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Effect of nitrate concentration on filamentous bulking under low level of dissolved oxygen in an airlift inner circular anoxic-aerobic incorporate reactor

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Abstract
This laboratory research investigated a possible cause of filamentous bulking under low level of dissolved oxygen conditions (dissolved oxygen value in aerobic zone maintained between 0.6–0.8 mg O₂/L) in an airlift inner-circular anoxic-aerobic reactor. During the operating period, it was observed that low nitrate concentrations affected sludge volume index significantly. Unlike the existing hypothesis, the batch tests indicated that filamentous bacteria (mainly Thiothrix sp.) could store nitrate temporarily under carbon restricted conditions. When nitrate concentration was below 4 mg/L, low levels of carbon substrates and dissolved oxygen in the aerobic zone stimulated the nitrate-storing capacity of filaments. When filamentous bacteria riched in nitrate reached the anoxic zone, where they were exposed to high levels of carbon but limited nitrate, they underwent denitrification. However, when nonfilamentous bacteria were exposed to similar conditions, denitrification was restrained due to their intrinsic nitrate limitation. Hence, in order to avoid filamentous bulking, the nitrate concentration in the return sludge (from aerobic zone to the anoxic zone) should be above 4 mg/L, or alternatively, the nitrate load in the anoxic zone should be kept at levels above 2.7 mg NO₃-N/g SS.

Key words: anoxic-aerobic system; low dissolved oxygen; filamentous bulking; low nitrate concentration; nitrate-storing capacity

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Introduction

Problems related to bulking sludge are commonly reported in biological nutrient removal (BNR) systems (Eikelboom et al., 1981; Jenkins et al., 1993). This has been estimated to affect at least 60% of activated sludge plants in the USA, resulting in 50% of plants failing to consistently meet effluent discharge standards (Michael, 2003). Over 30 years, the causes and controls of filamentous bulking have been extensively studied (Marten and Daigger, 1997; Ekama et al., 1996; Kruit et al., 2002), since Eikelboom and van Buijsen (1981) first published their identification guide to filamentous bacteria. It has been widely accepted that filamentous bulking does not completely depend on BNR conditions, but may be caused by other overlooked factors. Many operating parameters in BNR systems, such as low levels of dissolved oxygen (DO) (Guo et al., 2010, 2012; Martins et al., 2003b; Tian et al., 2011) and low food/microorganism (F/M) values (Casey et al., 1994, 1999) have been extensively investigated.

Apart from the two factors mentioned above, the presence of nitrate and/or nitrite at the time of transitions between anoxic and aerobic conditions may also have an influence on filamentous bulking, and this aspect has not yet received sufficient attention. Some studies have focused on the association between filamentous microorganism proliferation and nitrate/nitrite levels at the time of transition from anoxic to aerobic conditions. filamentous bulking is induced and ameliorated in laboratory BNR systems with high (> 5 mg) and low (< 1 mg NOx-N/L) concentrations of nitrate or nitrite during the transition from anoxic to aerobic conditions (Lakay et al., 1999; Musvoto et al., 1999). In some other studies, when nitrate and/or nitrite were present at the transition from anoxic to aerobic conditions, episodes of poor sludge settlement have been observed several times (Beeharry et al., 2001; Cronje et al., 2000).

Although it has received some attention in literature that nitrate and/or nitrite’s presence at the time of transition from anoxic to oxic conditions would influence the proliferation of filamentous bacteria, the possibility that nitrate’s presence at the time of transition from aerobic
to anoxic conditions might affect filamentous proliferation under low DO condition has acquired even less attention. Several recent reports (Ma et al., 2009; Tian et al., 2011; Zeng et al., 2010; Musvoto et al., 1999) have shown that the value of the sludge volume index (SVI) increases with the decrease of nitrate concentration within certain periods of time. Therefore, there is a stable relationship between SVI and the content of filaments (Schuler and Jassby, 2007; Gulez and De Los Reyes, 2009). It might be an appealing strategy for controlling filamentous bulking via controlling the nitrate concentrations during the transition from aerobic to anoxic conditions. However, up to now, research of the effect of nitrate concentration on filamentous bulking under low level of DO is scarce, and a comprehensive study about this effect will lend new insight into the design of wastewater treatment systems and the operation of wastewater treatment plant systems that want to minimize aeration costs.

The objective of this study is to demonstrate that low nitrate concentrations would affect sludge settle ability greatly in a laboratory-scale airlift inner circular anoxic-aerobic reactor. Batch tests were performed during the operating period to investigate the sludge’s denitrification. The mechanism responsible for the filamentous bulking was also explored.

1 Materials and methods

1.1 New airlift inner circular anoxic-aerobic reactor

This investigation involved the use of a laboratory-scale airlift inner circular anoxic-aerobic reactor with the reaction volume being 10 L (Fig. 1). The reactor is derived and modified from the wastewater treatment Bioreactor (Zhao et al., 2006). The reactor was inoculated with mixed liquor that was obtained from a secondary clarifier from the Quyang Wastewater Treatment Plant (anoxic/oxic) (Shanghai, China). The feed flow rate was controlled by a pump to achieve 9 hr hydraulic retention time, while the aeration rate was controlled by an airflow meter to retain DO concentration at 0.6–0.8 mg O$_2$/L. Temperature in the reactor was kept at (20 ± 1)°C by means of a heater and thermostat. The sludge retention time was maintained at 12–15 days by wasting an appropriate amount of settled sludge. The mixed liquor suspended solid concentration was 3000 mg/L, to achieve the value of food/microorganism at 0.13 g COD/g suspended solid.

1.2 Batch tests for exploring the mechanism of filamentous bulking

With the exception of the third test, the sludge used in batch tests was collected from the parent reactor. Firstly, a 1.5 L mixture was collected from the aerobic zone and centrifuged; the supernatant fluid was discarded before adding 1.5 L synthetic wastewater that did not contain any C, N, P or DO. Secondly, this new mixture was immediately filled to an airtight column container and a certain amount of sodium acetate was added to provide a shock concentration of HAc (0.185 g CH$_3$COONa). During the 2 hr anaerobic stage, a sample was collected every 15 min during the first hour, after which samples were taken every 30 min until the end. After the completion of the anaerobic stage, a certain volume of nitrate solution was added to create an anoxic condition (0.091 g NaNO$_3$). For batch tests 1 and 2, samples were taken every 15 min within the first 30 min of anoxic stage, followed by every 30 min until the third hour. For batch tests 3 and 4, additional three samples were collected at the eighth, twelfth and eighteenth hours. The concentrations of HAc, phosphorus, nitrate, intracellular nitrate, nitrite and mixed liquor suspended solids were monitored. For verifying tests, another two laboratory-scale reactors were seeded with the same sludge but cultivated with different nitrate concentrations in influent (reactor a, with 5 mg N/L; reactor b, without nitrate). However, the concentration of total nitrogen (TN) was kept at 40 mg N/L through changing NH$_4^+$-N content. Other components in influent and operating parameters were the same as those in Section 1.1.

1.3 Synthetic wastewater composition

The novel reactor was supplied with synthetic wastewater containing sodium acetate (125 mg/L), glucose (250 mg/L), ammonium chloride (36 mg NH$_4^+$-N/L), sodium nitrate (4 mg NO$_3^-$-N/L) and KH$_2$PO$_4$ (10 mg PO$_4^{3-}$-P/L). Other components consisted of MgSO$_4$-7H$_2$O (150 mg/L), CaCl$_2$-2H$_2$O (35 mg/L), EDTA (5 mg/L) and trace elements (1 mL/5 L). The composition of the trace element solution was composed as that described by Martins et al. (2003a).

Fig. 1 Schematic structure of airlift inner circular anoxic-aerobic reactor.
1.4 Analytical methods

The DO was measured by a WTW oxi340i oxygen probe (Germany). Chemical oxygen demand (COD), mixed liquor suspended solids, mixed liquor volatile suspended solids, SVI, and all nitrogen-containing compounds were measured according to standard methods (APHA, 1998). In order to measure intracellular nitrate concentration, several freeze-thaw cycles in liquid nitrogen were applied to break the cells. In addition, PO₄³⁻-P concentrations were analyzed according to Guo et al. (2010), and acetate content was measured by gas chromatography, according to Smolders et al. (1994). Olympus CX31-32C02 was employed to do microscopic examination.

1.5 PCR-DGGE analysis of the filamentous bacteria in activated sludge

Bacterial genomic DNA of activated sludge was first extracted as following steps: First, samples collected from the reactor were centrifuged and the supernatant was removed. Second, the settling was washed by sterile water and the mixture was recentrifuged. Third, the Fast DNA Spin Kit for soil (QBIogene, Carlsbad, USA) was used to extract total DNA from approximately 0.3 g settling.

The 16S rRNA genes from the mixed bacterial DNA were PCR-amplified with the primer set of 8f and 1492r as described by Bossard et al. (2000), and the 16S rDNA variable V3 region of extracted DNA was amplified with primers 341f with a GC-clamp and 534r according to the methods of Muyzer et al. (1993). PCR-amplification was also carried out according to the methods of Muyzer et al. (1993)

The PCR products were electrophoresed on 8% polyacrylamide gel with gradients ranged from 40% to 60% (100% denaturant: 7 mol/L urea and 40% (V/V) deionized formamide) in 1X TAE buffer at a constant voltage of 100 V for 10 hr at 60°C using a Dcode Universal Mutation Detection System (BioRad). After electrophoresis, DNA was stained with ethidium bromide and viewed with a BioRad Gel Documentation system. Bright bands were then excised from the gel and cleaned. Then the covered DNA was reamplified, purified, cloned top MD19-Tvector (TaKaRa, Japan) and sequenced via an ABIPRISM3730 automated DNA sequencer (Applied Biosystems, USA). The sequence from this study had been submitted to the GenBank data base under accession number JX178709, and the closest matching sequence was analyzed according to the result of Martins et al. (2003b). In addition, the 16S rDNA sequence from GenBank was FJ750467.1, and the identity was 100%, and it existed during the whole bulking period. Thus it was likely that the proliferation of *Thiothrix* sp. caused the increase of SVI, and this result was consistent with the result of Martins et al. (2003b).

1.6 Statistical analysis

All tests were performed in triplicate, and analysis of variance was used to test the significance of results and $p < 0.05$ was considered to be statistically significant.

2 Results and discussion

2.1 Performance of integrated nutrient removal by the reactor and the relationship between nitrate concentration and SVI

During more than two months’ operation period, the values of SVI and the removal rates of COD, TN, TP and NH₄⁺-N were recorded (Fig. 2). The entire operation process is divided into three parts, namely start, mid-run and end of run. Figure 2 illustrates the maintenance of the relatively high integrate nutrient removal rates under the condition of SVI values less than 300 mL/g, with the removal rates of COD, TN, TP and NH₄⁺-N at approximately 90%, 80%, 70% and 98% respectively. Furthermore, integrate nutrient removal rates within the limited bulking period were somewhat higher than those in normal period. The results were in line with other reports (Ma et al., 2009; Zeng et al., 2010; Tian et al., 2011). However, when the SVI value exceeded 300 mL/g, pollutant removal efficiency declined accordingly due to the marked proliferation of filamentous bacteria (Fig. 2). Moreover, according to the result of PCR-DGGE (Fig. 3), the newly appeared band 1 in sample S2 and S3 was *Thiothrix* sp. (the closely related sequence from GenBank was FJ750467.1, and the identity was 100%), and it existed during the whole bulking period. Thus it was likely that the proliferation of *Thiothrix* sp. caused the increase of SVI, and this result was consistent with the result of Martins et al. (2003b). In addition, the microscope images of nonbulking and bulking sludge in Fig. 4 also were in line with the DGGE result.

During the entire operation process, a strong association between nitrate concentration and filamentous bulking was also observed (Fig. 5). It was obvious that the values of SVI increased when nitrate concentrations in the aerobic zone fell below 4 mg/L (the dash line in Fig. 5), where nitrite in the reactor was beyond the detection limit, the phenomenon of which appeared four times (Fig. 5). Nev-
Nevertheless, the SVI values remained relatively stable while nitrate concentration was above 4 mg/L. This indicates that excellent sludge settleability could be maintained as long as nitrate concentration kept above certain level, such as 4 mg/L. In conclusion, the optimal nitrate concentration in the aerobic zone is decided to be slightly higher than 4 mg/L in a continuous-flow reactor with a completely mixed mode when operated under low DO conditions.

It was not accidental that a remarkably relationship was noted between nitrate concentration and SVI. A similar relationship between SVI and nitrate concentration has been observed in previous studies (Ma et al., 2009; Zeng et al., 2010; Tian et al., 2011). Currently the most widely-accepted theory explaining the morphology and ecology of bulking sludge is that, under non-bulking operation conditions, filamentous organisms grow inside the sludge floc, forming the backbone of the activated sludge aggregates (Krhutková et al., 2002). However, under conditions of low substrate availability, they would gain easy access to the limited substrate because of their preferential growth in one or two directions (Martins et al., 2003). It can therefore be assumed that filamentous organisms are also present in well-settled sludge and the uncontrollable proliferation of such organisms is mainly due to their morphological advantage stimulated and enlarged by unexpected continuously substrate-limited conditions. When nitrate concentration dropped below 4 mg/L, for whatever reason, a substrate-limited condition was created in the reactor. The reason that filamentous bacteria proliferate under low nitrate concentration condition will be further discussed in detail in the following sections. Nevertheless, with the removal of the stimulating condition, the growing advantage of the filamentous bacteria was simultaneously
lost, thus, the SVI value stopped increasing.

2.2 Exploring the mechanism of bulking sludge under low nitrate concentration conditions

To investigate the mechanisms underlying the association between nitrate concentration and filamentous bulking, anaerobic-anoxic batch tests with insufficient (batch 1) and sufficient (batch 2) carbon substrate were performed. After incubating with acetate anaerobically, the sludge (SVI of 200 mL/g) was exposed to anoxic conditions. For batch 2, it was given another spike of acetate at the beginning of the anoxic phase (0.095 g CH$_3$COONa). The results, summarized in Fig. 6, indicated that the different quantities of carbon source at the initial anoxic phase had different influences on the denitrification performance of the bulking sludge. During the anoxic stage, the difference between batch 1 and 2 was significant. In batch 2, nitrate was fully denitrified, with the additional acetate acting as an electron donor. However, in batch 1, not only was denitrification incomplete because of the lacking of an electron provider, but also occurred an unusual phenomenon, that was, nitrate was released twice. With these two specific phases (i.e., 2–2.25 hr and 4.5–5 hr, Fig. 6a), very little consumption of nitrite was observed, thus excluding the transforming of nitrate from nitrite. Therefore, the first release may have derived from the release of excessively absorbed nitrate, at the initial anoxic stage, by non-filamentous bacteria. According to the chemiosmotic theory (Peter, 1961), these bacteria produce a certain number of electrons during the synthesis of polyhydroxyalkanoates in the anaerobic stage. Once nitrate appears in the mixture, a large amount of nitrate would be immediately transported across the membrane. This theory was clearly affirmed by the big difference between theoretical (10 mg N/L) and actual (5 mg N/L) values of nitrate at the initial anoxic stage. The filamentous bacteria might also contribute to this discrepancy due to their absorbance of nitrate under low nitrate conditions, competing with nonfilamentous denitrifiers. Nonetheless, during the subsequent lacking of electron donors, the absorbed nitrate could not be denitrified and was thus released. In the opinion of the author, the second increase was mainly caused by the filamentous bacteria, which is to be discussed in more detail.

To explore the fundamental cause of the second increase (Fig. 6a), another series of tests were conducted. Batch tests (3 and 4) were set up for a long experimental time, up to 18 hr. The denitrification performances of wastewater treatment plant sludge (SVI 90 mL/g) and serious bulking sludge (SVI 450 mL/g) are illustrated in Fig. 7. During the anaerobic stage, acetate was taken up and transformed into polyhydroxalkanoates by bacteria such as polyphosphate accumulating organisms, glycogen accumulating organisms, or by other denitrifiers in the nonbulking sludge, with very little amount or no acetate utilized by filamentous bacteria in the bulking sludge. Moreover, the other main difference between batch 3 and 4 was identified related to their performance under anoxic conditions. In batch 3 (Fig. 7a), nitrate could be fully denitrified while in batch 4 (Fig. 7b) it could not be fully denitrified. What is more, the maximum increment of intracellular nitrate of two different sludges existed a large discrepancy. The intracellular nitrate content of nonbulking sludge increased by 1.43 mg N/g SS while it of bulking sludge increased by 6.1 mg N/g SS. It was much likely that filamentous bacteria had a nitrate-storing capacity. In batch 4, with the decrease of the acetate concentration to less than 10 mg/L, the nitrate concentration dropped from 7.2 to 1.7 mg N/L within half an hour. Meanwhile, intracellular nitrate content increased from 4.6 to 5.8 mg N/g SS. However, when the acetate concentration was above 10 mg/L, nitrate concentration only fell by 3.4 mg N/L and intracellular nitrate content remained stable within an hour. It was thus not likely that the filamentous bacteria used nitrate for denitrification but rather absorbed and stored nitrate when carbon substrate was limited. After 3 hr incubation
in the anoxic phase, the nitrate concentration was only 1.2 mg N/L, but with further incubation of another 6 hr and 12 hr, it reached levels of 2.4 and 5.6 mg N/L, respectively, which may have resulted from the release of excess absorbed nitrate, under carbon-limited conditions, by the filamentous bacteria. This speculation was well supported by the decline of the intracellular nitrate content from 5.8 to 0.08 mg N/g SS at the same time. It can thus be hypothesized that, when the carbon source was sufficient, filamentous bacteria would denitrify preferentially, but when carbon was limited they would preferably absorb and store nitrate. Based on this summary, together with the data of Fig. 6a, Fig. 8 schematically illustrated the nitrate’s dynamic transfer among different substances in batch 1 under anoxic condition.

It was reported previously that, certain types of the filamentous bacteria were able to use nitrate as an electron acceptor, reducing it to nitrite, like M. parvicella (Rossetti et al., 2002), S. natans (Pellegrin et al., 1999), and Thiothrix spp. (Shao and Jenkins, 1989; Williams and Unz, 1985). In the present study, nitrate was denitrified, but nitrite did not accumulate during the operation and the batch tests, which may suggest that filamentous bacteria or other denitrifying microorganism used nitrite as electron acceptors. This speculation, however, does not concur with previous studies, such as Casey et al. (1994) who proposed that floc-forming organisms were able to reduce nitrate to nitrogen gas, but filamentous bacteria were hypothesized to only be capable of reducing nitrate to nitrite. In addition, from the published data, the denitrification rate of the filamentous bacteria analysed so far (Type021N and Thiothrix spp.) was much lower (more than 80 times) than that of floc-forming bacteria (Zoogloea ramigera) (Shao and Jenkins, 1989). But in our batch tests 3 and 4, the maximum denitrification rates of bulking sludge were almost identical to those of the non-bulking sludge when a sufficient source of carbon was available. A significant difference with respect to substrate utilization performance was also noted between non-bulking and bulking sludge. During the anaerobic phase, non-bulking sludge had the capacity to store carbon source, which was not the case for bulking sludge. During the anoxic stage, non-bulking sludge had the capacity to utilize the stored carbon during denitrification while bulking sludge was only able to carry out denitrification when the carbon source was sufficient. When the carbon source was limited, it was likely that bulking sludge would preferably absorb nitrate. To the best of our knowledge, this is the first observation of a nitrate-storing phenomenon in a wastewater treatment system. Since nitrate-accumulating phenomena has been reported in association with sulphur oxidizing filamentous bacteria (Sweerts et al., 1990),
nitrate-accumulating sulphide-oxidizing, sulfur-oxidizing and sulphide-oxidizing filaments have also been discovered in freshwater, or marine, sediment surfaces and intensively examined (McHatton et al., 1996; Sayama, 2001; Sayama et al., 2005). Because of their capability to store nitrate, these filamentous bacteria could survive under carbon- and oxygen-limited conditions. In this study, the operating conditions of the BNR system were similar to those of the filaments in their natural habitat, thus, favoring for the growth of these bacteria. Their presence in the BNR system had a significant effect on the total nitrogen removal rate and on sludge settleability. Hence, it was worth studying at length.

### 2.3 Verifying the effect of low nitrate concentration on filamentous bulking

To verify the hypothesis that low nitrate concentration could cause filamentous bulking, another two laboratory-scale reactors were seeded with the same sludge but cultivated with different nitrate concentration in influent (reactor a with 5 mg N/L, Fig. 9a; reactor b without nitrate, Fig. 9b). However, the concentration of TN was kept at 40 mg N/L through changing NH$_4^+$-N content. During the 10-day processing time, the nitrate concentration in return sludge was kept below 4 mg N/L, with the nitrate load in the anoxic zone thus much smaller than 2.7 mg N/g SS. Results, in Fig 9a, indicated that the SVI value was retained below 150 mL/g and the intracellular nitrate content was about 2.1 mg N/g SS, meanwhile, in Fig. 9b, the SVI value increased steadily from 80 to 350 mL/g accompanied by the increase of intracellular nitrate content from 1.8 to 4.8 mg N/g SS. This confirmed our hypothesis that the minimum nitrate load in the anoxic zone should be 2.7 mg NO$_3^-$-N/g SS. The bulking process was also clearly illustrated in Fig. 10 under nitrate limited condition. In the aerobic zone, the diffusional limitation of oxygen in the floc facilitated the development of an internal anoxic microenvironment (Andreadakis et al., 1993). In addition, filamentous bacteria had the capacity to store a substantial amount of nitrate in anoxic microenvironment, due to the limited carbon source condition. When these filamentous bacteria rich in nitrate reached the anoxic zone with the influent, two very possibilities occurred, depending on specific environmental conditions. If the nitrate load in the anoxic zone was high, the filamentous bacteria would not acquire a competitive survival advantage, but if the nitrate load was low, then the filamentous bacteria would use newly-entering readily biodegradable COD and their interior nitrate to perform denitrification, while other bacteria were unable to carry out such reactions due to a lack of electron acceptors. Hence filamentous bacteria could prevail in the anoxic zone.

### 3 Conclusions

This laboratory research has investigated a cause of filamentous bulking under low dissolved oxygen conditions. The results showed that low nitrate concentrations played a significant role in the bulking process. When the food/microorganism value and DO concentrations were kept at 0.13 g COD/g SS and 0.6–0.8 mg O$_2$/L, respectively, and once the concentrations of nitrate in the aerobic zone was below 4 mg/L, or the nitrate load in the anoxic zone was below 2.7 mg NO$_3^-$-N/g SS, then the filamentous bacteria gained a competitive advantage.

![Fig. 10 Schematic diagram of bulking process.](image-url)
over other bacteria because of their nitrate-storing capacity. When these filamentous bacteria richen in nitrate arrived at the anoxic zone, where had high carbon source but limited nitrate was available, denitrification would occur, while denitrification by non-filamentous bacteria was restrained due to a lack of nitrate. Therefore, in such conditions, the filamentous bacteria would proliferate.

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