Characteristics of denitrifying granular sludge grown on nitrite medium in an upflow sludge blanket (USB) reactor

Xibiao Jin, Feng Wang, Guohong Liu and Yongdi Liu

ABSTRACT

While inoculating pre-acclimatized floccular sludge, nitrite-denitrifying granular sludge was obtained after approximately 40 days of cultivation in a 10 L upflow sludge blanket (USB) reactor. The nitrite removal efficiency was approximately 95% when the nitrite concentration was 50 mg L^{-1} at an influent flow rate of 20 L h^{-1}. The nitrite granular sludge had several notable features including good settleability (110 m h^{-1}), high ash content (79%), and high density (1.248 g cm^{-3}). The mixed liquor suspended solids (MLSS) of the sludge bed remained at 130.04 g L^{-1}, at a hydraulic upflow velocity of 2 m h^{-1}. These interesting characteristics were attributed to a high effluent pH (9.7) caused by the release of alkalinity during the nitrite denitrification process. The surfaces of the granules were dominated by cocci bacteria with a diameter of approximately 3 μm, which could be classified as *Nitrosomonas*-like species based on our analysis of 16 S rDNA sequences.

Key words | denitrification, granular sludge, nitrite, USB

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INTRODUCTION

In the treatment of wastewater with a high ammonia concentration or a low COD/NH_3-N ratio (less than 6–8), it is necessary to add an external carbon source to completely denitrify the NO_x^- produced in the nitrification process (Itokawa *et al.* 2001; Campos *et al.* 2002). In recent years, shortcut nitrification–denitrification (NH_3-N → NO_2^--N → N_2) has received special attention (Abeling & Seyfried 1992; Ruiz *et al.* 2003; Park *et al.* 2010) because this technique may result in a 25% reduction of oxygen demand in the nitrification step and a subsequent 40% reduction of the chemical oxygen demand (COD) required for post-denitrification (Ruiz *et al.* 2003). However, many researchers have focused solely on how to accomplish shortcut nitrification (NH_3-N → NO_2^--N) and have ignored the post-denitrification of nitrite. The anaerobic ammonium oxidation (Anammox) process, which transfers ammonium to dinitrogen gas by employing nitrite as an electron acceptor, is usually applied after nitritation. However, the Anammox process has a few drawbacks such as the slow growth rate and high sensitivity of anammox bacteria (Strous *et al.* 1998). In this paper, the heterotrophic denitrification of nitrite with granular sludge was studied using an upflow sludge blanket (USB) reactor.

To our knowledge, few researches have reported the single nitrite-denitrification process. The submerged biofilter (Rahmani *et al.* 1995) and sequencing batch reactor (SBR) (Torā *et al.* 2011) have been applied to treat nitrite wastewater. Some researchers have investigated the feasibility of removing nitrite by seeding with anaerobic granular sludge (Zhang & Verstraete 2001) or aerobic granular sludge (Adav *et al.* 2010). However, these works above did not involve granulation process or characteristics of nitrite-denitrifying granular sludge.

In previous years, many researchers have performed numerous studies on granular sludge using a variety of different conditions (i.e., anaerobic, anoxic, and aerobic). In 1974, anaerobic granular sludge was obtained for the first time by Lettinga *et al.* (1980) in an upflow anaerobic sludge blanket (UASB). Aerobic granular sludge was first reported by Mishima & Nakamura (1991) and Shin *et al.* (1992). Nitrate-denitrifying granular sludge was first reported in a USB by Miyaji & Kato (1975) and Klapwijk *et al.* (1979). However, few reports have discussed the cultivation of nitrite-denitrifying granular sludge and its characteristics. Therefore, in this study, floccular sludge was inoculated in a USB reactor to cultivate granular sludge using nitrite as the electron acceptor.

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This work had two objectives: (1) to investigate the cultivation process of granular sludge with nitrite as the electron acceptor by inoculating floccular sludge; and (2) to study the characteristics of nitrite-denitrifying granular sludge.

MATERIALS AND METHODS

Laboratory-scale upflow sludge blanket reactor

The experiments were carried out in a USB reactor made with transparent plexiglass as shown in Figure 1. The dimensions of the USB were 10 cm (length) × 10 cm (width) × 100 cm (height) with a total valid working volume of 10 L. Sampling ports were built along the height of column at 20 cm intervals. The cultivation experiment was conducted at 30 °C with continuous feed.

Seed sludge

The floccular sludge used in this study was obtained from a municipal wastewater treatment plant in Shanghai, China. Based on our experiences, at the start of cultivation of granules, channels and dead space were usually observed in the USB reactor due to the poor denitrifying activity, as the mixing effect in USB reactor mainly depends on the gas produced from denitrification. Therefore, before being inoculated into the USB reactor, the floccular sludge (approximately 200 g suspended solids (SS)) was put into a 20 L SBR to promote the nitrite-denitrifying activity. The influent NO_2^--N concentration ranged from 50 to 200 mg L^{-1} with CH_3OH as carbon source, a COD/NO_2^--N ratio of 2.2, and a P/NO_2^--N of 1/10. The SBR was operated with a volumetric exchange ratio of 50% and 24 h cycle period (anoxic reaction, 22 h; drain and fill, 2 h). A mechanical stirrer (15 rev/min) was installed in the SBR. No excess sludge was discharged during the acclimatization period in SBR. After approximately 1 month, the nitrite removal efficiency in SBR was over 90%, then the pre-acclimatized sludge was transferred to the USB reactor.

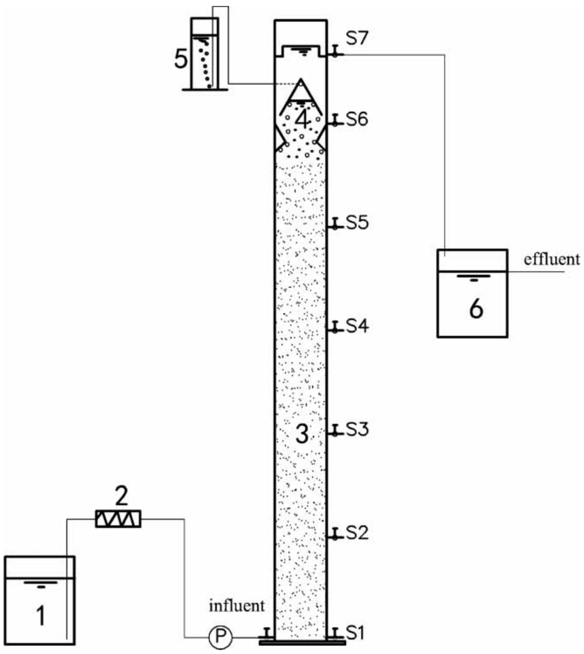

Figure 1 | A schematic diagram of experimental equipment. 1: feed tank; 2: thermostatic bath; 3: USB reactor; 4: three phase separator; 5: water seal; 6: effluent tank; P: metering pump.

Feed composition and reactor operation

The synthetic wastewater was a solution of $NaNO_2$, CH_3OH (COD/NO_2^--N = 2.2), and KH_2PO_4 (P/NO_2^--N = 1/10) in tap water. Nutrients and carbonates were provided by tap water. Before day 35, influent NO_2^--N was 50 mg L^{-1}; afterwards it increased to 75 mg L^{-1}.

During the first 10 days, influent flow rate was 10 L h^{-1}. Then, it increased to 20 and 30 L h^{-1} on day 11 and day 25, respectively. On day 35, the flow rate decreased to 20 L h^{-1}. Note: HRT is the ratio of working volume of USB (10 L) and the flow rate; upflow velocity is the ratio of flow rate and cross-sectional area of USB (0.01 m^2).

DNA extraction and sequencing and phylogenetic analyses

The granular sludge was washed several times with a phosphate buffer solution prior to the DNA extraction. The total community DNA was extracted from the resulting cell pellets via a lysozyme–proteinase K–sodium dodecyl sulphate (SDS) treatment followed by standard phenol–chloroform extractions (Murray *et al.* 1998).

The DNA samples were amplified by PCR using two sets of primers targeting the 16 S rRNA gene: 8f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′-TACGGYTACCTTGTTACGACTT-3′) (Invitrogen, USA). PCR was performed with a 'reconditioning PCR' program (Thompson *et al.* 2002), and six reactions were performed for each sample. The PCR products from the same sample were pooled to minimise PCR bias (Polz & Cavanaugh 1998). The pooled PCR products were purified with a PCR production purification kit (V-gene). The purified amplifications were cloned with a pUcm-T vector kit (Takara, Japan) according to the manufacturer's instructions. The sequencing and phylogenetic analyses were performed as described by Li *et al.* (2007).

Analytical methods

Nitrate, nitrite, COD, mixed liquor suspended solids (MLSS), and mixed liquor volatile suspended solids (MLVSS) were determined according to standard methods (APHA 1995). The settling velocity of the granules was determined in quiescent water in a 3 L plexiglass cylinder (100 cm in height). The scanning electron microscopy (SEM) on granules was carried out by HITACHI S-450 and PHILIPS XL-30. Calcium, magnesium, and phosphorus of granules were determined according to standard methods (APHA 1995), after dry sludge was weighed, acidified by diluted HCl, filtrated, and dissolved by deionised water.

RESULTS AND DISCUSSION

Cultivation process of nitrite-denitrifying granular sludge

Approximately 90 g SS of the pre-acclimatised sludge was added to the USB reactor, which resulted in a mean MLSS of 23.0 g L^{-1} in the reaction region. In the initial stage, the influent NO_2^--N concentration was 50 mg L^{-1} at an upflow velocity of 1 m h^{-1}; the COD/NO_2^--N ratio was maintained at 2.2 with methanol as the carbon source. In the first 10 days, a bulk of flocculant sludge and dispersed particles were washed out of the reactor with the effluent. NO_2^--N removal efficiency increased gradually during these days, reaching over 98% on day 11, and corresponding to an increase in gas production. Subsequently, the nitrite loading rate (according to the USB reactor volume 10 L, the same as below) was increased to 2.4 g N L^{-1} d^{-1} (Figure 2) to accelerate the formation of granular sludge. On day 19, fine granular sludge appeared in the reactor with a diameter of 0.5–1 mm, and the MLSS of the sludge bed reached 43.0 g L^{-1}. On day 24, the fine granules in the reactor turned yellowish brown and became irregularly spherical when the hydraulic upflow velocity was raised to 3.0 m h^{-1}. After the nitrite loading rate was increased from 2.4 to 3.6 g N L^{-1} d^{-1}, the nitrite removal efficiency decreased to 78%, with effluent nitrite increasing to 10.9 mg L^{-1}. Running at 3.6 g N L^{-1} d^{-1} in another 10 days, the effluent nitrite decreased gradually to approximately 5.6 mg L^{-1}. The effluent was clear, and the solid–liquid interface was distinct.

At approximately day 40, mature granular sludge formed in the USB reactor. The sludge was dense and wheat-coloured with a diameter of 1–3 mm. The MLSS was maintained at 130.04 g L^{-1}, at an upflow velocity (V_{up}) of 2 m h^{-1}.

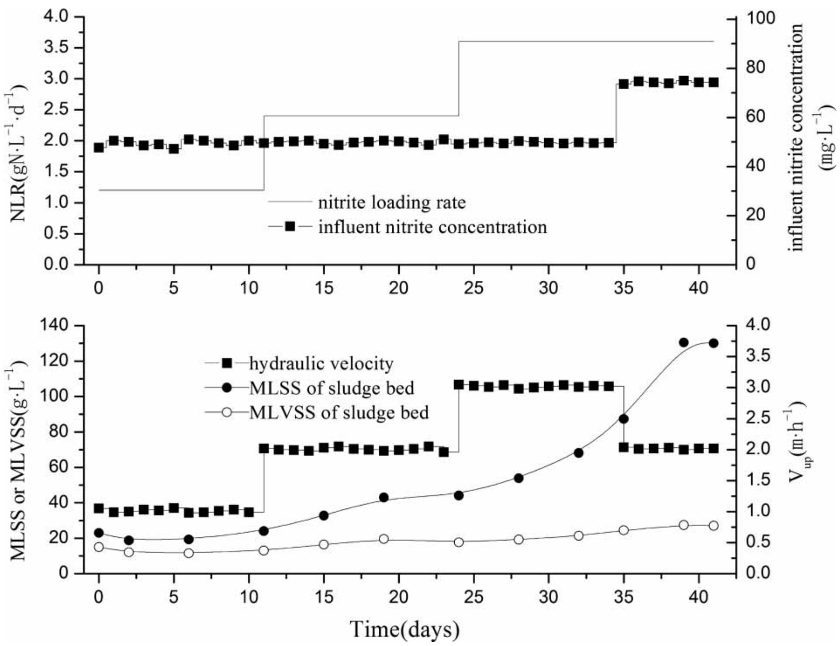

Figure 2 | Nitrite loading operation and biomass concentration of the reactor in the cultivation of nitrite granular sludge.

Denitrification activity of nitrite-denitrifying granular sludge

Based on the reactor performance and experimental objectives, the cultivation experiment can be divided into three periods (see Figure 3).

Phase 1, the first 10 days, was a start-up period. The influent NO_2^--N concentration was 50 mg L^{-1} at a flow rate of 10 L h^{-1}. The nitrite removal rate of the sludge bed reached 0.06 g N g volatile suspended solids $(VSS)^{-1}$ d^{-1} with a removal efficiency of 67.1% after 3 days. On day 10, the removal rate went up to 0.27 g N g VSS^{-1} d^{-1} with a removal efficiency over 98% and an effluent NO_2^--N concentration below 1 mg L^{-1}.

During Phase 2, from day 11 to day 34, sludge activity increased gradually. On day 11, when the flow rate was increased from 10 to 20 L h^{-1}, the nitrite removal efficiency was reduced to 81%. Next, the removal efficiency increased gradually alongside a significant increase in gas production. From day 11 to day 23, the removal rate of the sludge bed fluctuated between 0.25 and 0.3 g N g VSS^{-1} d^{-1}, with an NO_2^--N effluent concentration below 1 mg L^{-1}. After day 24, the nitrite removal rate of the sludge bed was approximately 0.22 g N g VSS^{-1} d^{-1} with a removal efficiency of 88–89% and an effluent NO_2^--N concentration below 6 mg L^{-1}, which indicated that the nitrite removal rate of the sludge bed was always relatively stable despite the increasing loading rate. The nitrite removal rate reported in this paper is comparable with previously published values. Adav *et al.* (2010) reported that aerobic biogranules can degrade 200 mg L^{-1} of NO_2^--N at a rate of 0.029 g N g VSS^{-1} d^{-1} (1.2 mg N g VSS^{-1} h^{-1}) with methanol as carbon source. In their experiments, aerobic biogranules were used for batch tests directly, and the nitrite-denitrifying activity has not been previously promoted. Thus, the nitrite removal rate obtained by Adav *et al.* (2010) was much lower than the one reported in this paper. Zhang & Verstraete (2001) studied the integration of denitrification from nitrite with methanogenesis in an expanded granular sludge bed (EGSB) reactor, where nitrite was denitrified for 97 to 100% at nitrite volumetric loading rate up to 0.9 g N L^{-1} d^{-1}. In this paper, the height of sludge bed is just 60–70% of USB reactor; thus, the nitrite removal rate of the sludge zone should be approximately 5.0 g N L^{-1} d^{-1} when nitrite loading rate of the USB reactor was 3.6 g N L^{-1} d^{-1}. It revealed that nitrite-denitrifying granular sludge can treat nitrite wastewater effectively under a relatively high loading rate.

In Phase 3, from days 35 to 42, the effect of increasing NO_2^--N concentrations on granular sludge was studied with the loading rate maintained at 3.6 g N L^{-1} d^{-1}. On day 35, when the influent NO_2^--N concentration was 75 mg L^{-1} and the flow rate was 20 L h^{-1}, the nitrite removal efficiency decreased to approximately 64%, and the sludge height decreased from 69 to 63 cm. For the next 5 days, the

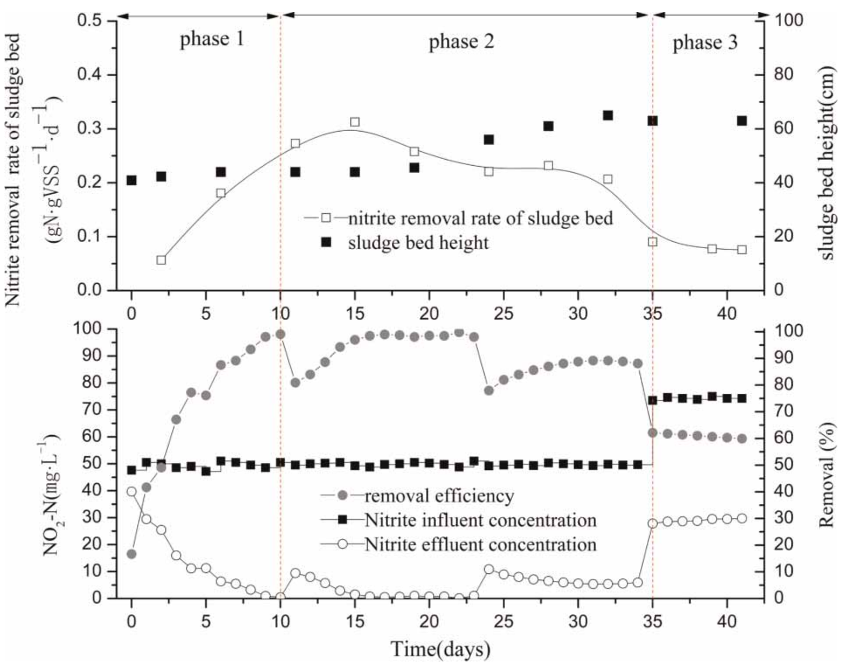

Figure 3 | Nitrite removal rate of nitrite granular sludge in the USB reactor.

NO_2^--N removal efficiency exhibited a remarkable decrease and fluctuated between 60 and 61%; the removal rate of the sludge bed was maintained at approximately 0.08 g N g VSS^{-1} d^{-1}. It suggested that the high concentration of nitrite had a toxic action on the microorganisms and decreased greatly the denitrifying rate of granular sludge (from 0.22 to 0.08 g N g VSS^{-1} d^{-1}). Zhou *et al.* (2011) revealed that nitrite denitrification was significantly inhibited at the range of 0.01–0.025 mg HNO_2-N L^{-1}. In this paper, the free nitrous acid concentration (2.8×10^{-5} mg HNO_2-N L^{-1}) is far below this range due to the high pH (approximately 9.7). However, it suggested that the nitrous acid inside the cells might be very high (no data), which has inhibited the nitrite denitrifiers.

Characteristics of nitrite-denitrifying granular sludge

The characteristics of mature granular sludge (obtained on day 42) are shown in Table 1; these characteristics vary significantly from other kinds of granules. The ash content of anaerobic granular sludge from Lettinga ranged from 10 to 70% (Lettinga *et al.* 1980), the larger value of which was obtained in conditions of high calcium ion concentrations. The ash content of nitrate granular sludge in our previous study (unpublished) was approximately 20%. In this work, the ash content of the nitrite granular sludge reached up to 75–80%. The SEM photographs revealed that the nitrite granules were irregularly spherical in shape with a rough surface (Figure 4(b)). The wet density of the nitrite-denitrifying granular sludge was approximately 1.248 g cm^{-3}, which was much larger than the wet density values reported for anaerobic granular sludge (1.025–1.080 g cm^{-3}; Kosaric *et al.* 1990) or aerobic granular sludge (1.020–1.078 g cm^{-3}; Etterer & Wilderer 2001). Reported settling velocities for granular sludge were in the range of 18 to 100 m h^{-1}, but satisfactory values were between 50 and 100 m h^{-1} (Schmidt & Ahring 1996). Nitrite granular sludge demonstrated a good settleability with a settling velocity of approximately 110 m h^{-1}; the MLSS of the sludge bed reached up to 130.04 g L^{-1}, at a hydraulic upflow velocity of 2 m h^{-1}. Therefore, the nitrite-denitrifying granular sludge exhibited notable features including high ash content, dense structure, and good settleability, among others. To our knowledge, these characteristics of nitrite-denitrifying granular sludge are reported first in this paper. As shown in Table 1, calcium was the major element of ash content, accounting for 29.4%, and magnesium and phosphorus occupied 9.4 and 2.2% of ash weight, respectively. Thus, $CaCO_3$ was the major component of ash content of granular sludge, accounting for 70–75% of ash weight, or 55–60% of dry sludge weight. Analysis of the samples (day 42) using SEM confirmed the existence of precipitated calcium carbonate crystals inside the granules (Figure 4(d)).

Table 1 | Physico-chemical characteristics of nitrite-denitrifying granular sludge[a]

Item	Result
Diameter (mm)	1.5–3.5
Settling velocity (m h^{-1})	110
Wet density (g cm^{-3})	1.248
Moisture content (%)	68.0
VSS/SS (%)	21.0
Ash content (%)	79.0
Calcium (%)	23.2
Magnesium (%)	7.4
Phosphorus (%)	1.6

[a]Ash content, calcium, magnesium, and phosphorus are percentage content of dry sludge weight.

The denitrification processes for nitrate and nitrite can be described by the following two equations if biomass yield is not considered:

Nitrate denitrification:

$$NO_3^- + \frac{5}{6}CH_3OH \rightarrow \frac{4}{6}HCO_3^- + \frac{1}{6}CO_3^{2-} + \frac{8}{6}H_2O + \frac{1}{2}N_2\uparrow \quad (1)$$

Nitrite denitrification:

$$NO_2^- + \frac{1}{2}CH_3OH \rightarrow \frac{1}{2}CO_3^{2-} + H_2O + \frac{1}{2}N_2\uparrow \quad (2)$$

Based on Equations (1) and (2), the effluent pH in the nitrite denitrification process will be considerably larger than that in nitrate denitrification due to the different ionisation constants of CO_3^{2-} and HCO_3^-, although their release of alkalinity at the end of the reactions would be the same. For nitrite denitrification, the effluent pH value can be calculated using Equation (3) because carbonate is a dibasic weak acid:

$$[H^+] = \sqrt{\frac{K_w K_{a2}}{C_s}} \quad (3)$$

where K_w, K_{a2} and C_s represent the water ion product (10^{-14}), secondary ionisation constant, and CO_3^{2-} concentration, respectively.

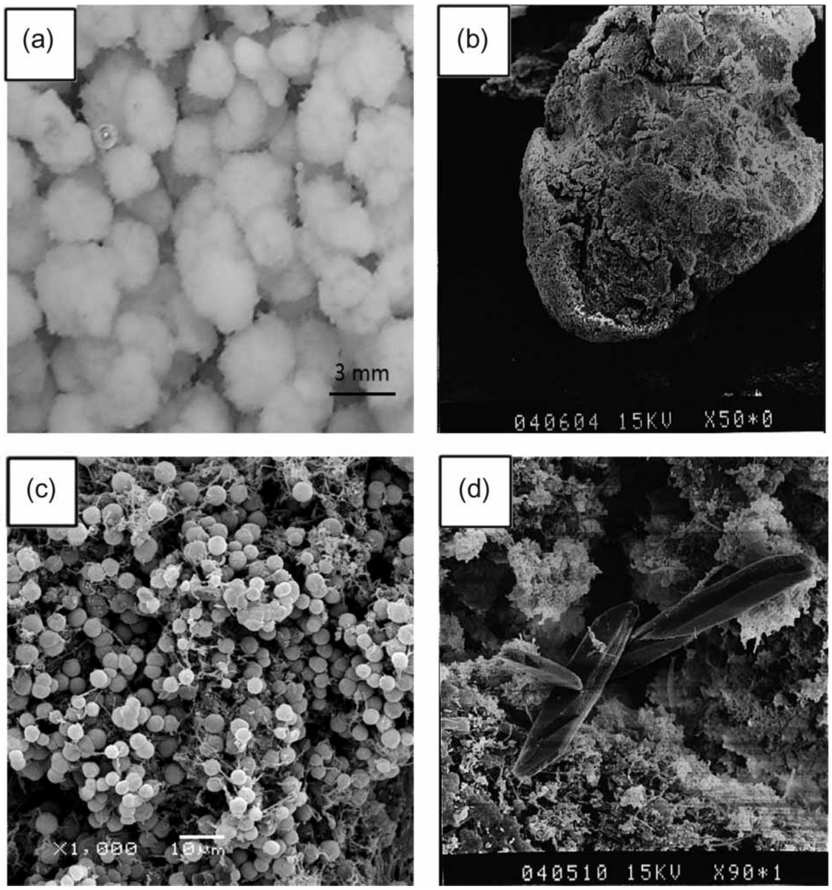

Figure 4 | Photographs of nitrite granular sludge on day 42. (a) An actual photo of the granules taken with a digital camera (Panasonic LX 3). (b–d) Scanning electron micrographs of the granular sludge (a: actual size; b: magnified 50 times; c: magnified 1000 times; d: magnified 900 times).

According to Equations (2) and (3), the theoretical value of effluent pH in this paper is calculated to be 10.7, which is close to the measured value (approximately 9.7). This result suggests that a high effluent pH enhanced the precipitation potential of calcium carbonate on the surface of the granule. Therefore, the features of nitrite granular sludge (i.e., high ash content, dense structure, and good settleability) were attributed to the release of alkalinity and subsequent increase in pH caused by nitrite denitrification. In fact, Green *et al.* (1994) also showed that a high pH (approximately 9.0) was a key factor for obtaining a heavy granular sludge in their denitrification studies using a USB reactor.

Microbial community of the granular sludge

Most of the bacteria on the surface of the sludge were cocci with a diameter of approximately 3 μm (Figure 4(c)). The volume of nitrite-denitrifying bacteria was nearly 20 times as large as for nitrate-denitrifying bacteria (rods, approximately 2×1 μm (Bhatti *et al.* 2001)).

Representative clones from the granule (taken on day 42) were sequenced and then underwent phylogenetic analysis. Clones HXW-1, HXW-2, and HXW-3 were closely related to *Planctomycete* KSU-1 (97% similarity), *Nitrosomonas europaea* ATCC19178 (97% similarity), and *Nitrosospira multiformis* ATCC 25196 (97% similarity),

Table 2 | Distribution of dominant sequence types from the microbial community of granular sludge (day 42)

Clone	Accession number	Closest cultivated species	Sequence number	Similarity (%)	Number of clones
HXW-1	HQ833404	*Planctomycete* KSU-1	AB057453	97	21/80
HXW-2	HQ833405	*Nitrosomonas europaea* ATCC 19178	AB070983	97	46/80
HXW-3	HQ833406	*Nitrosospira multiformis* ATCC 25196	AY123807	97	13/80

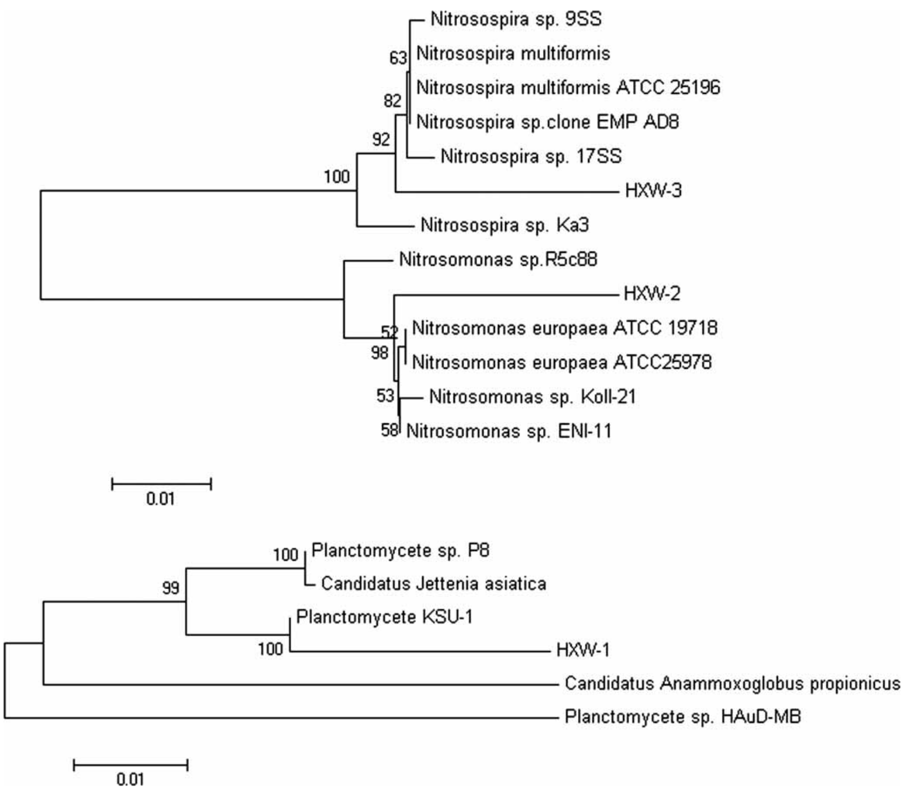

Figure 5 | Phylogeny of bacterial sequences obtained from nitrite-granular sludge on day 42 based on the neighbour-joining method.

which accounted for 26, 58 and 16% of clones examined from the granular sludge (day 42), respectively (Table 2). *N. eutropha* was shown to have the ability to use nitrite as electron acceptor, but such a capability in *N. europaea* has not been reported (Stein *et al*. 2007; Zhou *et al*. 2011). In this paper, the microorganisms on the granules had the ability to reduce nitrite to dinitrogen with methanol as electron donor in anoxic conditions. Therefore, we cannot confirm that the dominant isolates in the nitrite-denitrifying granular sludge are *N. europaea* ATCC19178. Rather, given the similarities, the nitrite denitrifier (cocci) obtained could be classified as a *Nitrosomonas*-like species (Figure 5).

CONCLUSIONS

- This study is the first report of the cultivation process and characteristics of nitrite-denitrifying granular sludge.
- The nitrite granular sludge had several notable characteristics such as good settling performance (110 m h^{-1}), high ash content (79%), and high density (1.248 g cm^{-3}).
- The high pH (9.7) of effluent caused by the release of alkalinity during the nitrite denitrification process was responsible for the dense, heavy characteristics of nitrite-denitrifying granular sludge.
- This paper provides an effective technology for nitrite treatment that could help with the application of shortcut nitrification-denitrification processes.

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REFERENCES

Abeling, U. & Seyfried, C. 1992 Anaerobic-aerobic treatment of high-strength ammonium wastewater-nitrogen removal via nitrite. *Water Sci. Technol.* **26** (5–6), 1007–1015.

Adav, S. S., Lee, D. J. & Lai, J. 2010 Enhanced biological denitrification of high concentration of nitrite with supplementary carbon source. *Appl. Microbiol. Biotechnol.* **85**, 773–778.

APHA 1995 Standard Methods for the Examination of Water and Wastewater, 19th edition, American Public Health Association, Washington DC, USA.

Bhatti, Z., Sumida, K., Rouse, J. & Furukawa, K. 2001 Characterization of denitrifying granular sludge treating soft groundwater in an upflow sludge-blanket reactor. *J. Biosci. Bioeng.* **91**, 373–377.

Campos, J., Mosquera-Corral, A., Sanchez, M., Méndez, R. & Lema, J. 2002 Nitrification in saline wastewater with high ammonia concentration in an activated sludge unit. *Water Res.* **36**, 2555–2560.

Etterer, T. & Wilderer, P. 2001 Generation and properties of aerobic granular sludge. *Water Sci. Technol.* **43** (3), 19–26.

Green, M., Tarre, S., Schnizer, M., Bogdan, B., Armon, R. & Shelef, G. 1994 Groundwater denitrification using an upflow sludge blanket reactor. *Water Res.* **28**, 631–637.

Itokawa, H., Hanaki, K. & Matsuo, T. 2001 Nitrous oxide production in high-loading biological nitrogen removal process under low COD/N ratio condition. *Water Res.* **35**, 657–664.

Klapwijk, A., Jol, C., Donker, H. & Milieutechnologie, S. 1979 The application of an upflow reactor in the denitrification step of biological sewage purification. *Water Res.* **13**, 1009–1015.

Kosaric, N., Blaszczyk, R., Orphan, L. & Valladares, J. 1990 Characteristics of granules from upflow anaerobic sludge blanket reactors. *Water Res.* **24**, 1473–1477.

Lettinga, G., Van Velsen, A., Hobma, S., De Zeeuw, W. & Klapwijk, A. 1980 Use of the upflow sludge blanket (USB) reactor concept for biological wastewater treatment, especially for anaerobic treatment. *Biotechnol. Bioeng.* **22**, 699–734.

Li, H., Yang, S. Z., Mu, B. Z., Rong, Z. F. & Zhang, J. 2007 Molecular phylogenetic diversity of the microbial community associated with a high-temperature petroleum reservoir at an offshore oilfield. *FEMS Microbiol. Ecol.* **60**, 74–84.

Mishima, K. & Nakamura, M. 1991 Self-immobilization of aerobic activated sludge – a pilot study of the aerobic upflow sludge blanket process in municipal sewage treatment. *Water Sci. Technol.* **23** (4), 981–990.

Miyaji, Y. & Kato, K. 1975 Biological treatment of industrial wastes water by using nitrate as an oxygen source. *Water Res.* **9**, 95–105.

Murray, A., Preston, C., Massana, R., Taylor, L., Blakis, A., Wu, K. & DeLong, E. 1998 Seasonal and spatial variability of bacterial and archaeal assemblages in the coastal waters near Anvers Island, Antarctica. *Appl. Environ. Microbiol.* **64**, 2585.

Park, S., Bae, W. & Rittmann, B. E. 2010 Operational boundaries for nitrite accumulation in nitrification based on minimum/maximum substrate concentrations that include effects of oxygen limitation, pH, and free ammonia and free nitrous acid inhibition. *Environ. Sci. Technol.* **44**, 335–342.

Polz, M. & Cavanaugh, C. 1998 Bias in template-to-product ratios in multitemplate PCR. *Appl. Environ. Microbiol.* **64**, 3724–3730.

Rahmani, H., Rols, J., Capdeville, B., Cornier, J. & Deguin, A. 1995 Nitrite removal by a fixed culture in a submerged granular biofilter. *Water Res.* **29**, 1745–1753.

Ruiz, G., Jeison, D. & Chamy, R. 2003 Nitrification with high nitrite accumulation for the treatment of wastewater with high ammonia concentration. *Water Res.* **37**, 1371–1377.

Schmidt, J. & Ahring, B. 1996 Granular sludge formation in upflow anaerobic sludge blanket (UASB) reactors. *Biotechnol. Bioeng.* **49**, 229–246.

Shin, H., Lim, K. & Park, H. 1992 Effect of shear stress on granulation in oxygen aerobic upflow sludge bed reactors. *Water Sci. Technol.* **26**, 601–605.

Stein, L. Y., Arp, D. J., Berube, P. M., Chain, P. S. G., Hauser, L., Jetten, M. S. M., Klotz, M. G., Larimer, F. W., Norton, J. M., Op den Camp, H. J. M., Shin, M. & Wei, X. 2007 Whole-genome analysis of the ammonia-oxidizing bacterium, *Nitrosomonas eutropha* C91: implications for niche adaptation. *Environ. Microbiol.* **9**, 2993–3007.

Strous, M., Heijnen, J., Kuenen, J. & Jetten, M. 1998 The sequencing batch reactor as a powerful tool for the study of slowly growing anaerobic ammonium-oxidizing microorganisms. *Appl. Microbiol. Biotechnol.* **50**, 589–596.

Thompson, J., Marcelino, L. & Polz, M. 2002 Heteroduplexes in mixed-template amplifications: formation, consequence and elimination by 'reconditioning PCR'. *Nucleic Acids Res.* **30**, 2083.

Torā, J. A., Baeza, J. A., Carrera, J. & Oleszkiewicz, J. A. 2011 Denitritation of a high-strength nitrite wastewater in a sequencing batch reactor using different organic carbon sources. *Chem. Eng. J.* **172**, 994–998.

Zhang, D. & Verstraete, W. 2001 The anaerobic treatment of nitrite containing wastewater using an expanded granular sludge bed (EGSB) reactor. *Environ. Technol.* **22**, 905–913.

Zhou, Y., Oehmen, A., Lim, M., Vadivelu, V. & Ng, W. J. 2011 The role of nitrite and free nitrous acid (FNA) in wastewater treatment plants. *Water Res.* **45**, 4672–4682.

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