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Short Communication

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Inactivation and adaptation of ammonia-oxidizing bacteria and nitrite-oxidizing bacteria when exposed to free nitrous acid

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Abstract: Inactivation and adaptation of ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) to free nitrous acid (FNA) was investigated. Batch test results showed that AOB and NOB were inactivated when treated with FNA. After an 85-day operating period, AOB in a continuous pre-denitrification reactor did not adapt to the FNA that was applied to treat some of the return activated sludge. In contrast, NOB did adapt to FNA. NOB activity in the seed sludge was only 11% of the original activity after FNA batch treatment, at 0.75 mg HNO_2-N/L. NOB activity in the pre-denitrification reactor was not affected after being exposed to this FNA level. *Nitrosomonas* was the dominant AOB before and after long-term FNA treatment. However, dominant NOB changed from *Nitrospira* to *Candidatus Nitrotoga*, a novel NOB genus, after long-term FNA treatment. This adaptation of NOB to FNA may be due to the shift in NOB population makeup.

Keywords: free nitrous acid (FNA); acclimation; ammonia-oxidizing bacteria (AOB); nitrite-oxidizing bacteria (NOB); nitritaion; *Candidatus Nitrotoga*.

1. Introduction

Nitrogen must be removed from wastewaters to control eutrophication in receiving water bodies. Conventional biological nitrogen removal technologies are widely used in wastewater treatment plants (WWTPs), where organic matter is consumed by denitrifiers, reducing the availability of organic matter for downstream methane production in an anaerobic digester (Ma et al., 2016; Ødegaard, 2016). The discovery of anaerobic ammonium oxidation (Anammox) bacteria introduced the possibility of autotrophic biological nitrogen removal. If anammox bacteria were applied in sewage treatment, more organic matter in sewage could be used to generate energy (e.g. methane), because it would not be needed to remove nitrogen. Furthermore, treating sewage with anammox could also reduce energy consumption because of the 60% reduction in oxygen demand for nitrification (Ma et al., 2016). The bottleneck of sewage treatment using anammox is a lack of stable nitritation (Ma et al., 2016; Yang et al., 2017), which could supply nitrite for anammox bacteria.

Recently, Wang et al. (2014) reported that nitrite-oxidizing bacteria (NOB) activity ceased after being exposed to FNA at 0.24 mg HNO_2-N /L for 24 h under anoxic conditions. In contrast, the ammonia-oxidizing bacteria (AOB) activity remained at approximately 70% after a similar treatment. Based on this, nitrogen removal via nitrite was achieved in the mainstream nitrification-denitrification reactor by treating part of sludge with a high level of FNA in a sidestream sludge treatment unit. Wang et al. (2016) achieved stable nitritation for mainstream deammonification by combining sludge treatment using FNA with dissolved oxygen (DO) control.

Free ammonia (FA) also has a more severe inhibition on NOB than on AOB (Vadivelu et al., 2007; Villaverde et al., 2000). After long-term exposure (6 months) to high FA levels in a

nitrifying biofilm reactor, however, NOB acclimated to FA (Villaverde et al., 2000). Similarly, if NOB can also adapt to a high FNA concentration, nitritation could not be maintained by treating sludge only with FNA. Therefore, studying the acclimation of NOB to FNA is crucial for optimizing sidestream sludge treatment using FNA and achieving stable nitritation in a mainstream wastewater treatment system.

The aim of this study is to evaluate the adaptation of AOB and NOB to FNA. This was achieved by measuring the effect of FNA on AOB and NOB in activated sludge that has not been exposed to FNA, then again after 85 days of operation with sidestream sludge treatment using FNA. Furthermore, the microbial community was characterized using Illumina high-throughput sequencing analysis.

2. Materials and Methods

2.1 Batch tests

Batch tests were used to investigate the effect of FNA pretreatment on AOB and NOB activities. The activated sludge collected from Beijing Gaobeidian WWTP was used as the seed sludge, which was treated in triplicate with different FNA concentrations (0.12, 0.25, 0.37, 0.50, 0.62, 0.75 and 0.87 mg HNO_2-N/L). The test with adding 0 mg HNO_2-N/L served as a control test. The mixed liquor suspended solids (MLSS) was adjusted to 4000 mg/L. The pH value was controlled at 6.0 ± 0.1 by manually adding 1.0 M HCL or 1.0 M NaOH. The test was conducted in an air-conditioned laboratory, with a steady temperature of 21± 2 °C. All experiments were carried out continuously for 6 h under strict anoxic conditions. After FNA batch treatment, activated sludge was washed for three times in a centrifuge at 4000 rpm for 5 min to remove nitrite. Then, AOB and NOB activities after FNA treatment were measured and represented as percentage of activities compared to the control test.

The effect of FNA treatment time on AOB and NOB was also investigated. The FNA treatment times were set at 0, 3, 6, 9, 12, 18 and 24 h, respectively. The test with a treatment

time of 0 h served as a control test. FNA concentration was 0.25 mg N/L. The other conditions were similar to the tests investigating the effect of FNA concentration.

To investigate AOB and NOB adaptation to FNA inactivation, the activated sludge, obtained from the An/O reactor on Day 85, was used in the following batch tests. FNA concentrations were 0, 0.12, 0.25, 0.75, 1.12, 1.37, 1.62 and 1.87 mg N/L, respectively. The other conditions were similar to the tests investigating the effect of FNA concentration.

2.2 Reactor set-up and operation

An anoxic/oxic (An/O) reactor with a working volume of 32 L was divided into nine equal chambers using baffles. The first three chambers were established as anoxic zones and the last six chambers were aerobic zones. The An/O reactor was operated in a temperature-controlled room (25 ± 1 ºC). Raw wastewater was collected from an university sewer line, and had the followig characteristics: soluble chemical oxygen demand ($COD_{soluble}$) =257.1 ± 7.4 mg/L, NH_4^+-N=60.2 ± 7.5 mg/L, NO_2^--N=0.5 ± 0.5 mg/L, and NO_3^--N=0.3 ± 0.5 mg/L.

An/O reactor was operated with part of the return sludge treated by FNA. The sludge retention time (SRT) was maintained at 15 d by controlling sludge wastage. The resulting MLSS was within the range of 2000-2500 mg/L. The returned sludge from the secondary clarifier (external recycle) and nitrate recirculation from the last aerobic compartment (internal recycle) were 0.5 and 2.0, respectively.

An/O reactor operation was divided into three phases: Phase I (Day 0–39): 3.2 L mixed liquor (10% of the total reactor volume) was collected from the last aerobic chamber over 1 h every day, and thickened to 0.5 L, resulting in a MLSS of approximately 15 g/L. The thickened sludge was treated in a FNA unit using 0.25 mg HNO_2-N/L (NO_2^--N = 100 mg/L, pH = 6.0, Temperature (T) =21 ºC) for 6 h. Therefore, the average sludge treatment frequency was 0.1 reactor volume per day (i.e. (3.2 L/d) /(32 L of total rector working volume)= $0.1d^{-1}$). Following the treatment, 0.5 L of FNA-treated thickened sludge was transferred to a sludge

storage unit and then recirculated to the first anoxic chamber of An/O reactor over one day. Both the FNA treatment and storage units were a 0.5 L Erlenmeyer flask, mixed using a magnetic stirrer. The influent flow rate was approximately 6.0 L/h, resulting in a hydraulic retention time (HRT) of 5.3 h. Phase II (Day 40–53): In the FNA treatment unit, nitrite concentration was increased from 100 to 400 mg/L on Day 40. This resulted in an FNA concentration increase from 0.25 to 1.00 mg HNO_2-N/L. The other An/O reactor operating conditions in Phase II were similar to those in Phase I. The influent flow rate increased to 8.0 L/h on Day 40, resulting in a HRT of 4.0 h. Phase III (Day 54–85): The average sludge treatment frequency was increased to 0.3 reactor volume per day on Day 54. The other An/O reactor operating conditions in Phase III were similar to those in Phase II.

2.3 Microbial community analysis

The microbial communities in the seeded sludge and in the An/O sludge were investigated using an Illumina high-throughput sequencing technique. The An/O sludge was collected from the An/O reactor on Day 85. DNA samples were extracted from 0.10~0.20 g dried sludge using the Fast DNA Kit (BIO 101, Vista, CA) according to the manufacturer's instruction. DNA concentrations were measured with a NanoDrop ND-1000 (NanoDrop Technologies, DE, USA). The V4–V5 region of the 16S rRNA gene was amplified using bacterial primers 515F (5'-GTGCCAGCMGCCGCGG-3') and 907R (5'-CCGTCAATTCM TTTRAGTTT-3'). The DNA amplicons were analyzed by gel electrophoresis using 2 % (w/v) agarose, recovered using an AxyPrep DNA Gel Extraction Kit (AXYGEN, China). Finally, the DNA library was constructed and run on the Miseq Illumina platform at Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). All the raw reads have been archived at NCBI Sequence Read Archive (SRA) database with accession number of SRR5462628 and SRR5462629. The trimmed sequences were grouped into operational taxonomic units (OTUs) using 97 % identity thresholds (i.e., 3% dissimilarity levels) by the Usearch software program.

The OTU numbers were counted for the sample as the species richness, and rarefaction curves and Shannon-Wiener were generated. The generated raw sequences of the sludge sample were assigned by Silva (http://www.arb-silva.de) to trim off the adapters and barcodes.

2.4 Analytical methods

Mixed liquor samples were filtered through 0.45 μm filter paper. SCOD, MLSS, and MLVSS were measured according to the standard methods (APHA, 1995). NH_4^+-N, NO_2^--N, and NO_3^--N were determined using a LACHAT quickchem 8500 Series 2 flow injection system.

3. Results and discussions

3.1 Inactivation of AOB and NOB by FNA treatment

AOB and NOB activities (expressed as a % of the original) both decreased after FNA treatment; the decrease was more significant for NOB (Fig.1A). For example, after being exposed to 0.25 mg HNO_2-N/L for 6 h, AOB activities was not affected, while NOB activity was only 35% of the activity seen with the control. AOB and NOB inactivation increased with FNA concentration. When FNA concentration increased from 0.12 to 0.87 mg HNO_2-N/L, the AOB activity decreased from 117% to 64% and the NOB activity decreased from 96% to 10%.

The inactivation also increased as the FNA treatment time lengthened. When the FNA treatment time was 18 h, AOB activity decreased to 47%; NOB all but ceased completely (Fig. 1B). These results indicate that NOB was more sensitive to FNA, and therefore, FNA treatments may suppress NOB growth to support nitritation in WWTPs. Optimizing FNA concentrations and treatment time is needed to reduce costs.

The percentages of dead cells increased as FNA concentrations increased in a sludge pretreatment unit, with viable cells decreasing to 20% when the FNA concentration was 2.04 mg HNO_2-N/L (Ma et al., 2015). This strong biocidal effect of FNA on microorganisms was also observed in previous studies (Jiang et al., 2011; Pijuan et al., 2012). As such, the FNA treatment effect was more likely attributed to FNA inactivation.

3.2 The effect of FNA treatment on An/O reactor performance

An/O reactor performance was investigated after applying the sidestream FNA treatment. Based on the results above, the FNA concentration was set at 0.25 mg HNO_2-N/L and the treatment time was set at 6 h in Phase I. At the same time, the average sludge treatment frequency was 0.1 reactor volume per day. The effluent nitrite accumulation rate (NAR, NAR = NO_2^--N/(NO_2^--N+ NO_3^--N)×100%) grew over the first 28 days (Phase I), reaching 75% on Day 28 (Fig.2A). However, the nitritation broke up on Day 32, and NAR suddenly dropped to 10% when the influent NH_4^+-N concentration decreased by 28%, resulting in excess aeration (aeration was still on after nitrotsation), which was thought to be the main cause of the NAR decrease (Gao et al., 2009). The following week, NAR did not increase, even though the influent NH_4^+-N concentration increased to 77.69 mg/L on Day 39. To rebuild nitritation, FNA concentration increased from 0.25 to 1.00 mg HNO_2-N/L on Day 40. This did not lead to an increase in NAR during phase II (Day 40 to 53).

On Day 54, the sludge treatment frequency increased from 0.1 to 0.3 reactor volumes per day. This change resulted in decreased AOB activity as the effluent NH_4^+-N concentration increased. However, effluent nitrite concentration did not increase, indicating that NOB activity did not significantly decrease. After Day 75, the influent COD concentrations decreased from 220-350 mg/L to 85-160 mg/L, resulting in an increased effluent NO_3^--N concentration (Fig.2B). After increasing the nitrite concentration in the FNA treatment unit and the sludge treatment frequency (Phases II and III), NAR still did not increase. This may suggest that the AOB and NOB in the An/O reactor have acclimated to FNA. As such, the effect of FNA treatment on AOB and NOB activities was investigated again using the activated sludge collected from the An/O reactor on Day 85.

3.3 The adaptation of AOB and NOB to FNA

FNA inactivated AOB when FNA was above 0.75 mg HNO_2-N/L after long-term An/O

reactor operation. However, NOB was not inactivated when the FNA ranged from 0-1.37 mg HNO_2-N/L (Fig. 3). The effect of FNA on AOB activity did not significantly change before and after long-term FNA treatment (Fig. 3A). However, the threshold FNA concentration for inactivating NOB, above which NOB activities decrease, increased significantly after applying FNA treatment (Fig. 3B). For example, NOB activity in the seed sludge was only 11% after FNA treatment at a level of 0.75 mg HNO_2-N/L. After long-term operation of the An/O reactor with the sidestream treatment using FNA, NOB did not lose activity after being treated by FNA at this level (Fig. 3B). This means that NOB become less sensitive to FNA as time passed; NOB had acclimated to FNA.

The relative abundance of NOB in the seed sludge was 2.53%, higher than AOB abundance, at 0.87%. After applying the FNA treatment in the An/O reactor, the relative abundance of NOB (4.62%) was still higher than AOB (2.53%). This helped to explain why nitrite did not accumulate in the An/O reactor effluent during Phase III. After FNA treatment, *Nitrosomonas* remained the dominant AOB. The relative abundance of *Nitrospira* decreased from 2.53% to 0.11%, which is consistent with Wang et al. (2016). However, *Candidatus Nitrotoga* increased from 0 to 4.51%. *Candidatus Nitrotoga* was previously unrecognized as the key NOB in full-scale WWTPs (Lucker et al., 2015). It was hypothesized that *Candidatus Nitrotoga* only grew at low temperatures of 4 °C and 17 °C(Lucker et al., 2015). However, *Candidatus Nitrotoga* was also found at room temperature in this study and during a study by Liang et al. (2015). Recently, Hüpeden et al. (2016) found that *Nitrotoga sp.* HW29 was successfully enriched under a low pH of 5.7 to 6.0, with an optimal temperature was 22 °C. However, *Nitrospira defluvii* displayed the highest nitrite oxidation rate at pH 7.3 and 32°C (Hüpeden et al., 2016). In this study, the temperature and pH were maintained at 21± 2 °C and 6.0 ± 0.1, respectively, during FNA treatment. These conditions may have benefited *Candidatus Nitrotoga* growth, and adversely impacted *Nitrospira* growth. Therefore, became

the dominant NOB in the An/O reactor after FNA treatment. These results indicate that NOB could adapt to high nitrite/FNA concentrations, driven by a shift in the NOB population from *Nitrospira* to *Candidatus Nitrotoga*.

Based on above results, controlling *Candidatus Nitrotoga* growth is very important for using FNA treatment to achieve mainstream nitritation in WWTPs. One possible way is to increase FNA concentration in the sidestream sludge treatment unit. Because *Candidatus Nitrotoga* activity decreased by 21% when FNA concentration increased to 1.87 mg HNO_2-N/L (the highest value in this study) (Fig. 3B). *Candidatus Nitrotoga* activity may continue to fall if FNA concentration increase to a more higher concentration. Another alternative approach is to optimize pH and temperature so as to control *Candidatus Nitrotoga* growth. However, more experimental studies are required to verify these two strategies.

4. Conclusions

AOB and NOB inactivation increased as the FNA concentration increased and exposure time lengthened. AOB did not adapt to the FNA treatment under the tested condition. In contrast, NOB did adapt to FNA, as the threshold FNA concentration for inactivating NOB increased greatly after long-term FNA treatment. The ability of NOB to adapt to FNA may be due to the shift in dominant NOB from *Nitrospira* to *Candidatus Nitrotoga*. Therefore, controlling *Candidatus Nitrotoga* growth is important for using FNA treatment to achieve mainstream nitritation in WWTPs.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at

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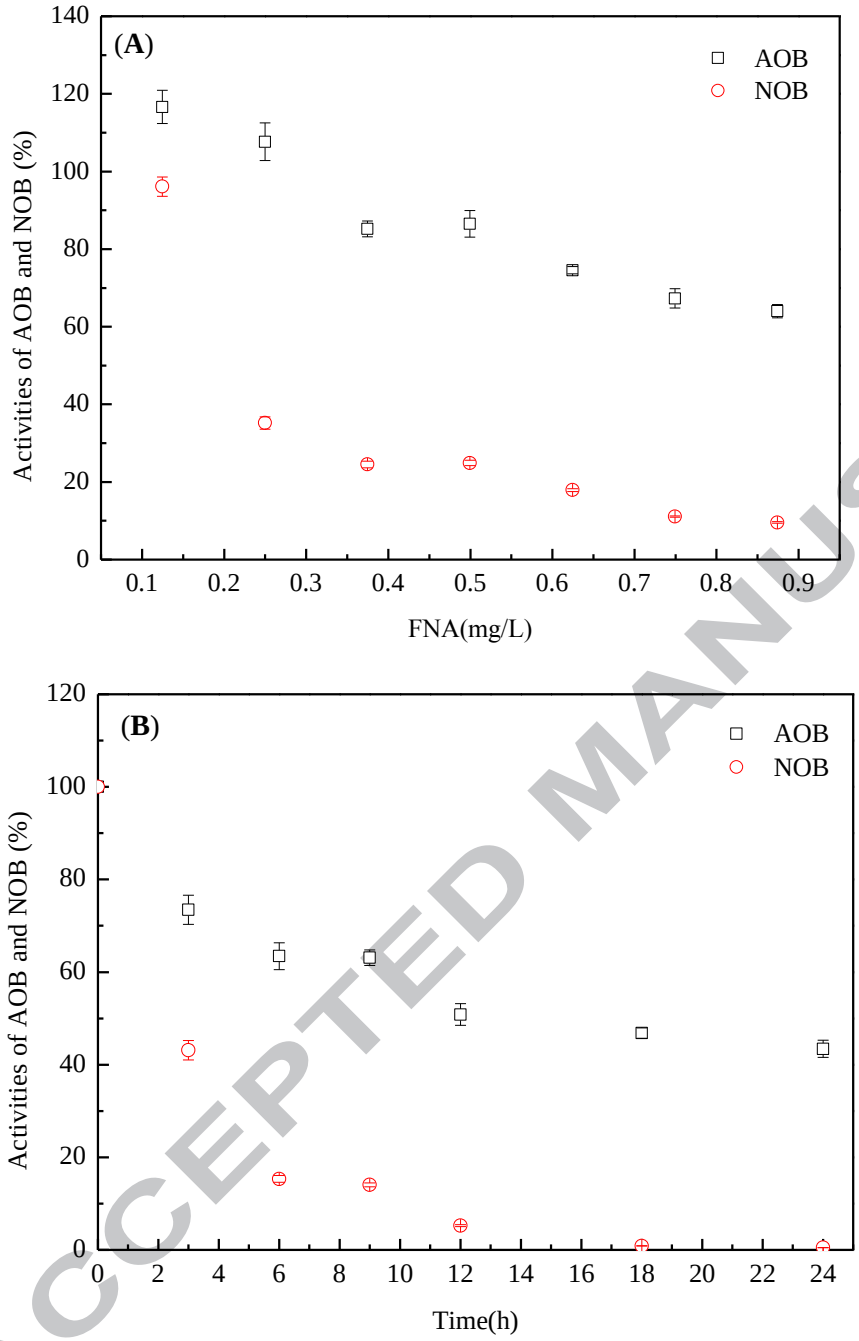
Figure captions

Fig. 1: The effect of FNA concentration on AOB and NOB activities in the seed sludge when temperature, treatment time and pH were 21 °C, 6 h and 6.0, respectively (A); the effect of FNA treatment time on these activities in the seed sludge when temperature, FNA concentration and pH were 21 °C, 0.25 mg HNO2-N/L and 6.0, respectively (B).

Fig. 2: The effluent nitrite accumulation rate (NAR) (A) and the influent and effluent nitrogen concentration (B) in the An/O reactor, where partial return activated sludge was treated in a sidestream FNA treatment unit.

Fig. 3: Comparison of the effect of FNA treatment on AOB and NOB activities in the seed sludge and the An/O sludge after 85 days. The seed sludge was obtained from Gaobeidian WWTP. The An/O sludge was obtained from the An/O reactor which had been operated for 85 days with partial return activated sludge treated by FNA.



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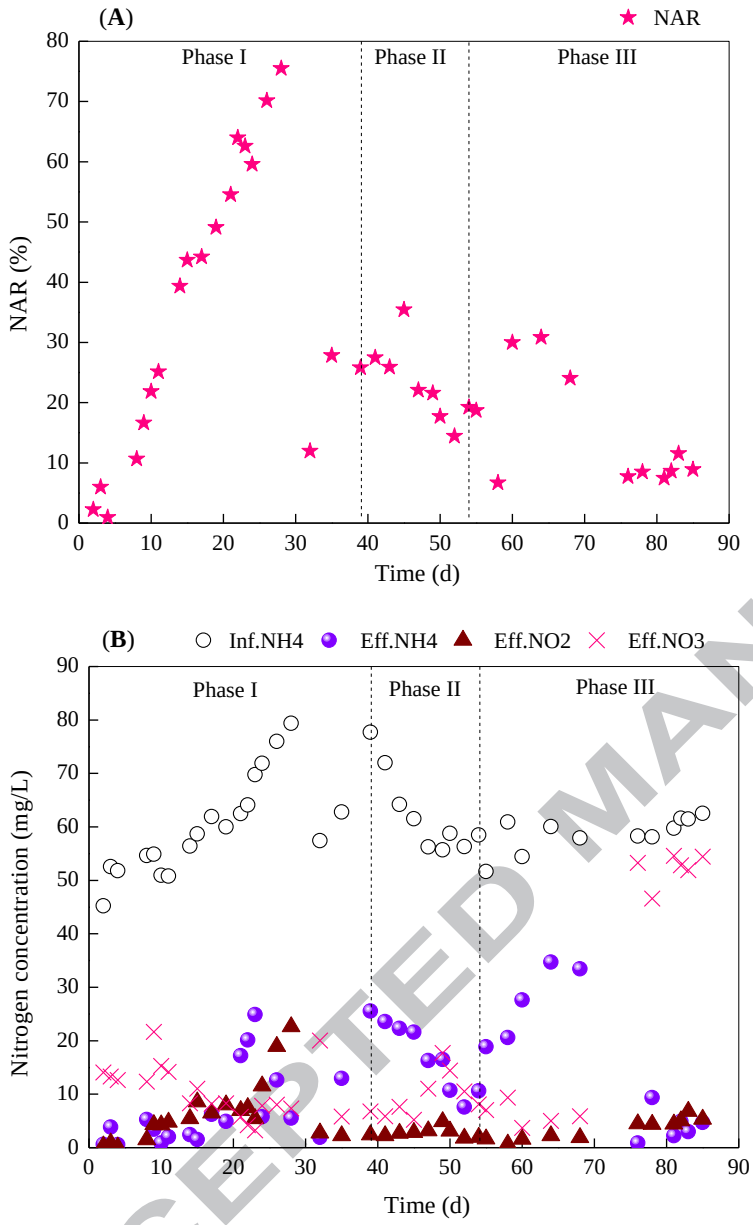

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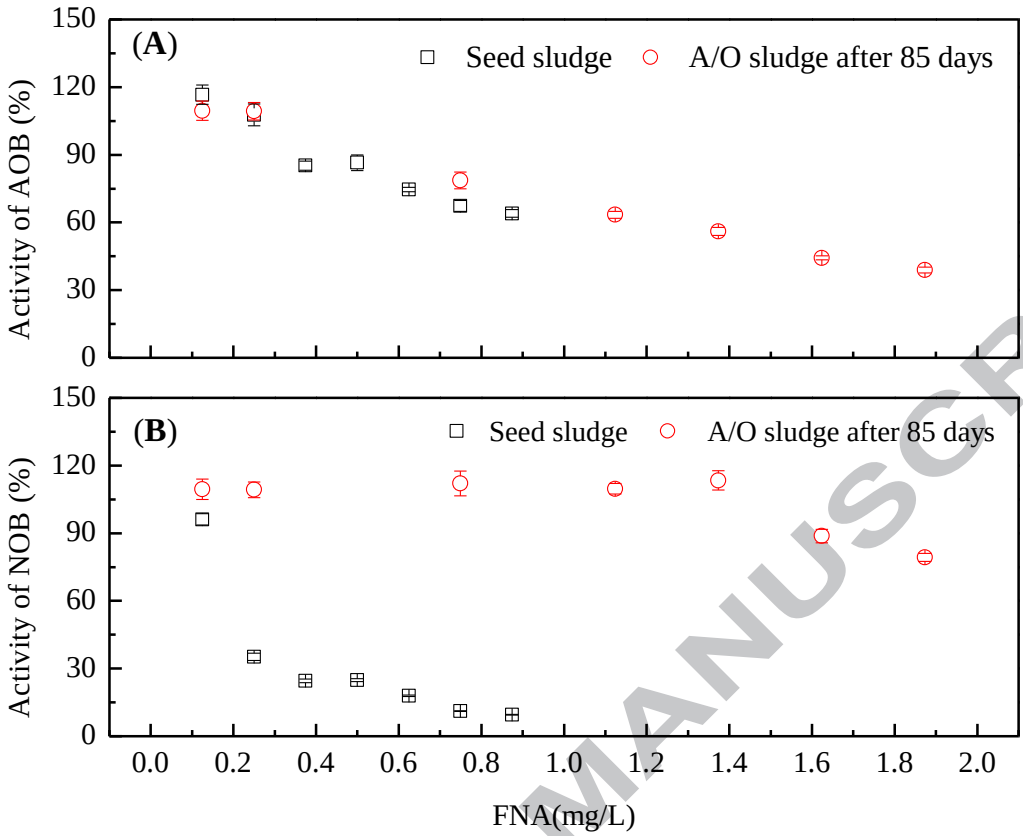

Fig. 3: Comparison of the effect of FNA treatment on AOB and NOB activities in the seed sludge and the An/O sludge after 85 days. The seed sludge was obtained from Gaobeidian WWTP. The An/O sludge was obtained from the An/O reactor which had been operated for 85 days with partial return activated sludge treated by FNA.

Research highlights

1) Inactivation and adaptation of AOB and NOB to FNA was investigated.

2) FNA caused a stronger inactivation effect on NOB than on AOB.

3) This inactivation increased as the FNA increased and exposure time lengthened.

4) AOB did not adapt to the FNA treatment. In contrast, NOB did adapt to FNA.

5) NOB adaptation may be due to its shift from *Nitrospira* to *Candidatus Nitrotoga*.