

# Study on Nitrite Accumulation Characteristics and Nitrifying Population Dynamics at Different Growth Environments

Yunxia Zhang, Jiti Zhou, Jianbo Guo, Xiuhong Zhang, Lihong Zhao, and Shouzhi Yuan

**Abstract**—Novel nitrogen removal technologies via nitrite pathway attract increasing interest in recent years. In this study, batch experiments were performed to investigate nitrite accumulation characteristics and shifts in nitrifying community structure at different growth environments including ammonia concentration, pH and alkalinity. It was found that nitrite accumulation ratios were maintained at around 95% at studied conditions, and the optimum pH and Alk/N (ratio between alkalinity and nitrogen) for ammonium oxidization were 8.5 and 8.33, respectively. Fluorescence in situ hybridization analysis of nitrifying bacteria showed that high free ammonia (from influent ammonium or caused by high pH) significantly altered the structure of nitrifying community, leading to abundance of ammonia-oxidizing bacteria (AOB), especially *Nitrosomonas*, and inhibition of nitrite-oxidizing bacteria (NOB). The results suggest that free ammonia plays more important role than other studied conditions on nitrite accumulation.

**Keywords**—Partial nitrification, Nitrite accumulation, Nitrifying bacteria, Fluorescence in situ hybridization (FISH).

## I. INTRODUCTION

THE biological removal of nitrogen by the combination of nitrification and denitrification has been widely used in the treatment of municipal and industrial wastewater [1,2]. Nitrification is known as a two-step process carried out by two groups of chemolithoautotrophic bacteria: the oxidation of ammonium to nitrite by AOB, represented by *Nitrosomonas* and *Nitrospira*, and the oxidation of nitrite to nitrate by NOB, represented by *Nitrospira* and *Nitrobacter* [3]. Since the nitrite is consumed by nitrification and formed again during denitrification, the nitrite oxidation becomes an unnecessary step. The novel partial nitrification process via nitrite pathway, which can be coupled with the Anammox process or with traditional denitrification, have economic advantages in saving costs of aeration (25% less), organic carbons (40% less) and investment [4]. Therefore, it has been recognized to be very promising for improved sustainability of wastewater

treatment. One challenge of partial nitrification is the maintenance of high and stable nitrite accumulation. Nitrite accumulation studies have been performed to assess the impact of several factors, such as dissolved oxygen (DO) concentration, substrate concentration, pH, temperature and the presence of inhibitory compounds [5-7]. However, the maintenance of low DO concentration to achieve high nitrite accumulation may be disadvantageous for ammonium oxidization because the overall nitrification rate could be decreased when DO is too low [6]. In addition, there is a significant disagreement on the effect of free ammonia (FA) concentration and pH on nitrite accumulation. Some reports conclude that high FA concentration is the main responsible factor causing the nitrite accumulation [8], whereas other investigations show that pH plays more important role [6]. The argument is possibly due to the different operating conditions, reactor configurations and microbial community structures. Furthermore, very little is known about the effects of different growth environments on nitrifying bacteria communities.

The objective of this study is to find out the crucial factor and the cause of nitrite accumulation by investigating changes of microbial activities and shifts in nitrifying community structure in response to different FA concentrations, pHs and alkalinity (expressed by  $\text{CaCO}_3$ ). The distribution of nitrifying bacteria was analyzed by fluorescence in situ hybridization (FISH), which has been successfully applied for phylogenetical identification and quantification in environmental and engineered systems [9,10]. These results could help better understand the behavior of the nitrifying bacteria, and further propose an effective approach to stable nitrite accumulation.

## II. MATERIALS AND METHODS

### A. Batch Experiments

The nitrifying activated sludge was obtained from the reactor with partial nitrification process in our laboratory. It was inoculated to 250ml Erlenmeyer flasks with 100ml inorganic medium to grow aerobically at 30°C in the dark. The batch experiments were performed at stepwise increased ammonium nitrogen concentrations (100, 200, 400, 600, 800, 1000 mg L<sup>-1</sup>), pH values (6.0, 7.0, 8.0, 8.5, 9.0, 10.0) and Alk/N (1.78, 4.17, 8.33, 16.66, 33.32, 49.98) for a month. The inorganic medium contained:  $(\text{NH}_4)_2\text{SO}_4$ , 0.5-5 g L<sup>-1</sup>;

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$\text{KH}_2\text{PO}_4$ , 0.7 g L<sup>-1</sup>;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.05 g L<sup>-1</sup>;  $\text{NaCl}$ , 1.2 g L<sup>-1</sup>;  $\text{NaHCO}_3$ , 0.3-8.4 g L<sup>-1</sup> and 2 mL L<sup>-1</sup> trace element solution. The composition of trace element solution (g L<sup>-1</sup>) was: EDTA, 50;  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 2.2;  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , 1.61;  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ , 5.06;  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 1.57;  $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ , 1.1;  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 5.54;  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 4.99. Hydraulic retention time (HRT) was kept at 2 days and the pH was regulated constantly at 8.0±0.2 by adding 0.5M  $\text{Na}_2\text{CO}_3$  solution with phenol red (0.05 mg L<sup>-1</sup>) as a pH indicator.

### B. Analytical Methods

The mixed liquor suspended solids (MLSS) of each batch experiment was 2.0±0.3 g L<sup>-1</sup>. The MLSS and alkalinity were analyzed based on the standard methods [11].  $\text{NH}_4^+\text{-N}$ ,  $\text{NO}_2^-\text{-N}$  and  $\text{NO}_3^-\text{-N}$  were measured by a spectrophotometer (JASCO, V-560, UV/VIS spectrophotometer). Dissolved oxygen and pH were measured by a DO meter (YSI, Model55, USA) and pH meter (PB-20, Germany), respectively. The FA concentration and the specific ammonium oxidation rate (SAOR) was calculated by Eq.(1) and Eq.(2), respectively.

$$\text{FA} = \frac{17}{14} \times \frac{(\text{Total } \text{NH}_4^+ - \text{N}) \times 10^{\text{pH}}}{e^{(6344/(273+T))} + 10^{\text{pH}}} \quad (1)$$

$$\text{SAOR} = \frac{S_{t-1} - S_{t+1}}{2X_t \Delta t} \quad (2)$$

where  $S_{t-1}$  and  $S_{t+1}$  represent the concentration of  $\text{NH}_4^+\text{-N}$  in the two consecutive samples,  $X_t$  represents biomass concentration in the batch experiment and  $\Delta t$  represents the time interval.

### C. Fluorescence in Situ Hybridization

FISH technique was used to investigate the microbial community of the ammonia-oxidizing and nitrite-oxidizing bacteria. All the hybridization experiments were performed according to methods previously described by Amann [9]. The biomass samples were taken from each batch culture on the 30th day and fixed in 4% freshly prepared paraformaldehyde solution for 2-3h at 4°C, then rinsed twice with phosphate buffered saline (PBS, pH 7.2). The samples were spotted on to gelatin-coated microscope slides (Cel-Line HT coating slide), air dried and then dehydrated by successive 50%, 80% and 98% (v/v) ethanol (3 min each). FISH probes used in the experiments were described in Table I. Oligonucleotides were synthesized and fluorescently labeled with a hydrophilic sulfoindocyanine dye (Cy-3) or fluorescein isothiocyanate (FITC) at the 5'end (Takara Biotechnology Co., LTD, Dalian, China). All in situ hybridizations were performed at 46°C for 3 h in 18µl of a hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl (pH 7.2), 0.01% (w/v) SDS, formamide at the percentage shown in Table I) and 2µl of the probe solution (50 ng µL<sup>-1</sup>). After hybridization, slides were treated in a stringent washing step at 48°C for 10 min with pre-warmed washing buffer (20mM Tris-HCl (pH 7.2), 0.01% SDS, NaCl at the concentration listed in Table I). Washing buffer was removed by rinsing the slides with distilled water. The slides were air dried, stained with 10 ng µL<sup>-1</sup> DAPI (4',6-diamidino-2-phenylindol-dihydrochlorid) for 8-10 min in the dark and rinsed again with distilled water. The cells were observed with a fluorescence microscope (BX-60; Olympus) equipped with a

cooled charge-coupled device (CCD) camera system (PXL1400; Photometrics). For quantitative analysis of FISH images, specific-probe-hybridized cell areas were used for the analyses of the proportions of probe-labelled cells to total cells by image pro-plus software (version 6.0). The average fraction was determined from eight to ten representative microscopic images.

## III. RESULTS AND DISCUSSION

### A. Effect of Ammonia Nitrogen Concentration on Nitrite Accumulation and Nitrifier Community

Free ammonia rather than the total ammonium is the real substrate for ammonium oxidization, so initial FA concentration, calculated by Eq.(1), was introduced in this study. At low FA (less than 18.7mg L<sup>-1</sup>), the SAOR increased slightly over time (Fig. 1). As FA increased, the SAOR was very low at the beginning and then increased gradually from 18.3 to 246.8 mgNH<sub>4</sub><sup>+</sup>-N g<sup>-1</sup>MLSS d<sup>-1</sup> at 74.8 mgNH<sub>3</sub> L<sup>-1</sup> and 13.4 to 201.5 mgNH<sub>4</sub><sup>+</sup>-N g<sup>-1</sup>MLSS d<sup>-1</sup> at 93.5 mgNH<sub>3</sub> /L. According to this study, the inhibition of ammonium oxidization started at 37.4 mgNH<sub>3</sub> L<sup>-1</sup> for inoculated nitrifying bacteria, however, for acclimatized sludge, no evident inhibition was observed at 93.5 mgNH<sub>3</sub> L<sup>-1</sup>, which suggests that nitrifiers might require a certain period of time for their physiological adaptation to new growth environment [16]. The nitrite ratio was observed over 97% at different FA concentrations (Fig. 2). The results indicate that high inputs of ammonia select a different nitrifying community that is perhaps specialized for high FA.

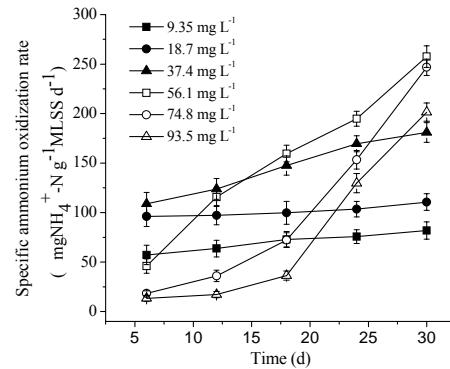


Fig. 1 Time-course profiles of the specific ammonium oxidation rate under different free ammonia concentrations (T, 30°C; pH, 8.0).

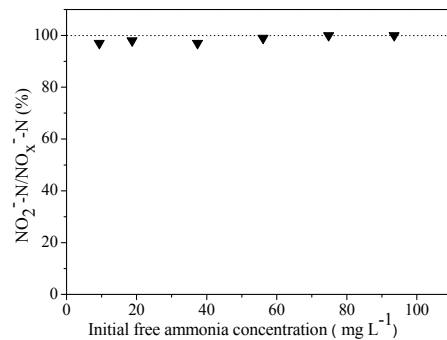


Fig. 2 Effect of initial free ammonia concentration on nitrite ratio ( $\text{NO}_2^-\text{-N}/\text{NO}_x^-\text{-N}$ )

The shift in nitrifying population caused by increased FA concentration was monitored by FISH analysis and its average fraction and respective standard deviations were shown in Fig. 3. Total AOB in the betaproteobacterial group (targeted by Nso190) accounted for 43.8% in inoculated sludge with the same fraction of *Nitrosomonas* and *Nitrospira*, and NOB averaged 3.2%. As FA was up to 56.1mg L<sup>-1</sup>, the fraction of AOB increased gradually from 43.8% to 62.9% and then decreased to 49.1% at 93.5 mgNH<sub>3</sub> L<sup>-1</sup>. Increased free ammonia significantly altered the structure of the nitrifying community. At FA 9.35mg L<sup>-1</sup>, *Nitrospira* was the dominant AOB with an average of 26.2%, and *Nitrosomonas* was 20.4%. However, as FA concentration increased (over 9.35mg L<sup>-1</sup>), *Nitrosomonas* outcompeted *Nitrospira* and began to be the dominant AOB (from initial 20.4% to 31.3%). This is consistent with findings from Mobarry et al. [13], who suggested that the predominant AOB in ammonium-rich systems was the members of the genus *Nitrosomonas*. Different population dynamics of AOB can be explained as following: *Nitrosomonas* is thought to be acted as r-strategists with a low substrate affinity but a remarkably high K<sub>s</sub> values and *Nitrospira* is K-strategists having a high substrate affinity and a relatively low reaction rate under high substrate concentrations [17]. This can also be proved by the evidence that similar SAOR was observed at both 56.1 and 74.8 mgNH<sub>3</sub> L<sup>-1</sup>.

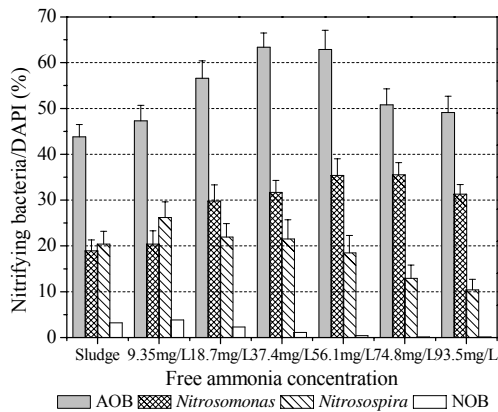


Fig. 3 Average fractions of total betaproteobacterial AOB, *Nitrosomonas*, *Nitrospira* and NOB on the 30th day at different free ammonia concentrations

### B. Effect of pH on Nitrite Accumulation and Nitrifier Community

The population of NOB (*Nitrobacter* plus *Nitrospira*) gradually decreased with increasing FA concentration (Fig. 3), suggesting that high FA had strong effect on the biomass levels of NOB. The inhibition of FA on NOB growth started at 9.35 mgNH<sub>3</sub> L<sup>-1</sup> and became evident at 37.4 mgNH<sub>3</sub> L<sup>-1</sup>. This observation is in agreement with Turk and Mavinic [18], who reports that acclimated NOB are capable of tolerating FA level as high as 48.57 mg L<sup>-1</sup>.

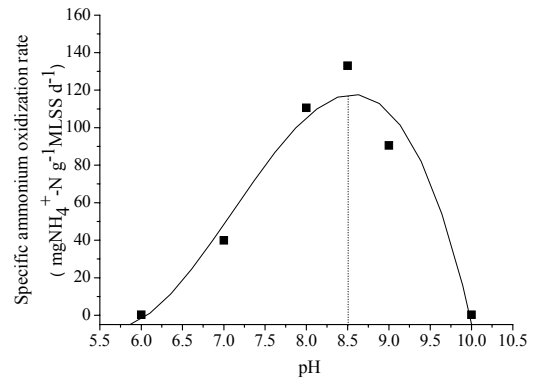


Fig. 4 Effect of pH value on the specific ammonium oxidation rate (T, 30°C; initial NH<sub>4</sub><sup>+</sup>-N, 200mg/L)

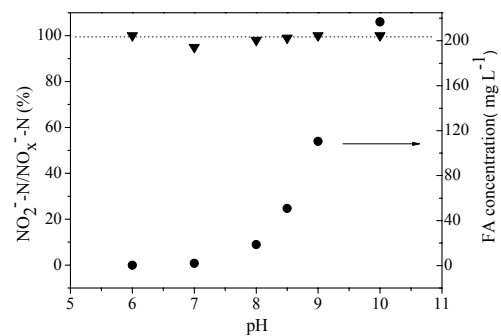


Fig. 5 Effect of pH value on nitrite ratio and free ammonia concentration

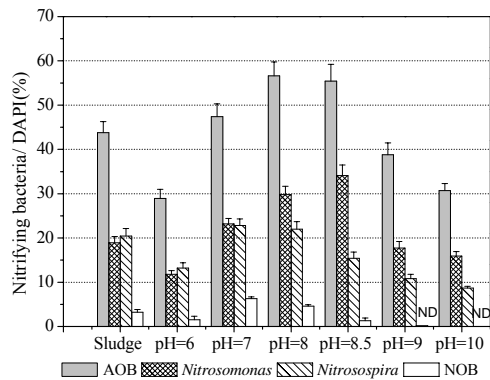


Fig. 6 Average fractions of total betaproteobacterial AOB, *Nitrosomonas*, *Nitrospira* and NOB on the 30th day at different pH values

At pH batch experiments, pH was changed in steps from 6.0 to 11.0, and the initial concentration of NH<sub>4</sub><sup>+</sup>-N was maintained at the level of 200mg L<sup>-1</sup>. The strong effect of pH on ammonium oxidation could be found in Fig. 4. The highest SAOR (110.6 mgNH<sub>4</sub><sup>+</sup>-N g<sup>-1</sup>MLSS d<sup>-1</sup>) was observed at pH 8.5 and the nitrification rate was retarded in pH extreme environments (6.0 and 10.0). Nitrite ratio was observed over 90% at studied pHs from Fig. 5. The optimum pH for AOB and NOB has been reported to be laid in the range of 8.2±0.3 and 7.9±0.4, respectively [19], so there is no significant

difference of AOB and NOB activity at pH range of 7.0-8.5. However, pH has a strong influence on FA concentration because it assigns the distribution of  $\text{NH}_4^+/\text{NH}_3$  equilibrium [20]. It can be seen from Fig. 5, FA concentration increased exponentially from 18.71 to 216.8  $\text{mg L}^{-1}$  at pH from 8.0 to 10.0. Therefore, high nitrite accumulation here attributes to the inhibition of FA concentration caused by high pH to NOB. This hypothesis can also be supported by the similar shift in nitrifying community structure observed compared with increased FA (Fig. 6). As a consequence, at pH range of 7.5-8.5, inhibition by FA on NOB caused by pH plays a more significant role on nitrite accumulation.

### C. Effect of Alk/N on Nitrite Accumulation and Nitrifier Community

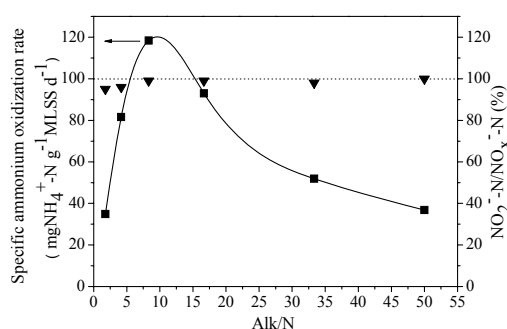


Fig. 7 Effect of different Alk/N on the specific ammonium oxidation rate and nitrite ratio (T, 30°C; initial  $\text{NH}_4^+-\text{N}$ , 200mg/L; pH, 8.0)

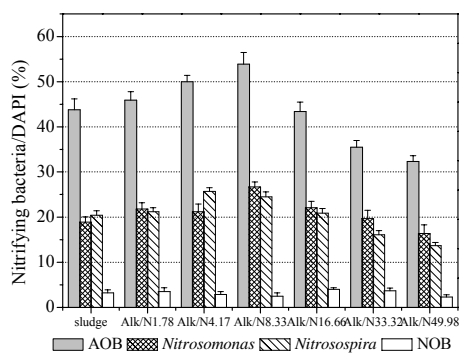


Fig. 8 Average fractions of total betaproteobacterial AOB, Nitrosomonas, Nitrospira and NOB on the 30th day at different Alk/N

According to the stoichiometry of ammonium oxidation, 1g of ammonium nitrogen consumes 7.14g of alkalinity. The effect of various Alk/N on the specific ammonium-oxidation rate (Fig. 7) showed that the SAOR was highest (118.4  $\text{mg NH}_4^+-\text{N g}^{-1} \text{MLSS d}^{-1}$ ) at Alk/N 8.33 and was lowest at Alk/N 1.78 and 49.98. And this suggests that enough alkalinity is necessary for nitrification to supply inorganic carbon resource, but excessive alkalinity can inhibit the activity of AOB (Fig. 8). Despite the significant variation in SAOR for different Alk/N treatments, nitrite ratio could remain higher than 95% and no evident variation at the AOB

community structure was observed. Meanwhile, NOB was not inhibited by high alkalinity.

### IV. CONCLUSION

In conclusion, this study provides the first high-resolution insight into the nitrifying population dynamics in response to increased FA concentration, pH and Alk/N, which is found to correlate well with the nitrifying activity. High FA level is suggested to be the most important factor contributing to high fraction of AOB. Nevertheless, AOB dominated at nitrifying community contributes to high and stable nitrite accumulation at all different conditions. The results would be a great improvement for optimization and control of partial nitrification.

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TABLE I  
16S RRNA-TARGETED OLIGONUCLEOTIDE PROBES USED IN THIS STUDY

Probe	Specificity	Probe sequence(5'-3')	%formamide/ NaCl (mM)	Reference
Eub338	Eubacteria	GCTGCCTCCCGTAGGAGT	20/225	[12]
Nso190	Ammonia-oxidizing $\beta$ -Proteobacteria	CGATCCCCTGCTTTTCTCC	55/20	[13]
Nsv 443	Nitrospira spp.	CCGTGACCGTTTCGTTCCG	30/112	[13]
Nsm 156	Nitrosomonas spp. and Nitrosococcus mobilis	TATTAGCACATCTTTCGAT	5/640	[13]
Nit3	Nitrobacter spp.	CCTGTGCTCCATGCTCCG	40/56	[14]
Ntspa662	Nitrospira genus	GGAATTCCGCGCTCCTCT	35/80	[15]