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ORIGINAL PAPER

Mechanical shear contributes to granule formation resulting in quick start-up and stability of a hybrid anammox reactor

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Abstract It appears that if suspended biomass washout can be reduced effectively, granule formation will be fastened in fluidized bed. Quicker reactor start-up can be anticipated especially for those system keeping slow growth bacteria such as anammox. A hybrid reactor combined fixed-bed with nonwoven fabrics as biomass carrier and fluidized bed with slow speed mechanical stirring was therefore developed, and its nitrogen removal performances was evaluated experimentally. Only in 38 days, the total nitrogen removal rate (NRR) reached to 1.9 kg(N) m^{-3} day^{-1} and then doubled within 17 days, with total nitrogen removal efficiency kept above 70%. After 180 days reactor operating, the NRR reached a maximum value of 6.6 kg(N) m^{-3} day^{-1} and the specific anammox activity was gradually constant in 0.32 kg(N) kg(VSS)$^{-1}$ day^{-1}. Biomass attached on nonwoven fabrics could additionally improve reactor nitrogen removal by 8%. The dominant size of granular sludge reached to 0.78 mm with stirring speed adjusted from 30 to 80 rpm and the hydraulic retention time (HRT) from 8 to 1.5 h during the whole operating time. Scanning electron microscope observation showed especially compact structure of granular sludge. A 70% of anammox bacteria percentage was identified by fluorescence in situ hybridization analysis.

Keywords Anammox · Hybrid reactor · Slow speed mechanical stirring · Granule formation

Abbreviations

Anammox	Anaerobic ammonuim oxidation
AOB	Ammonia oxidation bacteria (AOB)
DAPI	4,6-Diamidino-2-phenylindole
DO	Dissolved oxygen
FISH	Fluorescence in situ hybridization
FITC	Fluorescein isothiocyanate
HRT	Hydraulic retention time
MLSS	Mixed liquor suspended solids
MLVSS	Mixed liquor volatile suspended solids
NLR	Nitrogen loading rate
NRR	Nitrogen removal rate
PEG	Polyethylene glycol
PVA	Polyvinyl alcohol
SAA	Specific anammox activity
SEM	Scanning electron microscope
SS	Suspended solids
SVI	Sludge volume index
VSS	Volatile suspended solids

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Introduction

The anaerobic ammonium oxidation (Anammmox) process was considered to be a promising alternative to conventional biological wastewater treatment on nitrogen removal since it was firstly reported (Mulder et al. 1995). This novel autotrophic nitrogen removal process involved oxidation of ammonium with nitrite as electron acceptor and nitrate and nitrogen gas as production (Van de Graaf et al. 1995). Compared to traditional nitrification–denitrification process, this autotrophic process saved 100% biodegradable organic carbon and at least 50% oxygen (Tal et al. 2006). Therefore, a lower operating cost can be ensured (Jetten et al. 2005).

However, every wastewater treatment method had its limitation and the anammox process was no exception. Lots of researches heretofore focused on remedying difficulty in reactor start-up due to extremely low growth rate of anammox bacteria which double time was suggested to be 11 days (Strous et al. 1999; Isaka et al. 2006). The key point for cell-immobilization for slow growing microorganism such as anammox bacteria tied in forming microbial aggregates like biofilm or granular sludge. Various biomass carriers such as glass balls (Strous et al. 1997), nonwoven fabrics (Fuji et al. 2002; Furukawa et al. 2003), Polyvinyl alcohol (PVA) (Hsia et al. 2008) or Polyethylene glycol (PEG) gel (Isaka et al. 2007) and etc. were applied in fixed-bed reactors for biofilm attachment. Biomass retention could be ensured, but clogging and intense gas production became problems during the stable operational period of this kind of reactors (Furukawa et al. 2003). Completely bulking mixing aiming to enhance substrate transfer can be implemented in fluidized-bed reactors in which granular sludge often generated. The nitrogen removal capability was therefore kept at a higher level after granular sludge formation (Strous et al. 1998). From the viewpoint of mechanism, granules were generally considered to be shaped cooperatively by hydraulic, gas flow and mechanical shear which may be, respectively, dominant factor in up-flow reactor (Van de Graaf et al. 1996; Imajo et al. 2004; Tang et al. 2009), gas lift reactor (Sliekers et al. 2003; Depena-Mora et al. 2004; Jin et al. 2007; Arroji et al. 2008) and mechanical stirring SBR (Arroji et al. 2006; Depena-Mora et al. 2004). Considerable shear stress was required for granule formation which meant unstable fluidization would probably lead to excessive microorganism loss. Reactor start-up even failed (Strous et al. 1997) or sludge wash-out deteriorated nitrogen removal performances (Furukawa et al. 2003).

Up to the present, study on rapid, stable and efficient start-up of anammox process remained hot. Tsushima et al. (2007) had reported a maximum nitrogen removal rate (NRR) of 26 kg(N) m^{-3} day^{-1} in a fixed bed reactor using nonwoven fabrics after 247 operating days while during the initial 5 months, the NRR was under 1.0 kg(N) m^{-3} day^{-1}. It can be assumed that if lower suspended biomass washout can be ensured, granule formation will be fastened in fluidized bed resulting in quicker reactor start-up. A hybrid reactor combined fixed-bed using nonwoven fabrics as biomass carrier and fluidized-bed with slow speed mechanical stirring was therefore developed, and its nitrogen removal performances were evaluated experimentally.

Materials and methods

Experiment set up and operational strategy

The experimental reactor had an effective volume of 6.0 l and its working process was depicted in Fig. 1. An up flowing influent was supplied by peristaltic pump (Hitachi, Japan). A digital agitator with two rotators which had six stirring paddles for each made sludge and substrates mixed evenly in fluidized-bed. A three phase separator was designed to separate the mixture of sludge, substrate and gas production. The floating sludge would be rebounded and settled, and the gas production would be introduced along a glass tube and then collected by gas bag. Some flocculent sludge would attach on the nonwoven fabrics placed as fixed bed. Before effluent discharging, an overflow weir was set as the final barrier preventing sludge wash-out. The temperature in the reactor was controlled around 35°C by water jacket.

The reactor operating period of 180 days can be divided into four phases (see Table 1). The stirring initially followed an intermittent mode controlled by a timer that run 5 min then stopped 30 min. The stirring speed was about 30 rpm. Twenty-two days later, the continuous stirring started and stirring speed was kept at 30 rpm. From the day 39, the stirring speed was

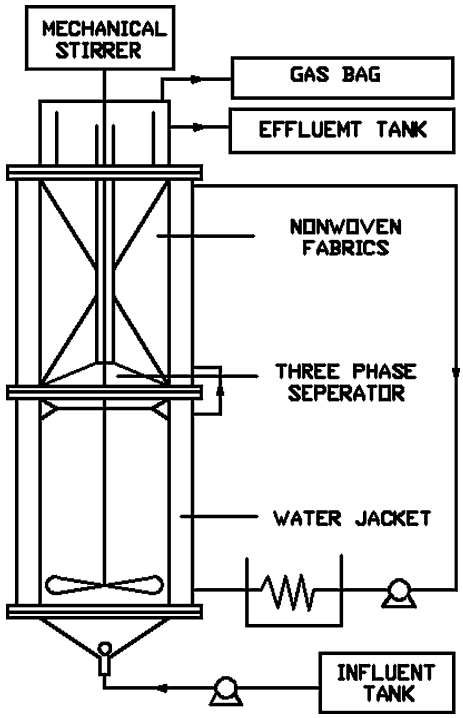

Fig. 1 Structure and working process of hybrid reactor

gradually increased from 30 to 80 rpm with HRT kept at 3 h. HRT reduction strategy was recovered after the day 96. During the whole operating period, the HRT was reduced from 8 to 1.5 h and the stirring speed was increased from 30 to 80 rpm, which suggested a range from 0.07 to 0.35 m h^{-1} of liquid up-flow velocity and a range from 0.2 to 0.5 m s^{-1} of agitator tangential velocity in this study.

Inoculums and feeding substrates

The hybrid reactor was inoculated with laboratory freezing stored anammox flocculent sludge which was immersed in 1.0 kg(N) m^{-3} KNO_3 solution at 4°C with sludge concentration of 4.2 kg(MLSS) m^{-3}. The seeding sludge was washed twice by $KHCO_3$ buffer solution and sparged nitrogen gas for 20 min before inoculation. After inoculums were poured in, nonwoven fabrics were placed on the top without being inoculated. The flocculent seeding sludge would float up when agitator run up and attach on the biomass carriers.

Synthetic wastewater was continuously fed in as influent with composition of $(NH_4)_2SO_4$ (50–300 mg(N) l^{-3}), $NaNO_2$ (50–300 mg(N) l^{-3}), KH_2PO_4 (189 mg l^{-3}), $KHCO_3$ (188 mg l^{-3}), $FeSO_4$·EDTA (1–1.5 ml l^{-1}, $FeSO_4$ 18 g l^{-3}, EDTA 10 g l^{-3}) and salt solution (2 ml l^{-3}, NaCl 0.5 g l^{-3}, $CaCl_2·2H_2O$ 0.7 g l^{-3}, KCl 0.7 g l^{-3}, $MgSO_4·7H_2O$ 0.5 g l^{-3}). The influent was sparged with nitrogen gas before being pumped into reactor to keep dissolved oxygen (DO) concentration under 1 mg l^{-3}.

Analytical method

The concentrations of nitrite and nitrate were determined colorimetrically by spectrophotometer (U-2010, Hitachi, Japan) according to standard methods (APHA 1995), using the *N*-1-naphthylethylenediamine spectrometric method and the Cadmium coated Copper reduction method respectively. While for ammonia, the modified phenate colorimetric method using ortho-phenyl phenol (Kanda 1995) was chosen for concentration determination. The concentrations of suspended solids (SS) and mix liquid suspended solids (MLSS) were qualified according to standard methods (APHA 1995) after samples dried at 105°C. And to get the concentration of volatile suspended solids (VSS), the samples should be continually heated to 600°C. The value of pH and DO were measured using a pH meter (B-211, Horiba, Japan) and a DO meter (D-55, Horiba, Japan) respectively.

Batch test was performed on day 182 (phase 4) according to the method described by Depena-Mora et al. (2004) to decide the specific anammox activity (SAA). Wet sludge of 6 g and 60 ml feeding medium which had similar composition concentration to

Table 1 Operational strategy

Phase	Time (days)	Stirring method	Stirring speed (rpm)	HRT (h)
1	0–21	Intermittent	30	8–5
2	22–38	Continuous	30	5–3
3	39–95	Continuous	30–80	3
4	96–180	Continuous	80	3–1.5

influent except $(NH_4)_2SO_4$ of 70 mg(N) l^{-3} and $NaNO_2$ of 70 mg(N) l^{-3} were filled in a 100 ml serum bottle, sparged with nitrogen gas to make anoxic, then sealed with butyl rubber stopper and aluminum cap. The sample bottles were softly shaken in a thermostatic shaker with temperature of 35°C and shaking frequency of about 1.5 Hz. Samples were taken once per hour and the concentrations of nitrogen compounds were analyzed colorimetrically. The concentration of MLSS and MLVSS were measured after batch test.

Granular sludge characteristics observation

Granular sludge size distribution was monitored by using a laser scattering particle size distribution analyzer (LA-920, Horiba, Japan). The sample analyzed was a mixture of which taken from the bottom, middle and top of fluidized bed when mechanical stirring was working. The sludge was microcosmically observed by scanning electron microscope (SEM, JSM-6390LV, JEOL, Japan). For SEM observation, two kinds of samples were taken including granular sludge from fluidized bed and biofilm attached on fixed bed. The samples were pre-fixed, post-fixed, dehydrated, gold sputtered and then observed.

FISH analysis

Fluorescence in situ hybridization (FISH) analysis was carried out for anammox bacteria identification and population evaluation according to standard FISH hybridization technique (Glockner et al. 1996; Schmid et al. 2005). Samples were immersed in 4% paraformaldehyde for 2 h at 4–8°C to fix bacteria cells. The 16SrRNA gene probes Amx820 (5′-CAAAACCCCT CTACTTAGTGCCC-3′) labeled with fluorescein isothiocyanate (FITC)-12-dUTP (green, Invitrogen, Tokyo) targeted the anammox bacteria. The total microbes were enumerated by 4,6-diamidino-2-phenylindole (DAPI) staining (Porter and Feig 1980). Hybridizations of the fixed biofilm samples were performed in 20 mM Tris–HCl buffer (pH 7.2) containing 0.9 M NaCl, 0.01% SDS, 30% formamide using the labeled probes as described by Amann et al. (1990) at 46°C, and then followed by washing with 20 mM Tris–HCl buffer (pH 7.2) containing 0.112 M NaCl and 0.01% SDS. According to FISH images, the anammox percentage was determined by image analysis software.

Results

Nitrogen removal performances

As described in "Experiment set up and operational strategy" section, the experiment was divided into four phases according to operational strategy. Since various microbial species coexisted in inoculums, aerobic and anaerobic microbes might competitively grow during initial time. It can be assumed that excessive fluidization will be adverse for anammox bacteria enrichment due to oxygen transfer enhancing. Mechanical stirring was therefore conducted intermittently during phase 1. The initial HRT was set at 8 h, which was considered to be a higher level of starting hydraulic shear to accelerate sludge granule formation and attachment on fixed bed. Only after 3 weeks, the nitrogen loading rate (NLR) quickly increased to 1.2 kg(N) m^{-3} day^{-1} and the total NRR to 0.9 kg(N) m^{-3} day^{-1} (see Figs. 2, 3). In phase 2, continuous stirring was carried out with stirring speed constant at 30 rpm and HRT was gradually adapted to 3 h. The NLR reached to 2.3 kg(N) m^{-3} day^{-1} and NRR to 1.9 kg(N) m^{-3} day^{-1} on day 38. The nitrogen removal performance doubled in 17 days. Mechanical shear contributing to nitrogen removal by affecting granular formation was studied in phase 3 (discussed later in "Mechanical shear contribute to nitrogen removal" section). The HRT was kept at 3 h whereas mechanical stirring speed increased from 30 to 80 rpm. A NLR of 4.6 kg(N) m^{-3} day^{-1} with NRR of 3.5 kg(N) m^{-3} day^{-1} was got on day 96. In phase 4, HRT restarted to be reduced from 3 h to a minimum of 1.5 h. A maximum NLR of 8.9 kg(N) m^{-3} day^{-1} with NRR of 6.6 kg(N) m^{-3} day^{-1} was obtained on day 168. During most of the operating time, the total nitrogen removal efficiency was kept above 70%. Batch test performed on day 180 revealed a SAA of 0.32 kg(N) $kg(VSS)^{-1}$ day^{-1} of anammox granular sludge in hybrid reactor.

Sludge characteristic assay

Granular sludge size tended to increase according to Figs. 3 and 4. In one aspect, the dominant granule size (granule size with maximum percentage) increased from 0.26 to 0.78 mm with relative percentage from 7.7 to 17.4%. In another aspect, size distribution curve became narrow and slender signifying homogenization

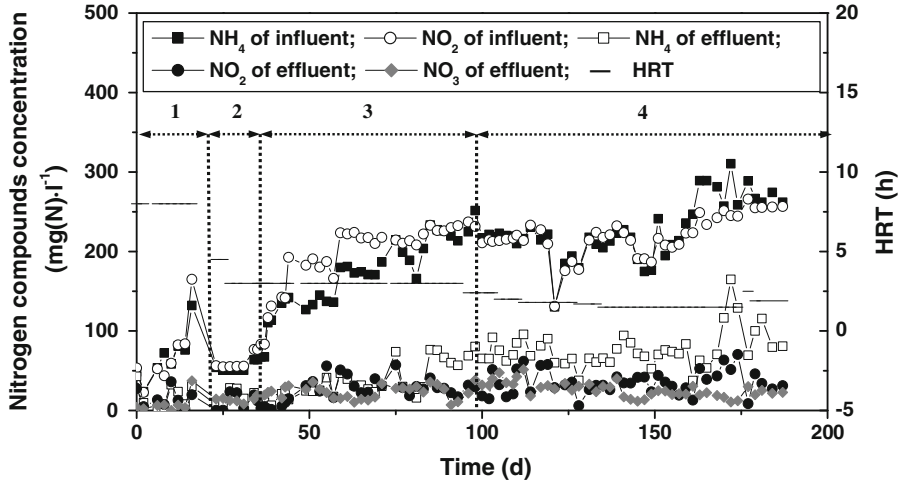

Fig. 2 Variation of nitrogen compounds concentration and HRT during operating days of hybrid reactor

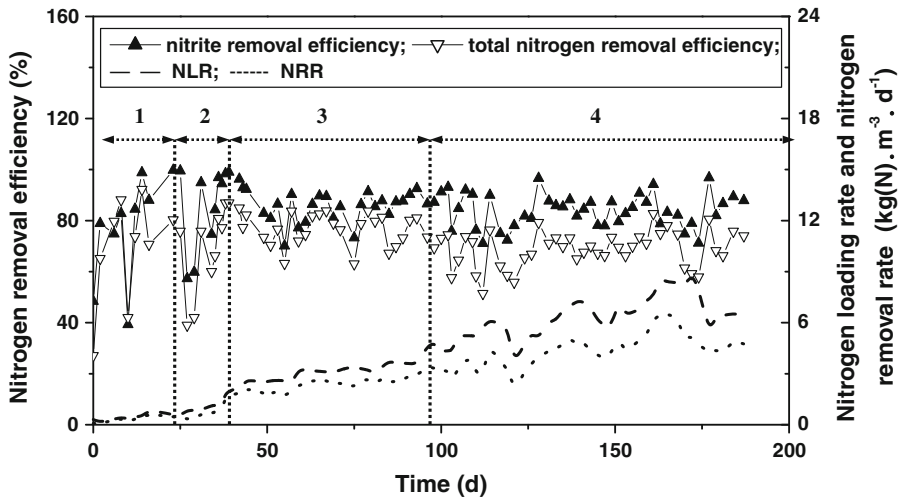

Fig. 3 Variation of nitrogen removal efficiency, NLR and NRR during operating days of hybrid reactor

of granule size (see Fig. 3). The sludge granules had a sharp increase on dominant granule size due to continuous stirring with speed maintained at 30 rpm in phase 2, while hardly any change in phase 3 during which HRT kept at 3 h whereas stirring speed increased from 30 to 80 rpm (see Fig. 4). It appeared that increase of mechanical stirring intensity did not contribute to the increase of dominant granule size but to the percentage (see percentage curve in Fig. 4). Size of dominant granules slightly grew again after HRT reduction strategy was resumed in phase 4 which suggested granule size increase would slow down when shear force increased above a certain level.

The SEM photos (Fig. 5a–c) revealed compact and complex structure feature of granular sludge taken from fluidized bed of hybrid reactor. The granule

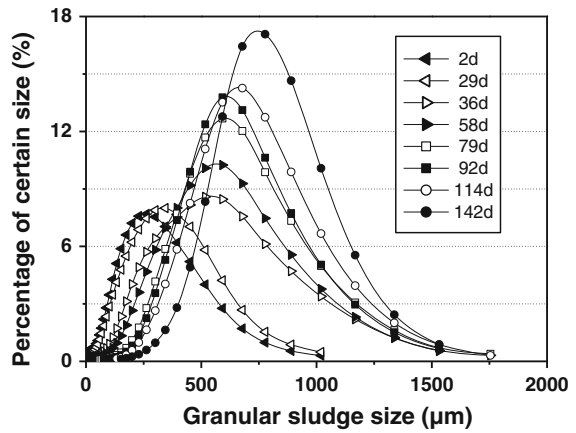

Fig. 4 Trend for granular sludge size distribution in hybrid reactor

surface was densely covered with particle aggregates (Fig. 5a). In granule interior, there seemed to be two interesting aggregating forms. One was particle clusters wrapped by membranous substance (Fig. 5b); the other was cavernous structure with a large number of pits densely distributing (Fig. 5b, c). Clustered particles were considered to be anammox aggregates in accordance with literatures (Strous et al. 1997; Arroji et al. 2006; Gong et al. 2007; Tang et al. 2009), while cavernous structure was assumed to be composed of ammonia oxidation bacteria (AOB) according to report by Gong et al. (2007) based on FISH analysis. Membranous substance may be shaped by internal extrusion. Meanwhile, internal extrusion made cavernous structure more compact and pushed out gas production with pit like vestige as evidence. Massive gas production accumulated and discharged from a gas channel (left side of Fig. 5b). Biomass attached on nonwoven fabrics gathered into cluster (Fig. 5d).

Fish analysis

For anammox population detecting, the16S rRNA gene probes Amx820 was labeled with FITC-12-dUTP (green) targeted the anammox bacteria including *C. Brocadia anammoxidans* and *C. Kuenenia stuttgartiensis* (Fig. 6b, d). By comparing to DAPI staining graph of total microbes (Fig. 6a, c), an anammox percentage of 70% can be confirmed. Tsushima et al. (2007) suggested that the bacteria coexisted with anammox in granular sludge was mostly like *Nitrosomonas eutropha*, *N. europaea* or *N. halophila*., some kinds of AOB which provided insulation from oxygen for anammox bacteria. Gong et al. (2007) had same opinion according to FISH analysis. Although the DO concentration was kept as low as possible in this study, AOB still seemed to survive. FISH graph of two randomly selected sections (Fig. 6) also displayed that anammox bacteria was not mainly existed in granule interior as described in some literatures (Gong et al. 2007), but scattered by form of clusters in this study. It appeared that if shear force was not enough, the aerobic bacteria would slowly and gradually cover the anaerobic bacteria. While in strong shear force condition, such as this study, granules were quickly formed in shorter time that aerobic and anaerobic bacteria randomly distribute within the granular sludge.

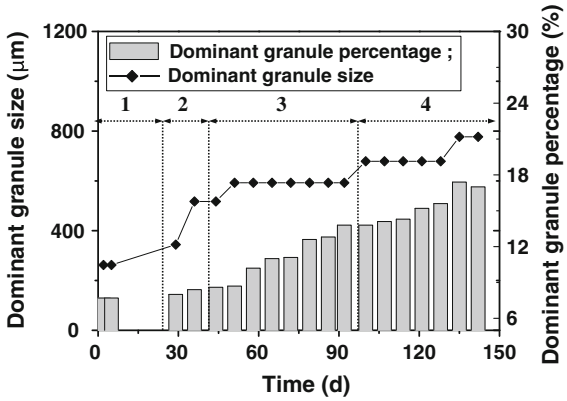

Fig. 5 Dominant granule size and percentage in every phase

Discussions

Mechanical shear contribute to nitrogen removal

It is believed that improvement of anammox performance relied on reactor ability of retaining biomass which is traditionally enhanced by forming biofilm or granules. Granule formation in anaerobic systems mainly depends on two factors: substrates concentration and shear force involving hydraulic, mechanical or gas shear. Moreover, the shear force appears to be more important selection mechanism (Trigo et al. 2006) that moderate shear force helps biomass aggregating while excess shear force will break big granules into small pieces. It can be assumed that granule size increase may slow down with shear force augment, according to that described in Fig. 4. However, mechanical shear force did enhance granule forming manifested in sudden increase of dominant granule size in phase 2 due to continuous stirring and percentage elevation in phase 3 due to stirring speed adjustment. Massive anammox bacteria can be fixed as aggregates in a relative short period of time resulting in nitrogen removal performance fast elevating simultaneously. Strous et al. (1998) reported a 15% higher enrichment degree of a mechanical stirring SBR than a fluidized bed reactor and considered it a result of higher shear force arousing efficient exchange between aggregates and suspended organisms. In this work, nitrogen treatment ability doubled in less than 40 days reaching an obviously higher NRR level of 1.9 kg (N) m^{-3} day^{-1}.

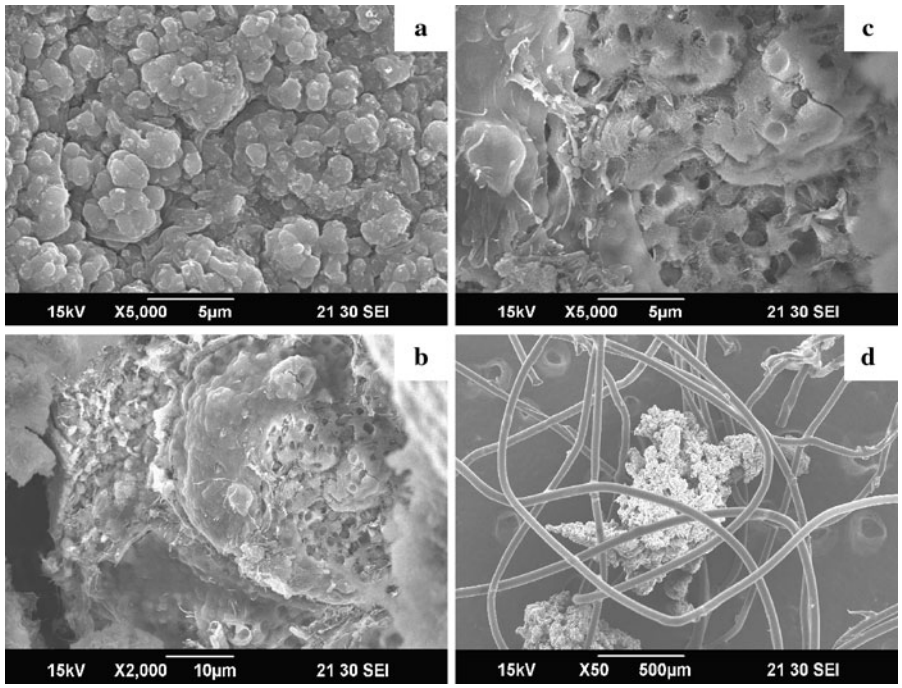

Fig. 6 The SEM graph of sludge samples taken from hybrid reactor: **a** surface of granule taken from fluidized bed; **b** inner of granule taken from fluidized bed (×2000); **c** inner of granule taken from fluidized bed (×5000); **d** sludge attached on nonwoven fabrics

Nonwoven fabrics enhance biomass retention

Granule size with corresponding bacteria activity under certain mechanical shear force showed similar results. Depena-Mora et al. (2004) reported an average diameter of 0.82 mm with SAA practically constant at 0.44 kg(N) kg(VSS)$^{-1}$ day^{-1} when stirring speed kept at 70 rpm in a SBR. According to feret diameter curve shown in Arroji et al.'s (2006) report, size range of 0.6–0.8 mm could be estimated with stirring speed increased from 60 to 90 rpm with SAA retaining around 0.40 kg(N) kg(VSS)$^{-1}$ day^{-1}. In this study, dominant granule size varied from 0.60 to 0.78 mm accompanying with stirring speed adjusting from 40 to 80 rpm and the SAA was found gradually to reach to 0.32 kg(N) kg(VSS)$^{-1}$ day^{-1}. In spite of relatively lower SAA, much higher maximum NRR of 6.6 kg(N) m^{-3} day^{-1} and average specific NRR of reactor (NRR/MLVSS) of 0.79 kg(N) kg(VSS)$^{-1}$ day^{-1} were acquired in hybrid reactor compared to 0.75 kg(N) m^{-3} day^{-1} of NRR (Depena-Mora et al. 2004) and 0.2 kg(N) kg(VSS)$^{-1}$ day^{-1} of average specific NRR of reactor (Arroji et al. 2006) as reported, suggesting long-term stability of hybrid reactor. This result may be due to sturdy granule taken shape in microbe rich environment specially existing in hybrid reactor combining fluidized and fixed bed. Suspended organisms raised by stirring will slow down due to flow resistance of fixed bed that more efficient combination with forming granule and suspended biomass can be ensured. SEM photos confirmed the structural compactness of anammox granule formed in hybrid reactor (Fig. 6a, b) which can be assumed to bear higher shear force and NLR impact. However, the more compact granule structure is, the higher substrate transfer resistance throughout granule becomes. The lower SAA in hybrid reactor can be explained accordingly.

The result of SS concentration monitored showed less than 30 mg l^{-1} sludge wash-out during the whole operational period suggesting good biomass retention. According to MLSS concentration results, biomass attached on nonwoven fabrics was about 10% of that of reactor. Attaching growth of biomass on fixed bed contributed to total nitrogen removal that the elevating degree was calculated to be 8%. Videlicet, nitrogen removal capacity was extra improved by 8% in hybrid reactor compared to that in single fluidized bed.

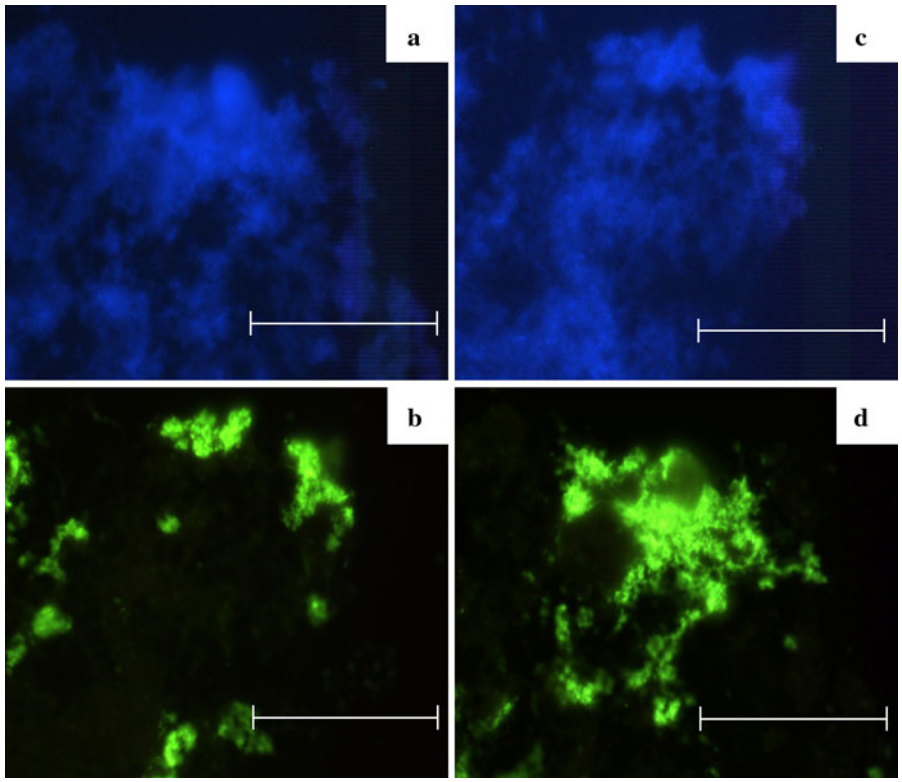

Fig. 7 FISH image of two randomly selected sections of granular sludge taken from hybrid reactor: **a** total microbes in section A; **b** anammox bacteria in section A; **c** total microbes in middle section B; **d** anammox bacteria in section B

Gas separation strategy

Nitrogen gas, as anammox reaction product, was responsible for granule flotation in fixed bed reactor observed by Strous et al. (1997). Other authors like Depena-Mora et al. (2004) also reported same phenomenon which led to SAA decline in a gas lift reactor and a SBR with sludge volume index (SVI) increasing from 90 ml g^{-1} to 110 ml g^{-1}. While in this study, flotation rarely happened during the operation time and the SAA kept growing gradually with SVI around 50 ml g^{-1} manifesting better granule settling performance. One of the contributing factors may be mechanical shear. Depena-Mora et al. (2004) suggested that mechanical shear appeared to be more effective to eliminate gas from granule compared to gas shear. What is more, FISH graphs (Fig. 7) illustrated that anammox bacteria did not mainly exist in granule interior but scattered by form of clusters, meaning that gas would not accumulate in the center. Gas elimination would be easier in hybrid reactor. Another factor improving floatation was using of gas separator. In terms of SBR, nitrogen gas producing was still nonstop even during the settling phase causing floatation (Depena-Mora et al. 2004; Arroji et al. 2006, 2008). In case of hybrid reactor, there was relative sliding velocity between granular sludge and gas after their mixture collided with gas separator. Gas product can be separated, collected and discharged continuously. However, sometimes there were still few of granule sludge washed out by abundant gas bubble. They would be intercepted by overflow weir and finally settled back to reactor.

Conclusions

According to experimental results, the quicker nitrogen removal performance start-up ability and long time stability of hybrid anammox reactor combined fixed bed and slow speed mechanical stirring fluidized bed were confirmed. The NRR reached to 1.9 kg(N) m^{-3} day^{-1} in only 38 days and then doubled within 17 days, with total nitrogen removal efficiency kept

above 70%. During 180 days operating time, a maximum NRR of 6.6 kg(N) m^{-3} day^{-1} was got and the SAA was gradually constant in 0.32 kg(N) $kg(VSS)^{-1}$ day^{-1}. Mechanical shear contributed to granule formation manifesting as size increase and structural compactness of granular sludge. The dominant size of granular sludge reached to 0.78 mm with stirring speed adjusted from 30 rpm to 80 rpm and the HRT from 8 to 1.5 h during the whole operating time. SEM observation showed especially compact structure of granular sludge. Nonwoven fabrics as biomass carrier enhanced biomass retention and attaching growing bacteria additionally improved nitrogen removal by 8%. A 70% of anammox bacteria percentage was identified by FISH analysis. Floatation was effectively prevented by gas separation strategy involving using mechanical stirring, gas separator and an overflow weir. The suspended solids (SS) concentration monitored was less than 30 mg l^{-1}. Compared to some fluidized bed reactor, the hybrid reactor may be an alternate according to its structure simplicity and effectiveness.

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