Nitrification of high strength ammonia wastewater and nitrite accumulation characteristics

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Abstract Biological nitrogen removal via the nitrite pathway in wastewater treatment is very important in saving the cost of aeration and as an electron donor for denitrification. Wastewater nitrification and nitrite accumulation were carried out in a biofilm airlift reactor with autotrophic nitrifying biofilm. The biofilm reactor showed almost complete nitrification and most of the oxidized ammonium was present as nitrite at the ammonium load of 1.5 to 3.5 kg $N/m^3 \cdot d$. Nitrite accumulation was stably achieved by the selective inhibition of nitrite oxidizers with free ammonia and dissolved oxygen limitation. Stable 100% conversion to nitrite could also be achieved even under the absence of free ammonia inhibition on nitrite oxidizers. Batch ammonium oxidation and nitrite oxidation with nitrite accumulating nitrifying biofilm showed that nitrite oxidation was completely inhibited when free ammonia is higher than 0.2 mg N/L. However, nitrite oxidation activity was recovered as soon as the free ammonia concentration was below the threshold level when dissolved oxygen concentration was not the limiting factor. Fluorescence *in situ* hybridization analysis of cryosectioned nitrite accumulating nitrifying biofilm showed that the β-subclass of *Proteobacteria*, where ammonia oxidizers belong, was distributed outside the biofilm whereas the α-subclass of *Proteobacteria*, where nitrite oxidizers belong, was found mainly in the inner part of the biofilm. It is likely that dissolved oxygen deficiency or limitation in the inner part of the nitrifying biofilm, where nitrite oxidizers exist, is responsible for the complete shut down of the nitrite oxidizers activity under the absence of free ammonia inhibition.

Keywords Biofilm reactor; FISH; inhibition; nitrification; nitrite accumulation

Introduction

Microbial nitrification, the sequential oxidation of NH_4^+ via NO_2^- to NO_3^-, followed by denitrification is the key process in the removal of ammonium from wastewater. In an integrated nitrogen removal system, it is beneficial if ammonium is only oxidized to nitrite and thereafter denitrified for two reasons: 1) an approximately 25% lower consumption of oxygen in the nitrification stage, and 2) an approximately 40% lower electron donor requirement in the denitrification stage.

Nitrite accumulation can be obtained by disequilibrium in numbers or activities between the ammonia oxidizers and nitrite oxidizers. The disequilibrium in numbers occurs when ammonia oxidizers outcompete nitrite oxidizers in a mixed nitrification system. Differences in the oxidation activities can be mainly imposed, for example, by an inhibition in the activity of the nitrite oxidizers linked to the presence of free ammonia and dissolved oxygen (DO) limitation in the nitrifying bacteria (Garrido *et al*., 1997; Hellinga *et al*., 1998; Joo *et al*., 2000; Kuai and Verstraete, 1998; Turk and Mavinic, 1989). Selective free ammonia (NH_3) inhibition of nitrite oxidizers usually occurs at 0.1 to 10 mg NH_3-N/L (Villaverde *et al*., 1997). Low DO also limits nitrite oxidation since the oxygen saturation coefficients (K_s) of ammonia oxidation and nitrite oxidation are known to be 0.3 and 1.1 mg/L, respectively (Wiesmann, 1994).

A biofilm airlift reactor was used for nitrification and nitrite accumulation, and sand

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(average diameter: 200 μm) was used as a nitrifying biomass carrier. Ammonium load and aeration condition were adjusted for the manipulation of free ammonia concentration and DO level. Batch oxidation experiments were also performed to determine the activities of the ammonia oxidizers and nitrite oxidizers of the nitrifying biofilm.

In situ microbial community structures of ammonia oxidizers and nitrite oxidizers in the nitrifying biofilm were analyzed by fluorescence *in situ* hybridization (FISH) with ribosomal RNA-targeted oligonucleotide probes. FISH has become a common tool for the direct, cultivation-independent identification of individual bacterial cells (Amann, 1995).

The purposes of this study were to find out the efficiency of the biofilm airlift reactor for nitrification and nitrite accumulation and the cause of nitrite accumulation by investigating the microbial activities and community of the nitrifying bacteria in a nitrite accumulating biofilm reactor. Specifically, the reasons for the decline and recovery of nitrite oxidizing activity in the biofilm reactor were examined by long term operation of the airlift reactor and batch tests. And the distribution of the ammonia oxidizers and the nitrite oxidizers in the nitrite accumulating biofilm reactor were determined by FISH with confocal laser scanning microscopy (CLSM). These results enable us to better understand the behavior of the nitrification reactor and to propose a solution to intensify the accumulation of nitrite with the goal of proposing a new process of nitrification-denitrification via nitrite pathway.

Materials and methods

Biofilm airlift reactor

A 3 litre laboratory scale concentric tube biofilm airlift reactor equipped with a three-phase separator was used. Its height, riser diameter and downcomer diameter were 100, 4 and 7 cm, respectively. It was inoculated with activated sludge of nitrifying wastewater treatment plant and already operated for 300 days before this research started. Ammonium load was adjusted by changing wastewater ammonium concentration. DO concentration was varied by changing air flow rate and air distributor type and monitored throughout the experiment. The pH was controlled at 7.5 by the supply of 1 mol l^{-1} $NaHCO_3$.

Sand with a mean diameter of 200 μm was the carrier material. The initial carrier concentration in the reactor was 45 g/L. Under these conditions, the sand and biofilm particles were homogeneously mixed and suspended in the reactor.

Batch nitrification kinetics

About 1.5 g VSS/L (dry weight basis) of the nitrifying biofilm from the biofilm airlift reactor was used for the batch experiment when nitrite was highly accumulated. The media were washed with buffered medium to remove the background concentrations of NH_4^+, NO_2^- and NO_3^-. Batch nitrification was performed with 300 ml batch medium with distilled water in 500 ml Erlenmeyer flasks. The batch nitrification medium has the same composition as the reactor wastewater except for the NH_4^+ and NO_2^- concentrations. For ammonium oxidation 100 mg/L NH_4^+-N was used, while 100 mg/L NO_2^--N was used for the nitrite oxidation experiments. All the batch experiments were performed in a shaking flask incubator at 28°C and 200 rpm. Dissolved oxygen concentrations were always between 7 and 8 mg/L so that dissolved oxygen limitation was negligible. All the batch kinetic experiments were performed with duplicate samples.

Wastewater and analysis

Synthetic wastewater without organic carbon source was prepared with tap water; the composition was as follows: $(NH_4)_2SO_4$, 300–750 mg N/L; $MgSO_4 \cdot 7H_2O$, 5 mg/L; KCl, 7 mg/L; K_2PO_4, 11 mg/L; $Na_2HPO_4 \cdot 12H_2O$, 29 mg/L; $FeCl_3 \cdot 6H_2O$, 1 mg/L; $CaCl_2 \cdot 2H_2O$, 7 mg/L; $NaHCO_3$ (as $CaCO_3$) 7.14 mg/NH_4^+-N mg. The analytical methods were based on

the *Standard Methods* (APHA, 1992). NH_4^+-N was measured by the Nesslerization method by reading absorbance at 425 nm by UV-Visible spectrophotometer (UV 1601, Shimadzu). Both NO_2^--N and NO_3^--N were measured by ion chromatograph (DX 500, Dionex).

Fixation and cryosectioning of biofilm samples

The biofilm samples were fixed in 4% freshly prepared paraformaldehyde solution for 2 to 3 h at 4°C. Then the biofilm samples were rinsed twice with phosphate-buffered saline (PBS). Each fixed biofilm was placed in a small aluminium cup, embedded in Jung OCT compound (Leica Int.) overnight and stored at room temperature to infiltrate the compound into the biofilm samples. Then the biofilm samples were frozen at –20°C, subsequently. The frozen biofilm samples were cut into 20 μm thick vertical slices with a cryostat (Reichart-Jung cryocut 1800; Leica Int.) at –20°C. Each slice was placed on a gelatin (0.1% gelatin + 0.01% chromium potassium sulfate) coated microscopic slide and dried overnight to allow optimal attachment of the biofilm sample to the slides. The specimen was finally dehydrated by successive 50, 80, and 98% ethanol washes (3 min each), air dried, and stored at room temperature. The ethanol dehydration procedure substantially reduced the inherent fluorescence, removed the OCT compound, and also increased probe penetration through the cell walls (Okabe *et al*., 1999).

In situ hybridization and microscopy

All *in situ* hybridizations were performed by the procedure of Manz *et al*. (Manz *et al*., 1992) and Amann (Amann, 1995). The probe concentration was approximately 20 ng/μl. Hybridization was followed twice by a stringent washing step at 48°C for 10 min with 50 ml of pre-warmed washing buffer. Washing buffer was removed by rinsing the slides with distilled water and dried. The slides were mounted to avoid bleaching and examined with microscopy.

Slides were examined with an Axioplan epifluorescence microscope (Carl Zeiss) and MRC-1024 (Biorad, UK) confocal laser scanning microscope (CLSM), equipped with Kr/Ar lasers (excitation wavelength 494 nm and 650 nm) and HeNe lasers (550 nm).

Result and discussion

Nitrification and nitrite accumulation in biofilm airlift reactor

The biofilm airlift reactor was started 300 days before this experiment. The average biomass concentration during the experiment was 12.5 g VSS/L. All sand carriers were covered with a biofilm except quartz-like particles in the sand (less than 5% of the particles). As the carriers were saturated with biofilm, granule formation was observed (particles of biomass without a carrier). The average diameter of the biofilm particles and granules were 550 and 500 μm, respectively. Thus, the average biofilm thickness was about 200 to 250 μm. Ammonium load was varied by adjusting inlet wastewater ammonium concentration, because the hydraulic retention time of the reactor was fixed to 5 hours.

Figure 1 shows the time courses of nitrification and nitrite accumulation with the variation of ammonium load and aeration condition in the biofilm airlift reactor. For case A, nitrification efficiency was 85–90% and the nitrite ratio (NO_2^--N/(NO_2^--N + NO_3^--N) × 100%) was about 100% at the ammonium load of 2.5 kg N/m^3·d. This means that most of the oxidized ammonium was present as nitrite while nitrite oxidation to nitrate was completely stopped. Free ammonia (NH_3-N) concentration was maintained between 1 and 3 mg/L, a range that has been known to inhibit nitrite oxidizers selectively. DO was kept around 1–2 mg/L. The low DO seemed to limit ammonia oxidation as well as nitrite oxidation and led to ammonium (free ammonia) in the effluent owing to incomplete nitrification. Both low DO and high free ammonia concentration were responsible for the high nitrite accumulation.

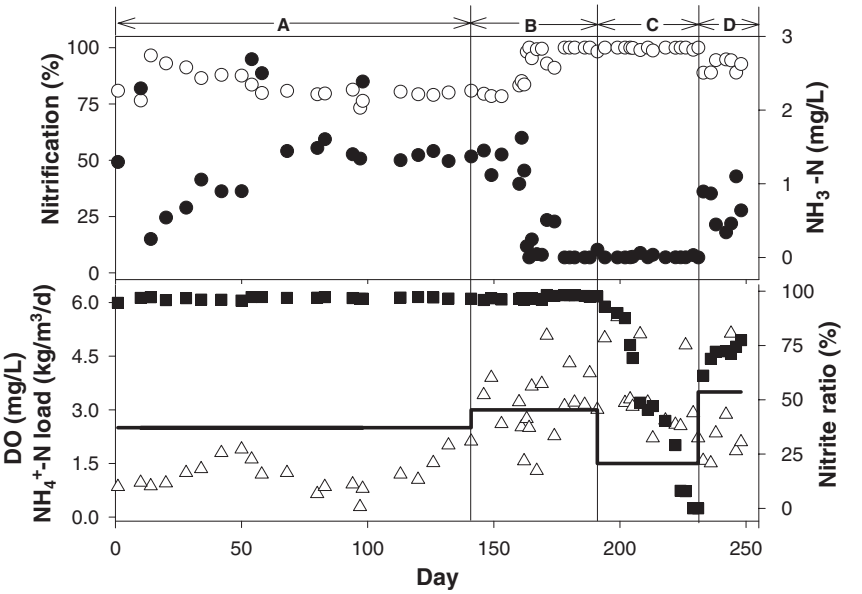

Figure 1 Profiles of free ammonia (NH_3-N, ●), dissolved oxygen (△), Nitrification efficiency (○), NH_4^+-N load (—), and Nitrite ratio (■) as function of time in the biofilm airlift reactor

For case B, ammonium load and average DO were increased to 3.0 kg N/m^3·d and 3 mg/L. Nitrification efficiency was gradually increased to more than 95% with the increase of the DO even though ammonium load was increased. Free ammonia concentration was decreased to well below 0.1 mg/L, which does not inhibit nitrite oxidizers, with the increase of nitrification efficiency. However, nitrite ratio remained at 100% despite the absence of free ammonia inhibition. The higher DO increased ammonia oxidation but not nitrite oxidation. In terms of nitrite ratio, Kuai and Verstraete (1998) obtained average nitrite ratio of 80% with a 1,000 mg NH_4^+-N/L suspension sequencing batch reactor under oxygen limiting conditions. Garrido *et al*. (1997) achieved a nitrite ratio of about 50% with 196 mg NH_4^+-N/L with an oxygen limited biofilm airlift reactor. In this study we achieved a nitrite ratio of about 100% with more than 90% nitrification efficiency.

We have observed the same phenomenon in a packed bed biofilm reactor (Han *et al*., 2001). It appears likely that the population decrease of nitrite oxidizers in the biofilm reactor due to long term inhibition on nitrite oxidizers growth by high free ammonia and oxygen limitation may be responsible for the higher nitrite ratio. The decrease of the growth rate of nitrite oxidizers in a continuously operated reactor may lead to population decrease and, in extreme cases, washout. In attached biofilm systems the microbial population decrease due to washout may not be as significant as in suspension systems, but it may affect the microbial population somehow.

For case C, ammonium load was decreased to 1.5 kg N/m^3·d to activate nitrite oxidizer activity by providing a greater chance of oxygen uptake. Complete nitrification was achieved and nitrite ratio was slowly decreased to 0% in 40 days after ammonium load reduction. This shows that the activity of nitrite oxidizer was recovered very slowly after the absence of free ammonia inhibition. At this time it is not clear whether the slow recovery of nitrite oxidizer activity was due to intrinsic characteristics or oxygen supply limitation to the layer of nitrite oxidizers in the biofilm.

For case D, nitrification efficiency decreased to 85% and free ammonia was between 0.5 and 1 mg/L, as ammonium load was increased to 3.5 kg N/m^3·d. Nitrite was accumulated

and nitrite ratio reached 75% as soon as free ammonia concentration was increased by quickly inhibiting nitrite oxidizers.

From the above results it can be summarized that free ammonia effectively inhibited nitrite oxidizer activity and oxygen limitation to nitrite oxidizers was also responsible for the nitrite accumulation in the absence of free ammonia. The reason for the high nitrite ratio in this study is still unclear and needs to be studied further.

Batch oxidation kinetics of ammonia and nitrite

In the biofilm airlift reactor it was difficult to keep the DO level high enough to completely exclude the possibility of oxygen limitation to nitrite oxidizers in the biofilm. Complete blocking of nitrite oxidation in cases A and B needs to be thoroughly examined to see whether washout of nitrite oxidizers from the reactor or free ammonia or oxygen limitation is responsible. In order to answer this question batch oxidations of ammonia and nitrite were carried out at high DO conditions (7–8 mg/L) with the nitrite accumulating biofilm from case A.

Figure 2 shows the results of ammonia oxidation [A] and nitrite oxidation [B] in batch experiments. In the case of ammonium oxidation, 100 mg/L NH_4^+-N was completely oxidized in 12 hours. During the ammonium oxidation, however, nitrite was hardly oxidized to nitrate for the first 8 hours. Nitrite oxidation began when NH_4^+-N was less than 30 mg/L (NH_3-N: 0.2 mg/L). Nitrite oxidation increased sharply after complete consumption of ammonium and its specific activity was comparable to ammonia oxidizers as shown in Figure 2-[A]. The result indicates that nitrite oxidation activity is completely inhibited in the presence of free ammonia concentration (higher than 0.2 mg NH_3-N/L), and it is recovered as soon as free ammonia concentration is below the threshold concentration. Figure 2-[B] shows the profiles of nitrite and nitrate in batch nitrite (100 mg NO_2^--N/L) oxidation with the biofilm. It shows the nitrite oxidizer activity of the biofilm in the absence of ammonium. As expected, nitrite was oxidized to nitrate without any lag time.

From the batch results, we can conclude the following: 1) free ammonia concentration higher than 0.2 mg/L completely inhibits nitrite oxidizers activity, 2) long term free ammonia inhibition and oxygen limitation did not eliminate nitrite oxidizers from the biofilm and the activity was quickly recovered as soon as the inhibition factor was removed, 3) the slow recovery of nitrite oxidizers activity in case C was due to oxygen limitation to the nitrite oxidizers.

Distribution of ammonia oxidizers and nitrite oxidizers in the biofilm by *in situ* hybridization

It is meaningful to see the *in situ* spatial distribution of nitrite accumulating nitrifying biofilm. In this study nitrifying biofilm taken from the reactor was fixed and thin sectioned, and hybridized with fluorescence labeled rRNA probes. Probe Alf1b labeled with CY-3

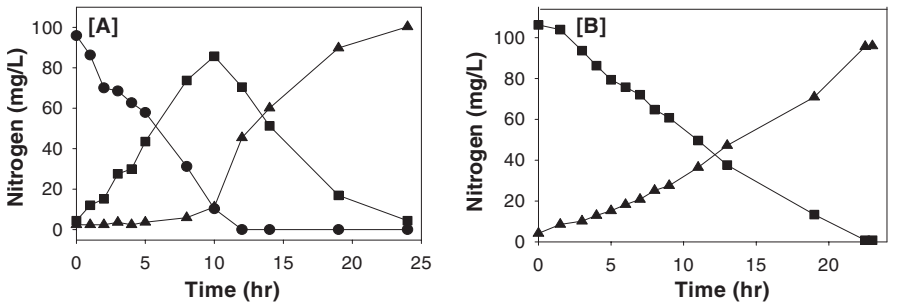

Figure 2 Batch nitrification kinetic analysis of nitrite accumulating biofilm from the biofilm airlift reactor ([A]: ammonia oxidation, [B]: nitrite oxidation, (●: NH_4^+-N; ■: NO_2^--N; ▲:NO_3^--N))

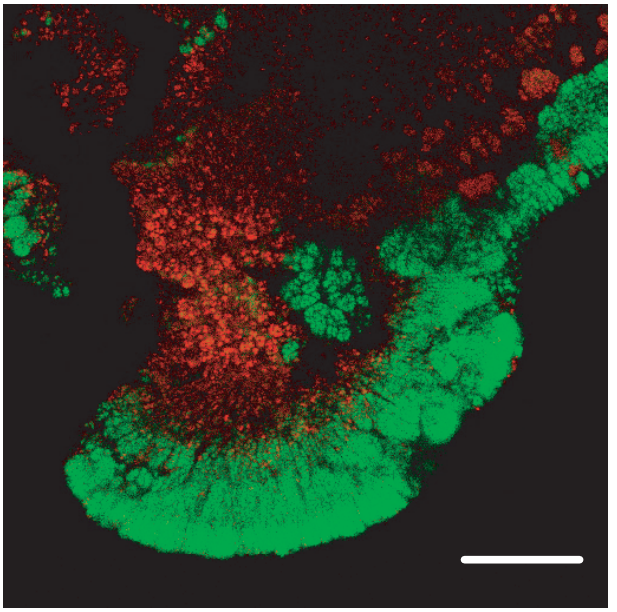

Figure 3 Simultaneous *in situ* hybridization of nitrite accumulating biofilm from the reactor with CY-3 labeled probe Alf 1b and FITC labeled probe Bet 42a. Cells of α-subclass of *Proteobacteria* are red (darker inner region); cells of β-subclass of *Proteobacteria* are green (brighter outer region). Bar = 50 μm

(red) is specific to cells of α-subclass of *Proteobacteria* where nitrite oxidizers belong, and probe Bet42a labeled with FITC (green) is specific to cells of β-subclass of *Proteobacteria* where ammonia oxidizers belong. Figure 3 shows an image of the spatial distribution of ammonia oxidizers and nitrite oxidizers in the nitrite accumulating autotrophic nitrifying biofilm. Densely packed ammonia oxidizers were found outside the biofilm. Most active ammonia oxidizers were present to 20 μm depth from the biofilm surface from the strong green fluorescence signal. In contrast, small clusters of nitrite oxidizers were surrounded by ammonia oxidizers and found in the deeper layer of the biofilm. The segregation of ammonia oxidizers and nitrite oxidizers in the biofilm has also been found in domestic wastewater treatment biofilm and autotrophic nitrifying biofilm where nitrite accumulation has not occurred (Beer *et al.*, 1997; Okabe *et al.*, 1999). The spatial distributions showed that nitrite oxidizers are more exposed to oxygen limiting conditions than ammonia oxidizers.

Conclusion

It has been shown that the biofilm airlift reactor was very effective for nitrification and nitrite accumulation with autotrophic nitrifying biofilm. The reactor obtained more than 98% nitrification efficiencies at up to 3.5 kg NH_4^+-N/m^3·d when sufficient aeration is provided. Nitrite was stably accumulated more than 95% for more than 200 days by selective inhibition on nitrite oxidizers by free ammonia and oxygen limitation. Stable 100% conversion to nitrite could be also achieved even under the absence of free ammonia inhibition on nitrite oxidizers.

Batch ammonium oxidation and nitrite oxidation with nitrite accumulating nitrifying biofilm showed that nitrite oxidation was completely inhibited when free ammonia was higher than 0.2 mg N/L. However, nitrite oxidation activity was recovered as soon as the free ammonia concentration was below the threshold level when dissolved oxygen concentration was not the limiting factor.

Fluorescence *in situ* hybridization analysis of cryosectioned nitrite accumulating nitrifying biofilm showed that the β-subclass of *Proteobacteria*, where ammonia oxidizers belong, was distributed outside of the biofilm whereas the α-subclass of *Proteobacteria*, where nitrite oxidizers belong, was found mainly in the inner part of the biofilm. The spatial distribution showed that nitrite oxidizers are more susceptible to oxygen limiting conditions than ammonia oxidizers.

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