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Effect of free ammonium and free nitrous acid on the activity, aggregate morphology and EPS distribution of ammonium oxidizing bacteria in partial nitrification

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Successful partial nitrification not only guarantees the inhibition of nitrite oxidation, but also does not excessively retard the ammonia oxidation rate. Therefore, the performance of ammonium oxidizing bacteria (AOB) during partial nitrification is fundamental to this process. In this study, two lab-scale partial nitrification bioreactors containing different inhibition conditions—one with free ammonium (FA) inhibition, the other with free nitrous acid (FNA) inhibition—were used to compare the differences between activity, quantity, aggregation morphology and extracellular polymeric substance (EPS) distribution of AOB. The results showed that although stable, long-term, partial nitrification was achieved in both reactors, there were differences in AOB activity, microbial spatial distribution and EPS characteristic. In the FA bioreactor, FA concentration was conducted at more than 40 mg/L, which had a strong impact on the metabolism of AOB. The activity and quantity decreased by 50%. Higher EPS (42.44 ± 2.31 mg g^{-1} mixed liquor volatile suspended solids [MLVSS]) and protein were introduced into the EPS matrix. However, in the FNA bioreactor, the FNA concentration was about 0.23 mg/L. It did not reach a level to affect AOB metabolism. The AOB activity and quantity were maintained at high levels and the total EPS content was 28.29 ± 2.04 mg g^{-1} MLVSS. Additionally, the microscopic results showed that in the FA bioreactor, AOB cells aggregated in microcolonies, while they appeared to be self-flocculating with no specific conformation in the other reactor. β-polysaccharides located inside sludge flocs in the FA bioreactor but only accumulated around the outer layer of activated sludge flocs in the FNA condition.

[**Key words:** Partial nitrification; Inhibition condition; Activity; Aggregated morphology; EPS distribution; Ammonium oxidizing bacteria]

Traditionally, nitrogen removal from wastewater is accomplished by a two-stage treatment of nitrification and denitrification (1). In this system, ammonium is firstly oxidized to nitrate by nitrifying bacteria under oxic conditions, and then to nitrogen gas by denitrifying bacteria under anoxic conditions. However, recently, a combination of partial nitrification and anaerobic ammonium oxidation (Anammox) has provided a new approach for nitrogen removal. Ammonium can be directly converted to nitrogen gas using nitrite as an electron acceptor in the absence of oxygen (2). Compared with traditional biological nitrogen removal, the aeration cost can be reduced by 25% and the demands on the carbon source are decreased by 40% (3,4). As it has unique cost-saving advantages, the partial nitrification–anammox process has been widely used in many developed countries for the treatment of carbon-limited wastewaters, such as sludge liquor, landfill leachates or piggery wastewater (5,6).

Based on the partial nitrification–anammox mechanism, ammonium and nitrite should be provided simultaneously in the partial nitrification process. Thus it is necessary to eliminate nitrite oxidizing bacteria (NOB) while retaining ammonium oxidizing bacteria (AOB). Previous studies have identified factors that result in the elimination of NOB including free ammonia (FA), free nitrite acid (FNA), temperature, dissolved oxygen (DO), sludge retention time (SRT) and inhibitors (7–11). Among them, the inhibitions resulting from FA and FNA are strong and complicated, because they significantly affect NOB metabolism. As such, they have gained much research attention (12). Vadivelu et al. (13) found that FNA at a concentration of 0.02 mg/L, or FA at a concentration of 7.5 mg/L, could completely stop the biosynthetic process of NOB. In support of this point, Chung et al. (3) reported that nitrite accumulation occurred at an initial FA concentration of 20 mg/L, with a high nitrite accumulation rate (90 ± 5%) in a continuous-flow reactor. Wang et al. (14) achieved long-term accumulation of nitrite in a side-stream treatment unit by maintaining the FNA concentration at around 1.35 mg/L.

However, AOB activity is also an important parameter for partial nitrification, which can directly determine the maximum treatment capacity and the stability of the process. Therefore, the successful partial nitrification should not only guarantee the inhibition

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of nitrite oxidation, but also does not excessively retard the ammonia oxidation rate (15). In several studies, researchers focused on the isolated effect of the FA and FNA concentration on the activity of AOB and found that negative effects on the performance of AOB have also been observed during the partial nitrification achievement. Hulle et al. (16) reported that the efficiency of total ammonium nitrogen (TAN, the sum of NH_4^+–N and NH_3–N) oxidation may decrease due to FA inhibition. In the work of Vadivelu et al. (13), extreme increases in FNA concentration caused the ammonium oxidation rate to decrease in reactors, which suggested an obvious drop of AOB activity. However, there still lack a comparison of the effects on the activity and proportion of AOB in the two systems.

Furthermore, microorganisms in reactors often form concentrated bioaggregates to achieve higher treatment efficiency, which is of great importance to the flocculability, settleability, and dewaterability for the activated sludge flocs and sludge retention (17). Meanwhile, extracellular polymeric substance (EPS) was believed to be mediated or enhanced the formation of bioaggregates (18). Therefore, a better understanding of these properties of the AOB-enriched sludge, such as AOB spatial distribution and EPS characteristics is very important to optimize the partial nitrification process. However, to the best of our knowledge, there has few papers describing the microbial spatial distribution characteristics and EPS distribution in the partial nitrification systems.

In this study, two partial nitrification bioreactors with the same operating parameters (hydraulic retention time, sludge retention time, temperature and DO concentration) and different inhibitors (one with FA and another with FNA) were employed to treat high nitrogen concentration wastewater, as a prior step for an anammox reactor. The main goal was to compare the differences on the activity, quantity, aggregated morphology and EPS distribution of AOB in the two different inhibition conditions.

MATERIALS AND METHODS

Experimental set-up and operational strategy The experiments were performed in two identical sequencing batch reactors (SBRs). Each had a working volume of 4.5 L. Mechanical stirrers were used to ensure a completely mixed status. Oxygen was supplied by an air pump from the bottom of the reactors and was controlled by a flow meter. The seed sludge was collected from the aeration tank of a full-scale biological nutrient removal wastewater treatment plant (WWTP), in Xi'an, China, which receives domestic wastewater from surrounding areas and operates with the anoxic/anaerobic/oxic process for both organic matter and nutrient removal. The initial suspended solids (SS) and volatile suspended solids (VSS) concentration in the reactor were 5.05 and 2.98 g/L, respectively, which corresponded to a VSS/SS of 0.59.

Reactors were automatically controlled by a programmable logical controller. The temperature was kept at 35 ± 1°C and pH was adjusted by $NaHCO_3$. The SBR cycle consisted of four phase: 435 min aeration, 30 min settling, 10 min discharging and 5 min idle. In the aeration phase, substrates were added to the reactor gradually within 360 min, namely fed-batch, to guarantee there was no substrate inhibition in the cultivation period (19). In each cycle, 1.5 L of wastewater was fed into the rector, which resulted in a hydraulic retention time (HRT) of 24 h. The sludge retention times (SRT) were kept at 15 days all the time. Synthetic wastewater containing ammonium chloride as a nitrogen source was used in this study and it was prepared as follows: NH_4^+–N, 25–1000 mg/L; KH_2PO_4, 22–877 mg/L; $CaCl_2{\cdot}2H_2O$, 25 mg/L; $MgSO_4$, 40 mg/L; $NaHCO_3$ (added as need) and trace element solution 1.0 ml/L [the composition of the trace element solution was (mg/L): $CuCl_2{\cdot}2H_2O$, 0.035; $NiCl_2{\cdot}6H_2O$, 0.036; EDTA, 0.05; $MgSO_4{\cdot}7H_2O$, 5; $FeCl_2{\cdot}4H_2O$, 6; $CoCl_2{\cdot}4H_2O$, 0.88; H_3BO_3, 0.1; $ZnSO_4{\cdot}7H_2O$, 0.1; $MnCl_2{\cdot}4H_2O$, 0.5; $(NH_4)MoO_2{\cdot}4H_2O$, 0.64].

Partial nitrification was achieved during two periods: enrichment and inhibition. During the enrichment period, the influent NH_4^+–N concentration was gradually increased from 25 to 1000 mg/L. Dissolved oxygen and pH were controlled at 2–3 mg/L and 7.5 ± 0.1, respectively. The complete nitrification should be guaranteed. Then, the nitrifying bacteria-enriched sludge was separated into two identical SBRs to achieve partial nitrification under different inhibition strategies. In the inhibition period, air supply was reduced gradually and finally at a flow rate of 0.3 L/min for controlling the dissolved oxygen concentration was 0.5 mg/L in both reactors. The pH in SBR_1 was maintained at 8 ± 0.2 and that in SBR_2 was conducted at 7 ± 0.2.

Activated sludge samples were taken from the seeding sludge (sample 1, Day 0), from sludge at the end of the enrichment period (sample 2, Day 72), and during the inhibition periods of SBR_1 (sample 3, Day 180) and SBR_2 (sample 4, Day 180). Aerobic batch tests were carried out for all the samples to determine the nitrifying activities. Fluorescence in situ hybridisation (FISH) was used to investigate the microbial community. Moreover, aggregated morphology and EPS distribution in samples 3 and 4 were further analysed by scanning electron microscope (SEM) and EPS staining.

Aerobic batch experiments Aerobic batch experiments were carried out to determine the activities of nitrifying bacteria. Sludge was taken from the operating SBRs and washed several times to remove all soluble substrates before testing. The initial $NH_4^+ - N$ and NO_2–N concentrations were 20 mg/L and 30 mg/L, respectively. During the whole test, temperature was maintained at 35 ± 1°C. $NaHCO_3$ was added to ensure a stable pH. Samples were taken every 10 min and NH_3–N and NO_2–N were measured to calculate the ammonium uptake rate (AUR) and nitrite uptake rate (NUR), respectively.

Analytical methods All chemical analyses were performed using standard methods (20). The DO concentrations were measured by a dissolved oxygen meter (SG6-FK10, Mettler). Ammonium, nitrite, nitrate, pH, and oxygen concentration were determined daily. Nitrification activity, biomass (as VSS) and microorganism population structure were periodically monitored. Free ammonium (FA), free nitrite acid (FNA) concentrations and nitrite accumulation rates (NAR) were calculated according to the following formulae (21,22):

$$[\mathrm{FA}] = \frac{17}{14}\frac{10^{\mathrm{pH}}}{e^{\left(\frac{6334}{273+T}\right)}+10^{\mathrm{pH}}} \times \left[NH_4^+ - N\right] \quad (1)$$

$$[\mathrm{FNA}] = \frac{47}{14}\frac{1}{e^{\left(-\frac{2300}{273+T}\right)}\times 10^{\mathrm{pH}}} \times \left[NO_2^- - N\right] \quad (2)$$

$$\mathrm{NAR} = \frac{C\left(NO_2^- - N\right)_{\mathrm{eff}}}{C\left(NO_2^- - N\right)_{\mathrm{eff}} + C\left(NO_3^- - N\right)_{\mathrm{eff}}} \quad (3)$$

where $[NH_4^+ - N]$ and $[NO_2^- - N]$ represent the actual concentrations of $NH_4^+ - N$ and $NO_2^- - N$ in the effluent respectively, and pH is the effluent pH value.

Fluorescence in situ hybridisation Sample fixation and hybridisation were performed according to the standard methods described by Amann et al. (23) and Daims et al. (24). The 16S rRNA-targeted oligonucleotide probes were listed in Table 1. Probe EUBmix (an equimolar mixture of EUB338, EUB338II and EUB338III), labelled with Cy5, was used to target almost all bacteria. Probe AOBmix (a mixture of Nso1225, NEU, NmV and Cluster6a192), labelled with Flous, was used to target the AOB. Probe NOBmix (a mixture of Ntspa662, NIT3 and Ntspa712), labelled with Cy3, was used to detect NOB. Nsm156 was specific for *Nitrosomonas* spp.; Nsv443 was specific for *Nitrosospira* spp.; Ntspa662 was specific for *Nitrospira* spp. and NIT3 was specific for *Nitrobacter* spp.

Confocal laser scanning microscope (CLSM; Leica SP8, Leica, Germany) equipped with one Ar-ion laser (488 nm, for detection of Flous) and two HeNe lasers (552 and 638 nm, for detection of Cy3 and Cy5, respectively) was used to examine the microbial community. Both stages of image combining and processing were performed with the process tools of the software delivered by the CLSM supplier. The image analysis was determined by using the software package PaintShopPro (Jasc, Eden Prairie, MN, USA). The proportions of the targeted nitrifiers were calculated according to Li et al. (25). The detail procedure was as flows: total 60 views were obtained for each sample (20 views for each well and three wells for each sample). All images were first processed with blur (or out-of-focus) removing, then three colours (Cy3-Red; Cy5-Blue; Flous-Green) were counted separately for each image. Based on the quantification of pixel areas of the 60 images, percentage of the targeted nitrifiers was calculated as follows:

$$\text{Percentage nitrifiers in biomass} = \frac{\text{Pixel aera of green (red) image}}{\text{Pixel aera of blue image}} \quad (4)$$

Scanning electron microscope The morphology of the bacteria was examined with SEM. The samples were pre-treated by fixing with 4% paraformaldehyde for 6 h. Subsequently, the samples were washed by PBS and dehydrated in a graded series of ethanol solutions (30%, 50%, 70%, 80%, 90%, 95%, and 100% [v/v]). The dewatered samples were dried by the critical point method and then sputter coated with gold for SEM observation.

EPS extraction and composition analysis Extraction of EPS was performed using the cation exchange resin (CER) method, which followed the same procedure as that of Frølund et al. (26). The dose of CER (Dower Marathon C, Na^+ form, 20–50 mesh, Fluka 91973) was adjusted to 70 g resin g^{-1} MLVSS. The mixing speed was set at 800 rpm. The extraction time and temperature were adjusted to 4 h and 4°C. The EPS was quantified in terms of protein and polysaccharide.

The Lowry method (26) was applied for protein determination and bovine serum albumin was used as the standard. Polysaccharide determination was determined by the phenol-sulfuric acid method (27) and glucose was used as the standard. The total EPS was defined as the sum of protein and polysaccharide.

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