inhibition by FA is critical for achieving nitrification in a nitrogen removal system.

### 2.1.2. Implementation of NOB inhibition by FA with real-time control

As shown in Fig. 1b, because nitrification produces acid, at the beginning of nitrification, pH decreased gradually. When ammonia was depleted, pH began to increase. Meanwhile, DO and ORP increased sharply as nitrification only required 25% as much of the oxygen as that of nitrification, ammonium valley, DO break point, and ORP break point appeared on the pH, DO and ORP profiles, respectively. During anoxic period, pH increased due to the production of strong base during denitrification and ORP decreased. At the end of denitrification, ORP decreased sharply whereas pH began to decrease. Nitrite apex on the pH profile and nitrite knee on the ORP profile indicated the completion of denitrification.

Thus, these key points correlated well with the conversion of nitrogen during biological nitrogen removal. Ammonia

valley and DO break point and ORP break point not only represented the end of nitrification exactly, but also maintained the nitrite accumulation rate by avoiding excessive aeration.

Gao et al. (2009) found that excess aeration (aeration is still on after ammonia oxidation) could promote the conversion of nitrification to nitrification. In other words, if the aeration is stopped as soon as nitrification finishes, the activity of the NOB population would be continuously inhibited by FA in the nitrification, which ensures that nitrite is no longer available for NOB. However, real-time control is available to prevent the occurrence of excess aeration, as indicated by these key points on DO, pH and ORP curves, respectively. In our study we were able to prolong favorable FA inhibition using real-time control to achieve the nitrite pathway in the SBR.

### 2.2. Start-up nitrification at low temperatures by means of FA inhibition and real-time control

The SBR was operated for approximately 10 months. FA inhibition on NOB activity and real-time control for achieving nitrogen via nitrite was applied throughout the experimental period. As Fig. 2 shows, the SBR operation consisted of two periods: start-up period (0–77 days) and maintenance period (78–310 days). The operational temperature in the SBR was  $13.0\text{--}17.6^\circ\text{C}$  and the NAR gradually increased from 0.3% to 91.7% during the start-up period. It is clear that at these low temperatures, nitrification was successfully started up in the SBR but operational temperatures increased slowly during this period. It is worth noting that at low temperatures nitrification was achieved but waste temperature increased slowly due to seasonal transition. On day 154, the water

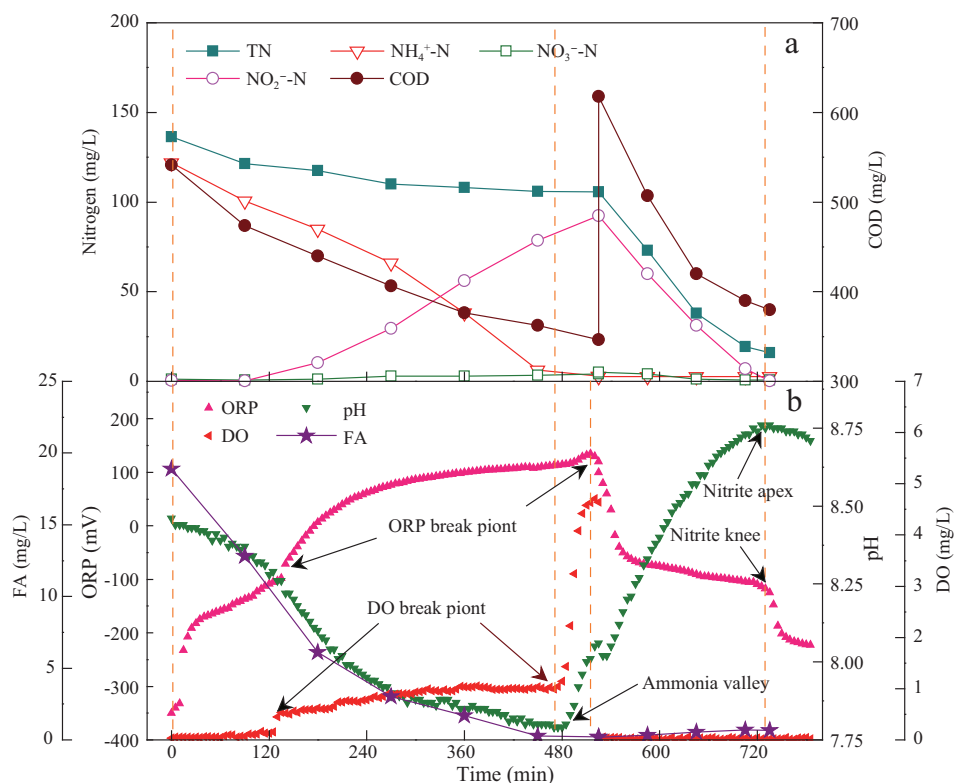


Fig. 1 – Mechanism of achieving nitrification in the SBR by FA inhibition and real-time control.

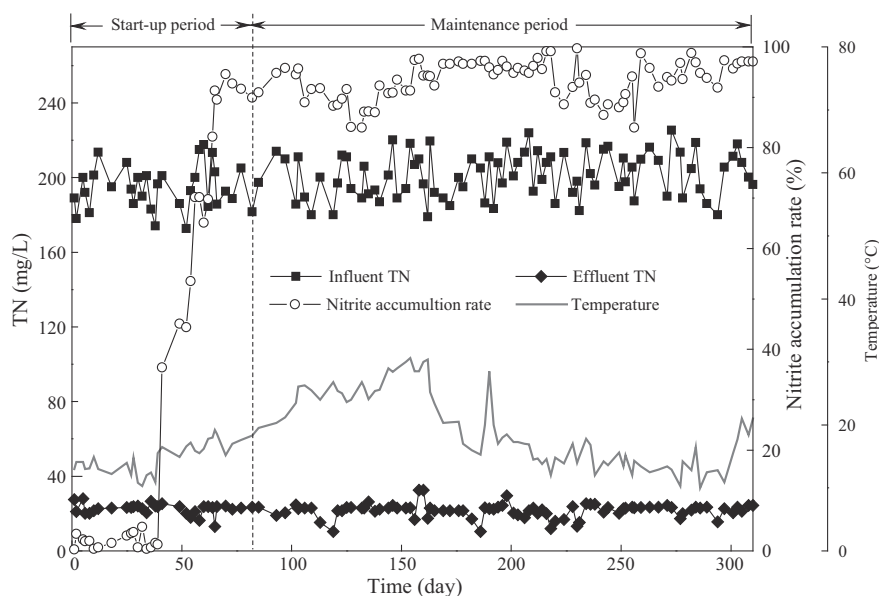


Fig. 2 – Dynamics of influent TN, effluent TN, nitrite accumulation rate and temperature during the whole operation.

temperature increased to 30.6°C, and then water temperature gradually decreased.

During the following maintenance period, the average NAR was steady at approximately 93.4% for 234 days until the end of this period, when the operational temperature in the SBR varied widely (10.0–30.6°C). It is worth mentioning that, from day 154 to 286, the nitrite accumulation did not deteriorate, even though the operational temperature gradually decreased to 10.0°C (the lowest level in our study) on day 286. The nitrification process in the SBR was well maintained by applying FA inhibition and real-time control. At the same time, the average effluent TN was below 21.8 mg/L, which indicates that advanced nitrogen removal was obtained in the system.

### 2.3. Optimization of nitrifying population in the sludge at low temperature

Optimization of nitrifying bacterial population is crucial for achieving nitrification in the SBR system. The nitrification process can be rapidly achieved when AOB is dominant nitrifying bacteria and NOB is eliminated from the system, even under these low temperatures (13.0–17.6°C). As noted previously, nitrification was maintained at low temperatures because the nitrifying bacteria population was optimized (Yang et al., 2007; Guo et al., 2010). Meanwhile, higher temperatures (>20°C) in the SBR should be kept in order to start up the nitrification process.

However, it was interesting to find, in our study, that it was possible to start up the nitrification at low temperatures and to optimize the nitrifying bacteria community. Fig. 3 shows that the microbial population composition was analyzed by FISH techniques. FISH quantification showed that the approximate sizes of AOB and NOB were 2.5%–3.0% and 1.5%–2.0% in the inoculated sludge, respectively. After applying FA inhibition on NOB and real-time control strategy for 77 days, the AOB

fraction increased to 4.0%–6.5% and the NOB fraction was almost negligible. Therefore, only AOB was present in the system.

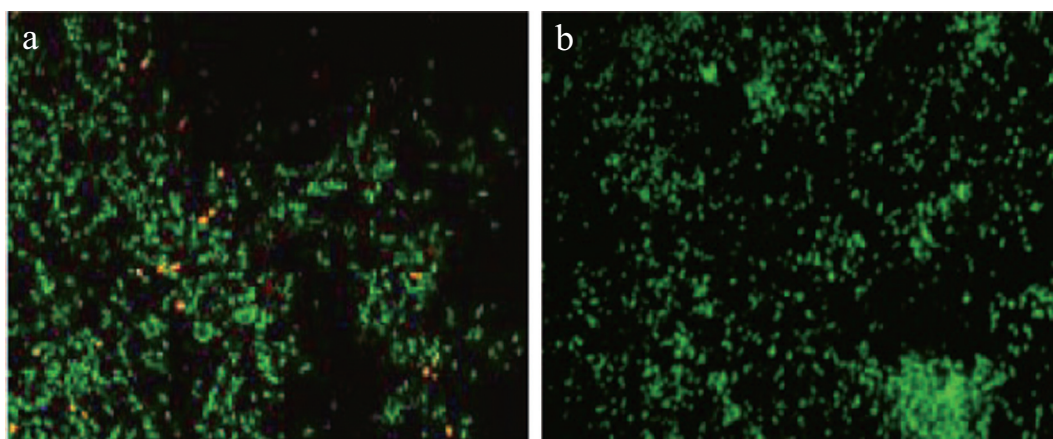
As shown in Fig. 4, ammonia oxidation worked well during the experimental period. At the end of aeration period, the nitrite concentration increased gradually from 0.7 to 109.8 mg/L and the nitrate concentration decreased from 88.3 to 4.9 mg/L. The energy and growth of AOB were stronger than that of NOB due to synergetic interaction of FA inhibition on NOB and robust real-time aeration control. In this case, we could have inferred that the NOB, which did not have sufficient growth potential and opportunities, were gradually eliminated due to the inhibition of their growth kinetics. Therefore, a stable nitrification process was successfully started up at this low temperatures range (13.0–17.6°C) in this system.

### 2.4. Mechanism of start-up nitrification at low temperatures

As shown in Table 1, previous publications have discussed the roles of FA and real-time control in achieving nitrification. FA was suggested as a contributing factor towards nitrification because the growth of NOB was inhibited by higher FA concentration while the growth of AOB was less inhibited or was not influenced. In such cases, AOB might be selected as the dominant nitrifying bacteria, while the NOB might still exist in the activated sludge system.

Real-time control was an effective method for achieving nitrification, especially in the SBR process, because that it would exert a negative effect on the NOB activity without having any harmful effects on the AOB. In such systems, AOB was optimized by real-time control, while NOB was washed out from the system.

Compared with literatures summarized in Table 1, it is noted that stable nitrification was successfully started up and maintained at low temperatures as long as 233 days in this study. Thus, the combination of FA inhibition and real-time



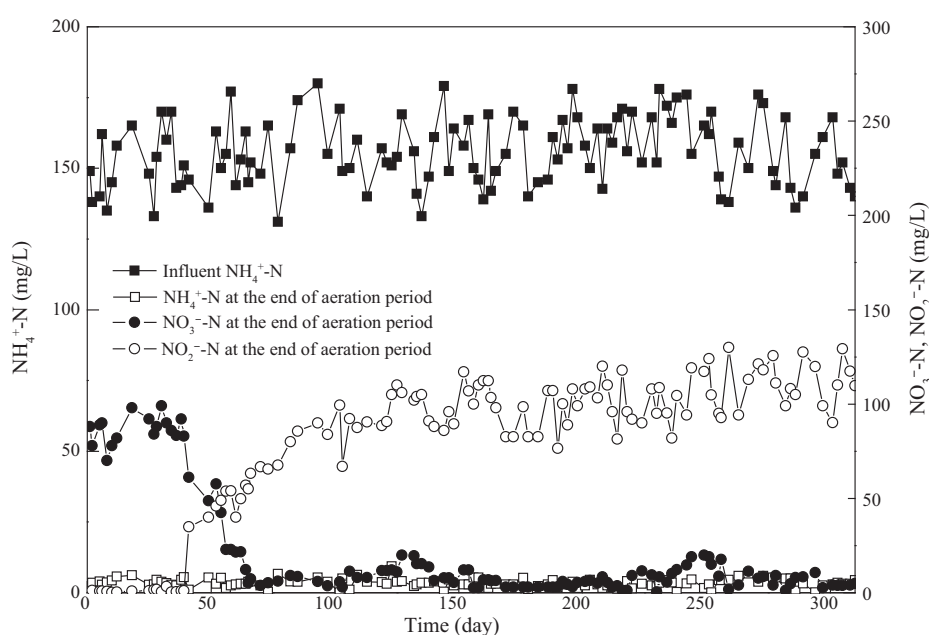
**Fig. 3 – Fluorescence in situ hybridization (FISH) results for AOB and NOB: (a) NSO1225 target for AOB; (b) NIT3 target for *Nitrobacter*, Ntspa662 target for *Nitrospira* (red color signifies AOB in panel a, green color signifies all bacteria in panels a and b, NOB was not detected in panel b).**

control is reliable and simple for achieving nitrogen removal via nitrite pathway from leachate.

Usually, nitrification was achieved at mild and high temperatures due to the specific growth rate of AOB ( $\mu_{AOB}$ ) being higher than that of NOB ( $\mu_{NOB}$ ). But why did, in our SBR system, nitrification started up at such low temperatures? Low temperatures ( $<20^{\circ}\text{C}$ ) would exert a negative impact on the activities of AOB and NOB, and  $\mu_{AOB}$  was lower than  $\mu_{NOB}$ . This means that AOB was unlikely able to out-compete NOB at temperature range of  $13.0\text{--}17.6^{\circ}\text{C}$  in this study.

However, AOB has been described to be much more tolerant to FA than NOB, so FA played a critical role in selectively inhibiting or limiting the growth of NOB. Moreover,

when real-time control was applied in the SBR, the aeration was stopped immediately as soon as nitrification finished, as indicated by these key points on the DO, ORP, and pH profiles during each aeration period. As a result, nitrite, the energy source for NOB, was reduced to nitrogen gas in the denitrification process, further limiting the growth of NOB to some extent. Therefore, in such system, NOB growth gradually weakened due to the FA inhibition and the reduction of their energy source. We conclude that through FA inhibition on NOB and robust real-time control, AOB gradually became the dominant nitrifying bacteria in the activated sludge and nitrification was started up at low temperatures. This implies that it is feasible to start up nitrification at an SBR wastewater



**Fig. 4 – Variations of  $\text{NH}_4^+\text{-N}$ ,  $\text{NO}_3^-\text{-N}$  and  $\text{NO}_2^-\text{-N}$  at the start and end of aeration period during the whole operation process.**



**Table 1 – Summary of main literature findings on the achievement of nitrification under normal/low temperatures.**

The operational process and wastewater	Temperature (°C)		Days		Approach of achieving nitrification	Mechanism for achieving nitrification	Reference
	Start up nitrification	Maintain nitrification	Start up nitrification	Maintain nitrification			
A lab-scale two-stage UASB-A/O system treating landfill leachate	17–25	25–30	35	120	The NOB inhibition by FA aeration time control based on pH	The FA inhibited NOB growth, but almost had no impact on AOB	Peng et al. (2008)
A 10.8 L lab-scale swim-bed reactor treating synthetic wastewater containing high ammonium	20–30	10–30	60	240	The synergetic inhibition of FA and FNA on NOB	The NOB activity was inhibited by FA and FNA, AOB was confirmed to be the dominant nitrifying bacteria	Qiao et al. (2010)
Two 2.5 L lab-scale A/O SBR with and without step-feed treating synthetic wastewater	28	28	44	85	The combination of FA inhibition on NOB, step-feed strategy and nitrifying granules	The NOB activity was inhibited by FA, step-feed and granules was favorable for nitrite accumulation	Wang et al. (2012)
A 7.0 m <sup>3</sup> pilot-scale SBR treating campus wastewater	11–16	17–26	40	140	Real-time control strategy (based on blower frequency and pH) coupled with SND activity	Nitrifying bacteria community was optimized: AOB was dominant and NOB was washed out	Gu et al. (2012)
A 54 m <sup>3</sup> pilot-scale SBR treating real municipal wastewater	17.3–25	11.9–26.5	120	177	A real-time control (based on DO, pH and ORP) coupled with step-feed pattern	Nitrifying bacteria community was optimized: AOB was dominant and NOB was washed out	Yang et al. (2007)
A 7 L lab-scale SBR treating abattoir wastewater	18–22	18–22	50	90	Aeration phase length control and step-feed operational pattern	The NOB were eliminated from the sludge system due to the gradual reduction of their energy source of nitrite	Lemaire et al. (2008)
An 8.0 L lab-scale SBR treating real landfill leachate	13.0–17.6	10.0–30.6	77	233	The NOB inhibition by FA and process control (based on DO, pH and ORP)	NOB activity was completely suppressed by FA inhibition and the reduction of available energy source of nitrite for NOB growth	This study

Upflow anaerobic sludge bed (UASB), anoxic/oxic (A/O), nitrite-oxidized bacteria (NOB), ammonia-oxidizing bacteria (AOB), free ammonia (FA), free nitrous acid (FNA), sequencing batch reactor (SBR), simultaneous nitrification and denitrification (SND), dissolved oxygen (DO), and oxidation–reduction potential (ORP).

treatment plants in winter, and nitrite pathway could be established without assuming that NOB have poorer growth kinetics than AOB.

### 3. Conclusions

Nitrification process can be achieved rapidly at low temperatures (13.0–17.6°C) in 77 days and was maintained stably for as long as 233 days by applying FA inhibition on NOB coupled with a real-time control. In such systems, AOB became dominant nitrifying bacterial population, but NOB was eliminated due to the inhibition of their growth kinetics. The reduction of the NOB growth kinetics would help to achieve nitrite pathway. The end-point of ammonium oxidation can be reliably detected from the on-line DO, pH and ORP signals. Therefore, the real-time control could implement NOB inhibition by FA to speed-up the onset and establishment of the nitrite pathway.

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