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Selective inhibition of nitrite oxidation by chlorate dosing in aerobic granules

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ABSTRACT

Partial nitrification was successfully achieved with addition of 5 mM $KClO_3$ in the aerobic granules system. Batch tests demonstrated that $KClO_3$ selectively inhibited nitrite-oxidizing bacteria (NOB) but not ammonia-oxidizing bacteria (AOB). During stable partial nitrification, the influent pH was kept at 7.8–8.2, while the DO and temperature were not controlled in the SBR. When the NH_4–N and COD levels were kept at 100 mg/l and 400 mg/l in the influent, the NH_4–N and COD removal efficiencies reached 98.93% and 78.65%, respectively. The NO_2–N accounted for 92.95% of the NO_χ–N (NO_2–N + NO_3–N) in the effluent. Furthermore, about 90% of the chlorate was reduced to nontoxic chloride, thus it would not cause environmental problem. SEM showed that the main composition of the aerobic granules was bacilli and coccus bacteria. FISH analysis revealed that AOB became the dominant nitrifying bacteria, whereas NOB were detected only in low abundance. Chlorate could be used to control the development and maintenance of aerobic granules sludge for partial nitrification.

1. Introduction

Partial nitrification is the oxidation of ammonia to nitrite but not to nitrate, and then the produced nitrite would be removed by the subsequent anaerobic ammonium oxidation (anammox) or heterotrophic denitrification. This new biological nitrogen removal process could save much energy and operational cost, especially in treatment of ammonium-rich or low C/N ratio wastewaters [1,2]. The nitrogen removal via nitrite not only reduces the aeration consumption by 25% during nitrification but also saves the organic matter requirement by 40% and 100% for the subsequent denitrification and anammox process, respectively.

The key of maintaining partial nitrification is to accumulate AOB and inhibit or wash out NOB. Several process control parameters, such as low dissolved oxygen (DO) [3,4], high free ammonia (FA) and free nitrous acid (FNA) [5], short sludge retention time (SRT) [6], high temperature, real-time control [7] and inhibitors, have been used to achieve partial nitrification. Chlorate has not been studied as a parameter for partial nitrification so far. Related experiments about the specificity of chlorate, which selectively inhibited NOB but not AOB, had just been conducted with pure cultures of AOB and/or NOB in culture medium, soil and sediment slurries [8–10].

As a novel wastewater treatment method, aerobic granulation technology has been extensively researched in the sequencing batch reactor (SBR). Compared to conventional activated sludge, aerobic granules characterize as a dense structure, excellent settle ability, high biomass retention and wide diverse microbial species, as well as the high tolerance to medium toxicity and high organic loading rates. Therefore this technology has been applied in treatment of different wastewaters, such as domestic wastewater, high strength wastewater, toxic wastewater and wastewater containing heavy metals or dyes [11,12]. However, in case of treating ammonium-rich or low C/N domestic wastewater, it is necessary to control and investigate partial nitrification process in the aerobic granular sludge system.

Partial nitrification is difficult to achieve by control of low DO in the granular sludge system because of the requirement of high-level aeration. Aeration exerts hydraulic shear stress which plays a crucial role in the granulation and maintenance of sludge granules [13]. In addition, low DO would result in low nitrification rate. Bao et al. [14] indicated that NOB were washed out of the reactor and partial nitrification took place in aerobic nitrifying granules at low temperature (10 °C), whereas the ammonia removal efficiency decreased. FA was also used to cultivate aerobic granules with capacity of simultaneous partial nitrification and organic carbon removal using ammonium-rich synthetic wastewater [15,16]. However, aerobic granules formed only when the concentration of FA was less than 23.5 mg/l, and nitrification was completely inhibited at a FA concentration greater than 10 mg/l [17]. In summary, it is difficult to achieve stable partial nitrification for aerobic granules sludge through these normal control parameters. Therefore, chlo-

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Table 1
Characteristics of mature aerobic granules.

Parameter	SVI (ml/g)	MLSS (mg/l)	VSS (mg/l)	SOUR ($mg\,O_2\,g^{-1}MLSS\,h^{-1}$)	Mean diameter (mm)
Value	31.42	3850	1120	2.57	2.85

rate dosing was considered in this experiment for the development of aerobic granule sludge capable of partial nitrification.

The main objectives of this study were to identify the effectiveness of $KClO_3$ on the cultivation of aerobic granules for partial nitrification and the composition of microbial community in the aerobic granules treating wastewaters with low C/N ratio. Moreover, the chlorate and its products in effluent were also analyzed due to their potential environmental toxicity.

2. Materials and methods

2.1. Synthetic wastewater

NH_4Cl and CH_3COONa were used as nitrogen source and carbon source in the synthetic wastewater, respectively. The synthetic wastewater also contained $CaCl_2$ (45 mg/l), KH_2PO_4 (22 mg/l), $MgSO_4{\cdot}7H_2O$ (25 mg/l), $FeSO_4{\cdot}7H_2O$ (20 mg/l), EDTA (20 mg/l), $FeCl_3{\cdot}6H_2O$ (1.5 mg/l) and trace elements: H_3BO_3 (0.15 mg/l), $CuSO_4{\cdot}5H_2O$ (0.03 mg/l), KI (0.03 mg/l), $Na_2Mo_7O_{24}{\cdot}2H_2O$ (0.15 mg/l), $CoCl_2{\cdot}6H_2O$ (0.058 mg/l), and $ZnCl_2$ (0.12 mg/l). The influent also contained around 400 mg/l COD and 100 mg/l NH_4–N with a low C/N ratio ($\leq$4). $NaHCO_3$ was used as buffering agent and potential inorganic carbon source for the nitrification process. The pH of the influent was set at 7.8–8.2.

2.2. Experimental design

Aerobic granules were cultivated in an internal-cycle sequencing batch reactor (SBR; working volume of 2.5 l; 100 cm in height and 8 cm in diameter for down-comer; 70 cm in height and 4 cm in diameter for riser) at room temperature. The air was introduced from the bottom of the reactor through a fine-bubble diffuser. Effluent was discharged at the middle part of the reactor, thus the liquid exchange ratio was about 50%. The reactor was operated sequentially in 6-h cycles, each composed of four periods: influent filling (2 min)), aeration (355 min), settling (2 min)) and effluent withdrawal (1 min)). In addition, in order to provide high hydraulic shear stress and high level of DO, the superficial upflow air velocity in the riser was controlled at around 1.8 cm/s.

The reactor was inoculated with activated sludge taken from Wastewater Treatment Plant of Lingshui River (Dalian, China). It took 85 days for the cultivation of mature aerobic granules and their characteristics were shown in Table 1.

2.3. Batch experiments

In order to examine the effect of different concentrations of chlorate on a granular sludge system, batch experiments were performed in glass bottles with 200 ml working volume. Condition similar to that in the SBR was applied for the batch experiments. Mixed liquor suspended solids (MLSS, about 3800 mg/l), DO (above 9 mg/l), room temperature and reaction time (6 h for one cycle) were kept nearly the same in every bottle. The initial medium was kept the same as that in the SBR except $KClO_3$ concentrations (1, 5 and 10 mM). Besides, the NH_4–N and COD were kept at 50 mg/l and 250 mg/l, respectively. Aeration was performed from the bottom of the bottles through small diffusers.

2.4. Analytical methods

MLSS, SVI and concentrations of the effluent nitrogen compounds and COD were analyzed by standard methods [18]. pH was measured with a digital pH meter-muti 340i (WTW Company, Germany). DO was detected with an YSI-55 Dissolved Oxygen Meter (USA). Chloride was titrated with standard silver nitrate solution. Chlorate and chlorite were measured by the five-step iodometry method [19].

2.5. Scanning electron microscope detection

Microbial morphology and structure of granules were observed using scanning electron microscope (SEM). The granule samples were fixed with 2.5% glutaraldehyde for 3 h and then rinsed in 0.1 M phosphate buffer (PBS, pH 7.2) for 3 times. Subsequently, the samples were dehydrated with series of ethanol (25%, 50%, 75%, 90% and 100%). After rinsed 3 times with tert-butyl alcohol, the dewatered samples were gold-coated by a sputter and then observed with SEM (JEOL JSM-5600LV, Tokyo, Japan).

2.6. Fluorescent in situ hybridization analysis

Samples fixation and hybridization steps were carried out according to the standard hybridization protocol [20]. The treated granules were cut into 20 μm thick slices at −20 °C with a cryostat. The following 16S rRNA-targeted oligonucleotide probes fluorescently labeled with a hydrophilic sulfoindocyanine dye (Cy-3) or fluorescein isothiocyanate (FITC) (TAKALA Company, Dalian, China) on the 5′ end were used for in situ detection of the bacteria (Table 2).

3. Results and discussion

3.1. Effect of chlorate on the partial nitrification in batch test

The ammonium oxidation to nitrite was not inhibited severely by chlorate, but the nitrite oxidation to nitrate was completely blocked by chlorate. Fig. 1a shows that the NH_4–N removal efficiency reached 98.72% with addition of 1 mM chlorate, which was similar to that (98.17%) in the control bottle (with no $KClO_3$ addition). In the other two bottles with 5 mM and 10 mM $KClO_3$, the NH_4–N removal efficiencies just reached 78.75% and 72.45%, respectively. The NO_2–N oxidation to NO_3–N was completely inhibited and no NO_3–N was detected with chlorate additions (1, 5, and 10 mM), whereas the accumulated NO_3–N (19.15 mg/l) accounted for 80.60% of the produced NO_χ–N without $KClO_3$ addition. The result was consistent with that obtained by Lees and Simpson [8], who indicated that 10^{-3} to 10^{-2} M $KClO_3$ completely blocked the oxidation of nitrite. Similarly, Belser and Mays [9] also demonstrated that 10 mM $KClO_3$ had no effect on the

Table 2
Probes for the FISH detection.

Probe	Dye	Specificity	Reference
NSO190	Cy3	β-Proteobacterial AOB	[21]
Nsm156	Cy3	*Nitrosomonas* spp.	[21]
Nsv443	Cy3	*Nitrosospira* spp.	[21]
Nit3/CNit3	FITC	*Nitrobacter* spp.	[22]
Ntspa662	FITC	*Nitrospira* spp.	[23]

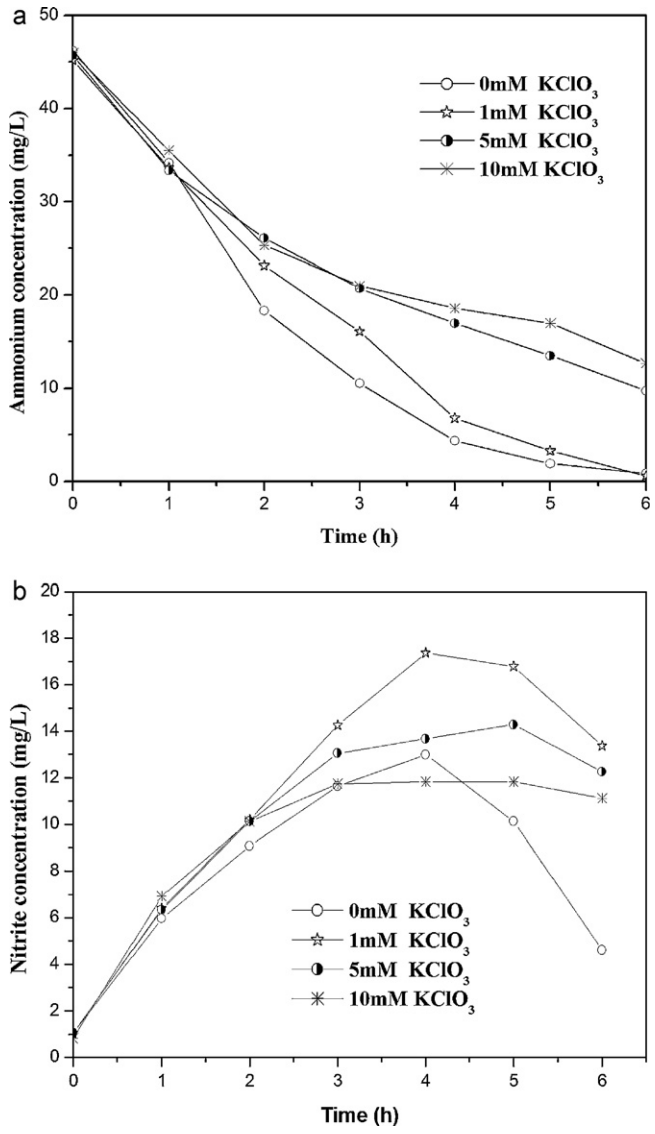

Fig. 1. Profiles of NH_4–N and NO_2–N during one cycle in the batch experiment. (a) Profile of effluent NH_4–N; (b) profile of effluent NO_2–N.

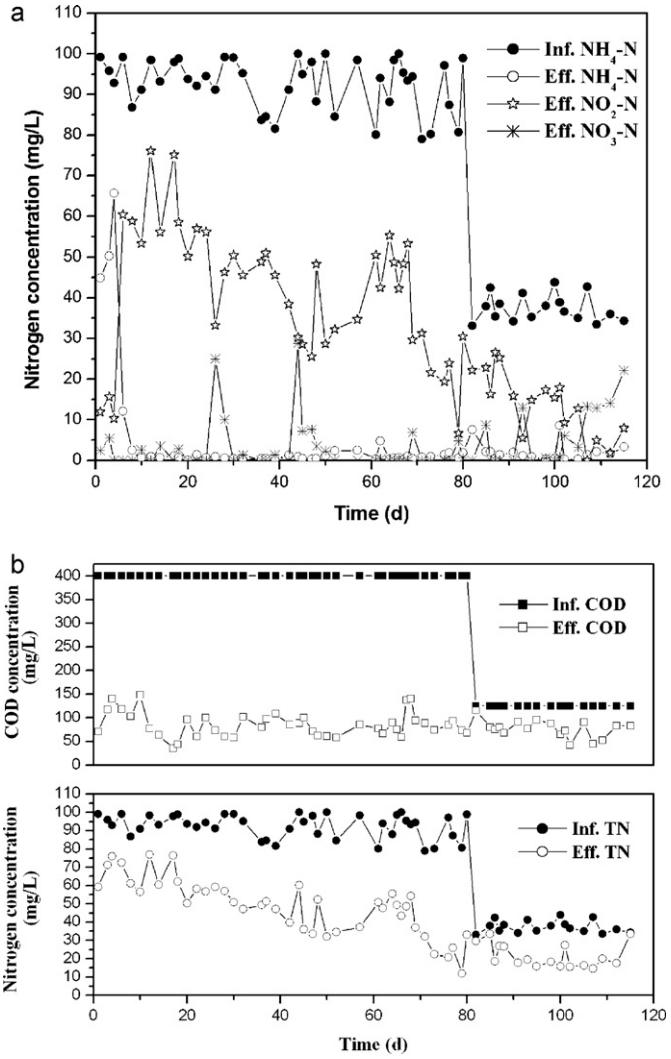

Fig. 2. Profiles of nitrogen compounds and COD during stable partial nitrification process. (a) Time courses of influent NH_4–N and effluent NH_4–N, NO_2–N and NO_3–N; (b) variations of TN and COD concentrations in the SBR reactor.

activity of AOB, but it would completely block the activity of NOB.

High concentration of chlorate not only prevented the NO_2–N oxidation but also reduced the NH_4–N oxidation rate in the batch test. At the end of the reaction, more than 20% of the NH_4–N left in bottles with 5 mM and 10 mM $KClO_3$ additions. The AOB in the tested bottle with 10 mM $KClO_3$ were inhibited more seriously than those in the others. Correspondingly, the maximum concentration of NO_2–N (11.88 mg/l) accumulated in this bottle was less than those (17.37 and 14.29 mg/l) in the bottles with 1 mM and 5 mM $KClO_3$ (Fig. 1b). It demonstrated that high concentration of $KClO_3$ inhibited both NOB and AOB in the short time. Results proposed by Hynes and Knowles [10] proved that the oxidations of both NH_4–N and NO_2–N were inhibited in the presence of 10 mM chlorate after 2-h lag period, during which time *N. winogradskyi* reduced ClO_3^- to ClO_2^- and the undetected ClO_2^- inhibited the mixed *N. europaea* and *N. winogradskyi* cell suspensions.

The NO_2–N removal (including nitrite oxidization and denitrification) was inhibited with the increase of $KClO_3$. In the control experiment, the NO_2–N level started to decrease sharply from the 4th hour (Fig. 1b), due to further oxidation to NO_3–N and denitrification. However, with 1 mM $KClO_3$ addition, the NO_2–N removal from the 4th hour was attributed mainly to denitrification in the case of the inhibition on nitrite oxidation, resulting in a slower removal rate. It illustrated that denitrification via nitrite still occurred with 1 mM $KClO_3$ addition. Fig. 1b also shows that, the NO_2–N level almost remained unchanged from the 3rd hour with 10 mM $KClO_3$ addition, which indicated that high concentration of chlorate inhibited both nitrite oxidation and denitrification.

The above results showed that partial nitrification and denitrification via nitrite could occur simultaneously with suitable chlorate addition in the aerobic granule sludge. 1–5 mM $KClO_3$ additions had less impact on ammonium oxidation and nitrite denitrification in the granule sludge, but apparently inhibited further oxidization of NO_2–N. Accordingly, it is possible to successfully achieve simultaneous partial nitrification and denitrification by suitable chlorate addition.

3.2. Continuous performance of nitrogen removal and stability of partial nitrification by chlorate dosing in the SBR

Based on the results of batch experiments, 1 mM $KClO_3$ was chosen to incubate aerobic granules capable of partial nitrification in the SBR. After 2 weeks, almost no NO_2–N was detected, thus the dosage of $KClO_3$ was adjusted to 5 mM, and day 1 in Fig. 2 represents the first day when adding 5 mM $KClO_3$.

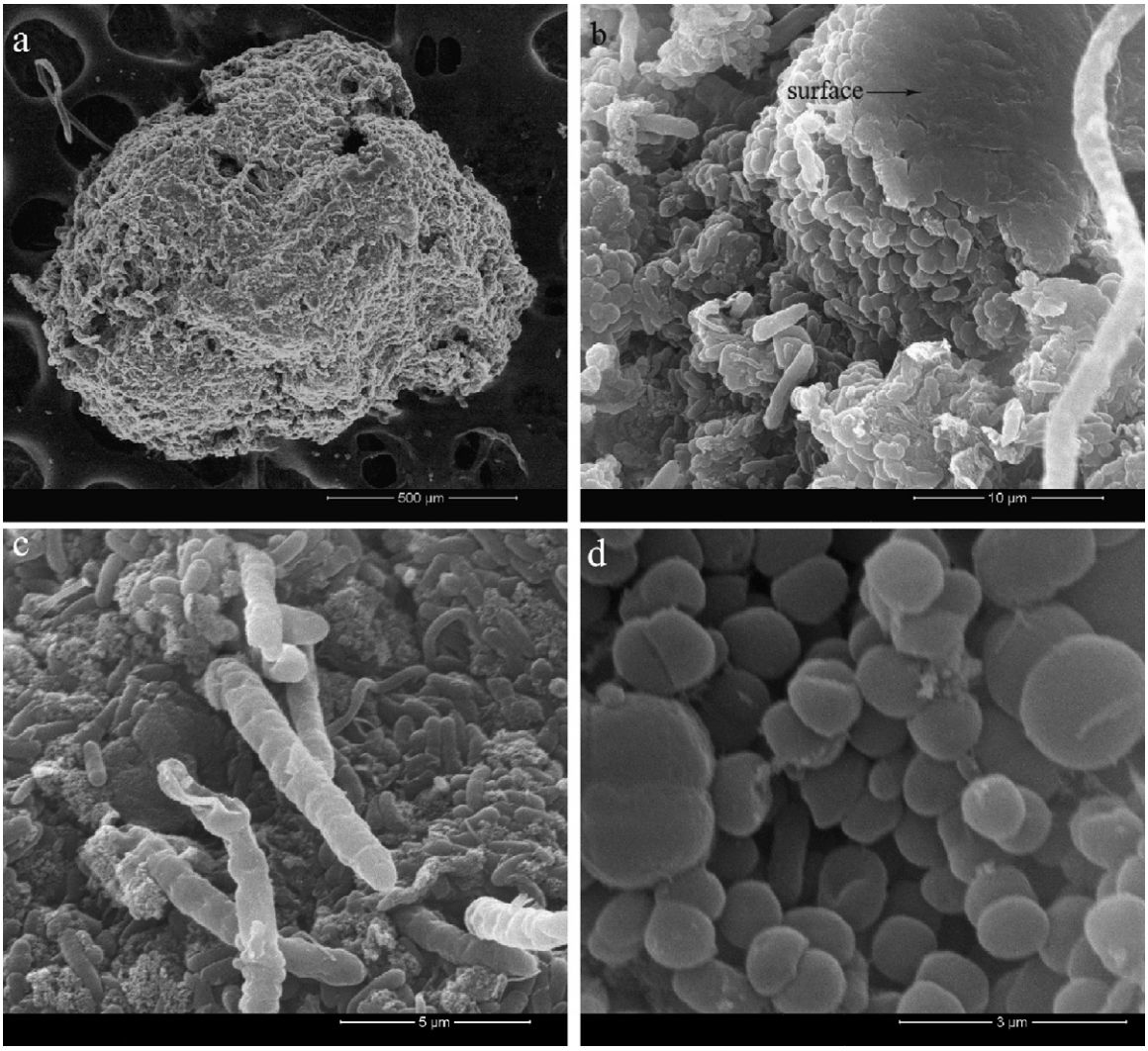

Fig. 3. SEM images of the aerobic granules. (a) Full-profile of the sludge; (b) cross-profile of the sludge; (c) surface of the sludge; (d) interior of the sludge.

Before $KClO_3$ dosing, complete nitrification process was carried out within a pH range of 7.8–8.2 and without any temperature or DO (above 9 mg/l) control. NO_3–N was the dominant product in the effluent. No parameters were changed in the following experiment except the $KClO_3$ addition. Nitrification was deteriorated but nitrite accumulation was observed immediately with the $KClO_3$ dosing. During the first 3 days, 5 mM KClO3 was directly added into the influent. On day 3, the effluent NO_2–N and NO_3–N levels reached 15.58 mg/l and 5.49 mg/l, respectively. Afterwards, the $KClO_3$ was directly dosed into the reactor at a level of 5 mM every 8–20 cycles. On day 6, the effluent NO_2–N increased to 60.37 mg/l, whereas NH_4–N decreased to 12.12 mg/l and no NO_3–N was detected. The ammonia oxidation was increased quickly by the intermitted dosing strategy of chlorate. From then on, the oxidation of NH_4–N was maintained at a high level. When the NO_3–N was reduced by nitrite oxidation inhibition, the average NO_2–N/NO_χ–N was near to 100% during the start-up of the partial nitrification (Fig. 2a). Based on these results, the activity of NOB was inhibited thus the oxidation of NO_2–N to NO_3–N was terminated. $KClO_3$ dosing was beneficial for NO_2–N accumulation.

Long-term monitoring of partial nitrification was conducted with $KClO_3$ dosing. Fig. 2a also shows that the NH_4–N removal was not inhibited by $KClO_3$. During the first 81 days, the concentrations of influent NH_4–N and COD were set at 100 mg/l and 400 mg/l, respectively. The NH_4–N removal efficiency reached about 98.93% and the NO_2–N accounted for 92.95% of the effluent NO_χ–N. Furthermore, very little NO_3–N was produced in most cases.

Meanwhile, the TN and COD removal efficiency in this granular sludge reached 47.23% and 78.65%, respectively (Fig. 2b). These COD and TN removals clearly indicated that chlorate did not inhibit the growth of heterotrophic anaerobes and denitrifiers.

During the last month, even though the influent NH_4–N and COD were adjusted to 35 mg/l and 125 mg/l, respectively, similar results were obtained. However, the accumulated NO_2–N reduced to 70.57% of the effluent NO_χ–N. With the decrease of nutrient load, the granules began to disintegrate from day 113. The sludge disintegration might be caused by the superfluous growth of filamentous owing to low pollutant load [24].

3.3. Robustness of partial nitrification conducted by chlorate dosing in SBR

In order to investigate the robustness of partial nitrification established by chlorate dosing in the SBR, the nitrogen removal process with no chlorate dosing was examined. 5 mM $KClO_3$ was added into the influent for 3 days and stabilized partial nitrification occurred immediately. From then on, no chlorate was added into the influent, but partial nitrification could still be sustained. During this period, more than 99% of the NH_4–N was removed and the product was NO_2–N but not NO_3–N (data not shown). Most

of the chlorate-sensitive NOB were inactivated and washed out of the reactor. Though the chlorate dosing was not persistent, the NOB populations increased slowly because of their small bacterial base. However, from day 25, as rapid growth of NOB, the NO_3–N accumulation gradually increased. Finally, partial nitrification was shifted to complete nitrification. Though long-time partial nitrification could be maintained by chlorate dosing, the inhibition of nitrite oxidation would disappear easily in the absence of chlorate. Consequently, regular chlorate dosing was necessary to maintain partial nitrification in the aerobic granular system.

3.4. Ecotoxicity analysis of chlorate

Chlorate is toxic to the fungi, algae, invertebrate, fish, and higher plants [25,26], but it is easily biodegraded by nitrate reductase in the organic-rich environment. In this experiment, the ecotoxicity of chlorate was also considered. Chlorate and its products were measured, but only chlorate and chloride could be detected in the effluent. After 6 h reaction, more than 90% of the chlorate was reduced to chloride, and the residual chlorate was kept about 0.6 mM. However, the level of effluent chlorate might occasionally reach to 1.5 mM owing to the absence of chlorate-reduced bacteria. Previous research showed that undetected chlorite was the supposed toxic compound rather than its reactant chlorate [27]. However, it was not confirmed that it was just the chlorite inhibited nitrite oxidation. The chlorite would inhibit both NOB and AOB, but only inhibition of nitrite oxidation was detected in this experiment. Therefore, it might be the chlorate itself inhibited the nitrate accumulation because it was a competitive substrate of nitrate in the nitrogen catabolism, and then nitrogen starvation would take place.

There was no chlorite in the effluent, so it would not bring any toxicity to the environment or the bacteria in the subsequent wastewater treatment processes. To eliminate the potential toxicity of effluent chlorate, some measures could be taken to reduce residual chlorate to non-toxic chloride in the case that a small amount of chlorate was detected. Appropriate amounts of reductant could be added into the reactor to fully eliminate potential pollution when the nitrification process ended, because pH would decrease during this period and chlorate is easily reduced to chloride by the nitrate reductase in the condition of weak alkaline or acid [28].

3.5. Effect of chlorate on sludge morphology and community

The NOB growth was blocked by chlorate and the biomass decreased. The average MLSS decreased from 3850 mg/l to 2850 mg/l in the first week. With the chlorate-insensitive bacteria growth, the MLSS gradually recovered over 3500 mg/l. The median aggregate size changed a little and returned quickly to above 2.8 mm.

SEM examination was conducted to observe the morphology and microstructure of aerobic granules on day 60. The apparent structures of granules sludge were not affected by the addition of $KClO_3$, except that a few of them were broken into pieces. The aerobic granules sludge had a very compact structure initially, but the granules structure became a little looser after the addition of chlorate. It might be ascribed to the decrease of the NOB biomass in the granules. Fig. 3 shows that the aerobic granules were mainly consisted of bacilli and cocci, as well as a lot of micropores. These pores could enhance substrate and metabolic products transfer between the bulk and the granules.

Sludge samples were taken for FISH detection to evaluate microbial composition of the granules on the 80th day. Hybridizing cells with Cy3-labeled probes, including NSO190 specific for β-proteobacterial AOB, Nsm156 specific for *Nitrosomonas* spp. and Nsv443 specific for *Nitrosospira* spp., produced strong red signals (Fig. 4a). The green signal of bacteria hybridized with two FITC-labeled probes, including Nit3 specific for *Nitrobacter* spp. and Ntspa662 specific for *Nitrospira* spp., was quite weak (Fig. 4b). It clearly indicated that AOB became the dominant nitrifying bacteria. The NOB were severely inhibited by chlorate and most of them were washed out of the granules system. Thus, the nitrite accumulation would not been obviously effected by the residual NOB in short term. However, nitrite oxidation would gradually revive in the absence of chlorate.

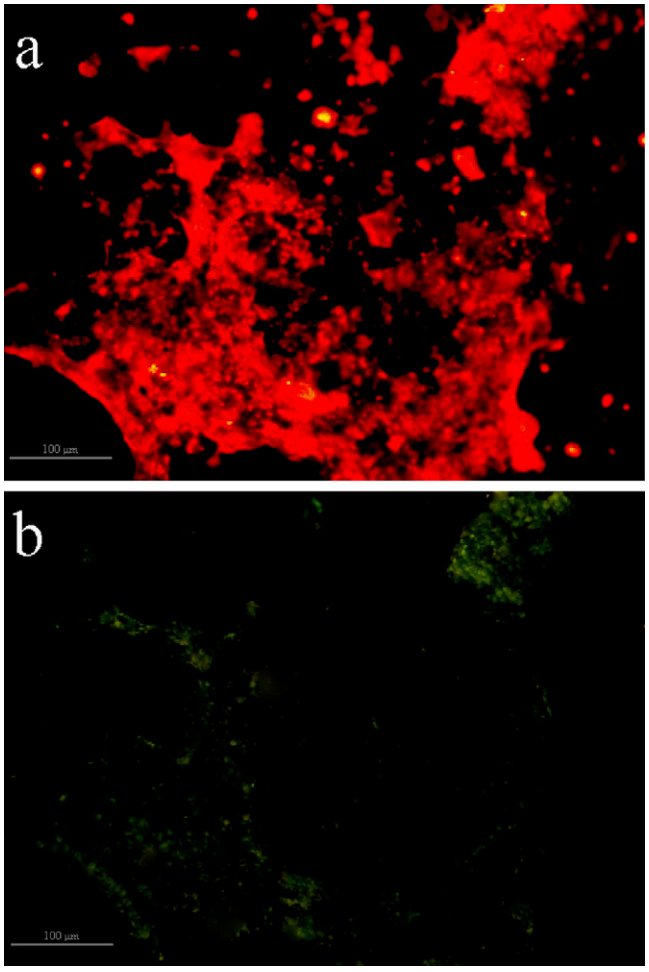

Fig. 4. In situ identification of nitrifying bacteria distribution in the aerobic granules. (a) Image of the AOB cells hybridized with Cy3-labeled probes (red) NSO190, Nsm156, Nsv443; (b) image of the NOB cells hybridized with FITC-labeled probes (green) Nit3 and Ntspa662. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

4. Conclusions

In this study, stable partial nitrification/denitrification was successfully achieved and maintained by $KClO_3$ dosing in the aerobic granular system. The chlorate selectively inhibited the activity of NOB but not AOB, so nitrite became the dominant product. Moreover, most of the chlorate was translated to nontoxic chloride, thus poisonous effect would not be produced on the bacteria of the following process or the water bodies. Since a few of chlorate-sensitive bacteria, mainly including NOB, were inhibited and washed out of the SBR, partial nitrification in this aerobic granule system were developed and maintained for a long period.

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