Bioresource Technology 133 (2013) 475–481

Contents lists available at SciVerse ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Ecological characteristics of seeding sludge triggering a prompt start-up of anammox

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HIGHLIGHTS

- Pre-inoculation to three external MBRs harvested the different initial seedings.
- Initial relative abundance and concentration trigger a prompt start-up of anammox.
- A high initial concentration and even anammox population benefits the start-up.

ARTICLE INFO

Article history:
Received 23 November 2012
Received in revised form 24 January 2013
Accepted 29 January 2013
Available online 6 February 2013

Keywords:
Anaerobic ammonium oxidation (Anammox)
Microbial community
Evenness
Ecological factor
Membrane bioreactor

ABSTRACT

Anammox start-up can be limited by the availability of seeding biomass in some areas. Previous studies have listed suitable alternative seeding sludge for anammox start-up such as anaerobic digestion sludge and conventional activated sludge (CAS), the ecological reasons behind has long been ignored. In this study, the inherent ecological factors that trigger a prompt start-up of anammox were identified, focusing on the initial relative abundance and concentration of anammox bacteria. An external membrane bioreactor was utilized as an enriching tool due to its suitability of retaining cells. Results revealed that a high initial concentration of anammox bacteria benefitted the start-up, meanwhile an even community seeding sludge (Gini coefficient < 0.25) gained a more than three-time higher anammox activity compared to the uneven one (Gini coefficient > 0.5). The discovery reminds to select the seeding sludge that is ecologically appropriate rather than to only care for the type of sludge in general.

1. Introduction

Anaerobic ammonium-oxidizing bacteria (anammox bacteria) belong to the *Planctomycetes* that are capable of autotrophically oxidizing ammonium with nitrite as electron acceptor (Kuenen, 2008). After having been discovered over 20 years, anammox has reached to a stage of full-scale application, treating ammonium rich wastewater with low demand of energy and cost (Joss et al., 2009; Kartal et al., 2010). Due to the slow-growing characteristics of anammox bacteria, the start-up from activated sludge is time-consuming and hence is not recommended (van der Star et al., 2007). Although lab-scale studies from different continents have reported successful start-up of anammox inoculated with local sources (Kieling et al., 2007; Park et al., 2010; Tang et al., 2011), considering the huge demand of anammox seed and the lack of experiences on efficiently enrichment, the full-scale application is still limited by the availability of anammox biomass in some areas (Araujo et al., 2011). Even for the cases where anammox seed were available, the mixed-in of high percent of extra sludge (Park et al., 2010) would decrease the initial concentration of anammox bacteria, possibly bringing uncertainties to start-up.

The comparison study based on previous experiences (Joss et al., 2009; Park et al., 2010; van der Star et al., 2007) indicates that the sole anammox start-up seeded with partial/all anammox biomass is faster than the one with complete conventional activated sludge (CAS). The probable reasons may be that anammox seed has: (1) higher relative abundance of anammox bacteria (higher proportion in a given community with the unit of %), and/or (2) higher anammox bacteria concentration (with the unit of gene copies per milliliter). Theoretically, a high abundance means being (one of) the dominant species in a microbial community. Previous studies have pointed that it is important to limit the growth of anammox competitors, for example ammonium-oxidizing bacteria (AOB), nitrite-oxidizing bacteria (NOB) and denitrifying bacteria (Dapena-Mora et al., 2004; Tao et al., 2011). Hence, it is conceivable that a seeding community with less proportion of anammox competitors would have a faster start-up. On the other hand, given the fact that high

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http://dx.doi.org/10.1016/j.biortech.2013.01.147

concentration of anammox bacteria will also present a high anammox activity (in kg N m^{-3} d^{-1}) (Hu et al., 2010; Shen et al., 2011), the high concentration of anammox bacteria in seeding sludge may also lead to a quick start-up. However, there is very limited experimental proof to these hypotheses that are based on comparison studies. From an ecological perspective, details concerning the characteristics of the favoring seeding sludge for anammox start-up are also scarce to date.

Membrane bioreactors (MBRs) combines suspended biomass with filtration membranes. From an engineering perspective, MBRs are ideal for localized and decentralized sewage treatment. From a microbiological perspective, since MBRs are able to (almost) completely keep biomass from washing-out, they are feasible tools for enriching slow-growing microbes such as methane-oxidizing sulfate-reducing microorganisms (Meulepas et al., 2009b), methanotrophic archaea (Meulepas et al., 2009a) and anammox bacteria (van der Star et al., 2008). In this study, high retention capability was used to identify the ecological factor of seeding sludge that triggers a prompt anammox start-up. Two ecological factors (relative abundance and bacterial concentration) are compared through a successful manipulation of initial community structure of the seeding sludge.

2. Methods

2.1. EMBR reactor

External MBRs (EMBRs) were used to start the anammox process, which is quite different from the previous studies that used submerged EMBRs (Suneethi and Joseph, 2011; Wang et al., 2009). An EMBR would circumvent the potential break of the strictly anaerobic environment caused by replacement of membrane module, which would benefit the start-up from conventional sludge. Although most biomass was returned to EMBRs after washing in buffer, the loss of some biomass during the replacement was still inevitable (about 150–200 mg volatile suspended solids (VSS) lost) due to their adhesive attachment to the membrane surface with the help of their metabolic products. Three EMBRs had the same size (volume of 3.0 L and 80% as effective volume) and configuration. The equipped membrane module had an effective volume of 0.19 L with a core of 15 sheets of polyethylene hollow fiber membrane (Mitsubishi Rayon Co., Ltd. Tokyo, Japan; mean pore size 0.4 μm). The bulk liquor was completely mixed by a mechanical stirrer (150 rpm). The EMBRs were covered with opaque fabrics to prevent phototrophic conversions. The EMBRs were fed with synthetic medium that has been described previously by Tao et al. (2011). The three reactors shared the same operational conditions (Table 1).

2.2. Seeding sludge and Pre-inoculation

EMBR1 was inoculated with about 10 g of VSS of CAS (described as ***control sludge***) from an anoxic tank of a full-scale wastewater treatment plant (WWTP) located in Harbin, China. The CAS was not washed using anammox medium before inoculation because the settlability of it was unsatisfied (SVI ~ 155 mL/gMLSS), which could be due to relatively low temperature when sampled (16 °C). So too much suspended biomass, which could play an important role in this study, could be washed out during washing process.

EMBR2 was firstly inoculated with the same type and amount (10 g VSS) of CAS as EMBR1. Then a 10 days pre-inoculation was applied (Fig. 1A) in order to achieve a microbial community with the similar concentration of anammox biomass to EMBR1 (the same order of magnitude in gene copies per milliliter biomass)

Table 1
Operational conditions.

Operational parameters	Value and unit
Temperature	33 ± 1 °C
pH of Influent	8.0 ± 0.3
pH in Reactor	7.3 ± 0.4
DO of influent	<0.08 mg L^{-1}
DO in reactor	<0.02 mg L^{-1}
TOC in reactor (stable stage[a])	0.5–11 mg L^{-1}
HRT	2 d
Retention time of sludge in the membrane module[b]	1.1 min
Effective life-span of the membrane[c]	23–40 d

[a] The stable stage means the time after day 60, when the fluctuations of TOC and VSS in the EMBRs were little.
[b] Since the effective volume of the membrane tube was 188 mL and the flow rate of recycling pump was 170 mL/min, the sludge stayed in the tube for about 1.1 min.
[c] The period from the start to the appearance of severe membrane fouling, by when the permeate flow rate could not match the influent flow rate. This value is relevant to both biomass concentration and bacterial metabolism.

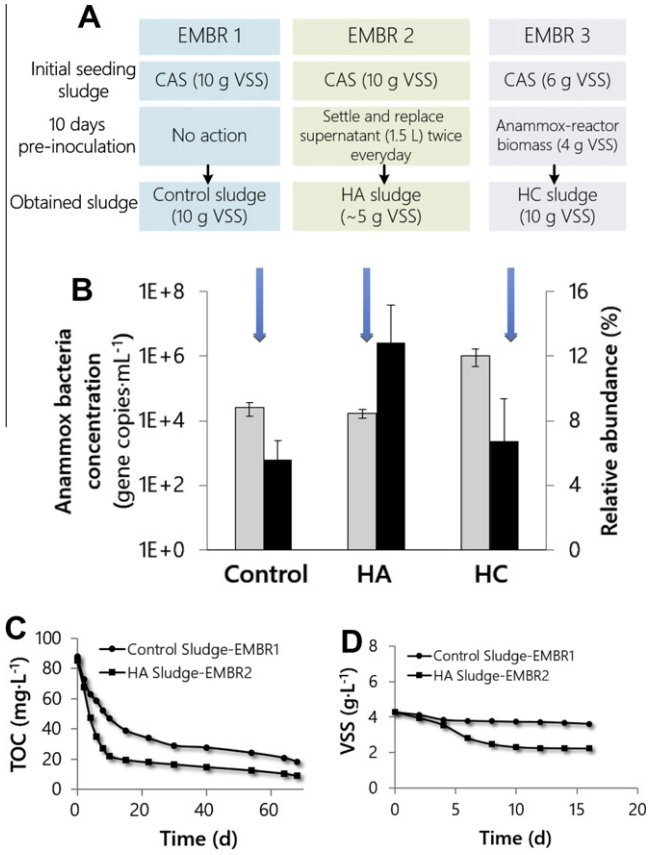

Fig. 1. Pre-inoculation to each EMBR and its result. (A) Operation scheme of the 10-day pre-inoculation process, CAS stands for conventional activated sludge. (B) Anammox bacteria concentration and the relative abundance after pre-inoculation, grey bars stand for the anammox bacteria concentration based on qPCR results and the black bars show the relative abundance based on T-RFLP analysis, error bars indicate the range of data from the triplicated tests. (C) The variation of total organic carbon (TOC) concentrations of EMBR1 and EMBR2. (D) The variation of the volatile suspended solids (VSS) concentrations of the sludge from EMBR1 to EMBR2.

but higher anammox abundance (percentage of anammox bacteria out of the total species). For every 12 h during pre-inoculation, EMBR2 was shut down for 30 min until most sludge settled and then the supernatant was moved out and replaced by new anammox medium (Fig. 1A). Since the medium was organic-matter free, the total organic carbon (TOC) concentration in EMBR2 rapidly decreased from 80 down to 20 mg L^{-1} and kept low (Fig. 1C). Thus,

the organic availability in EMBR2 was strictly controlled and heterotrophs were gradually deactivated and eliminated in both total amount (about 1/3 decrease in VSS, Fig. 1D) and microbial diversity (Fig. 2), leading to a microbial community with higher abundance of anammox biomass (named ***HA sludge***, Fig. 1B).

EMBR3 was firstly inoculated with 6 g VSS of WWTP CAS (the same to EMBR1 and EMBR2) and then 4 g VSS of anammox biomass were added (Fig. 1A). The anammox sludge was collected from a pilot-scale anammox UASB (upflow anaerobic sludge bed – response to general 1) that treated dewatering streams of digested sludge in a WWTP of Beijing, China. The pilot-scale anammox UASB has a volume of 17 m^3 and is started using the combination sludge of aerobic granular sludge, anaerobic granular sludge, oxidation ditch sludge and nitritation sludge since July of 2009. The start-up process was about one year. The UASB was fed with dewatering streams of digested sludge after partial nitrification and ammonium chloride was added in order to keep high ammonium concentration. After start-up the nitrogen removal capacity reached to 0.8 kg $NH_4^- - N\ m^{-3}\ d^{-1}$. Anammox bacterium *Kuenenia stuttgartiensis* was dominant. The mixed seeding sludge had higher concentration of anammox bacteria but the similar (about 15% in difference) abundance to control sludge and is named ***HC sludge***.

2.3. Sampling, DNA extraction, polymerase chain reaction (PCR) and terminal restriction fragment length polymorphism (T-RFLP)

Biomass was sampled from EMBR2 and EMBR3 after pre-inoculation to compare the microbial community to control sludge (from EMBR1). Five milliliters samples for molecular tests were collected and stored at −70 °C until DNA extraction. Nucleic acids were extracted using *Aqua-SPIN* Gel Extraction Mini Kit (WATSON Biotechnologies, Inc., Shanghai, China) according to the manufacturer's protocol. A forward primer of *Bact0009f* (GGTTTGATCGTGGCTCAG) with the 5′ end labeled with dye 6-carboxyfluorescein, and a reverse primer of *Bact1492r* (ACGGYACCTTGTTACGACCTT) were used for PCR analysis. PCR was performed using one denaturation step at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 45 s, extension at 72 °C for 90 s and a final extension at 72 °C for 8 min. Fluorescently labeled PCR products were purified using a QIAquick PCR purification kit (Qiagen Inc., Canada). One part of the purified PCR products were sequenced by a commercial service (Sangon Biology Engineering Technology & Services Co. Ltd, Shanghai, China) and submitted for comparison to GenBank database using BLAST algorithms. Another part of the purified PCR products were digested with the restriction enzyme *Rsa I* at 37 °C overnight. Digested PCR products were precipitated with ethanol and re-suspended in 15 μL of Hi-Di formamide with 500LIZ standard (Applied Biosystems, Foster City, CA). Samples were denatured at 95 °C for 5 min, followed by rapid chilling on ice. The samples were run on an ABI PRISM 3130 Avant Genetic Analyzer (Applied Biosystems, Foster City, CA) in the GeneScan mode and analyzed with the GeneMapper program version 3.0 (Applied Biosystems, Foster City, CA). Only fragment lengths in the range of 50–600 bps were considered for analysis to avoid the detection of primers and uncertainties of size determination. The R package (TRAMPR) was applied to identify T-RFs (Fitzjohn and Dickie, 2007) in order to link T-RFLP raw peaks with the cloning and sequencing library. A minimum combination of 2 enzymes per primer set coupled with an acceptable error of 2 bp was utilized (between sample T-RF location and library sequence T-RF).

2.4. Fluorescence in situ hybridization (FISH) analysis

A sample (1.5 mL) was harvested and fixed in paraformaldehyde. The probe Amx 820 (S-∗-Amx-0820-a-A-22, specific for *Candidatus Brocadia anammoxidans* and *Candidatus Kuenenia stuttgartiensis* (Schmid et al., 2005), purchased from TaKaRa, Dalian, China) was labeled with Cy3. The hybridizations with fluorescent probes were performed according to a previously-described protocol (Schmid et al., 2000). The samples were counterstained using mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI). A confocal laser-scanning microscope (CLSM, Carl Zeiss, Oberkochen, Germany) equipped with an Ar ion laser (488 nm) and He–Ne laser (543 nm) was used for observation.

2.5. Quantitative real-time PCR (qPCR)

Anammox specific qPCR was conducted using hydrazine oxidoreductase (*hzo*) gene primer pair: hzocl1F1 (5′-TGYAAGACYTGYCAYTGG-3′) and hzocl1R2 (5′-ACTCCAGATRTGCTGACC-3′) (Schmid et al., 2008). Reactions (25 μL) contained 12.5 μL 2 × SYBR

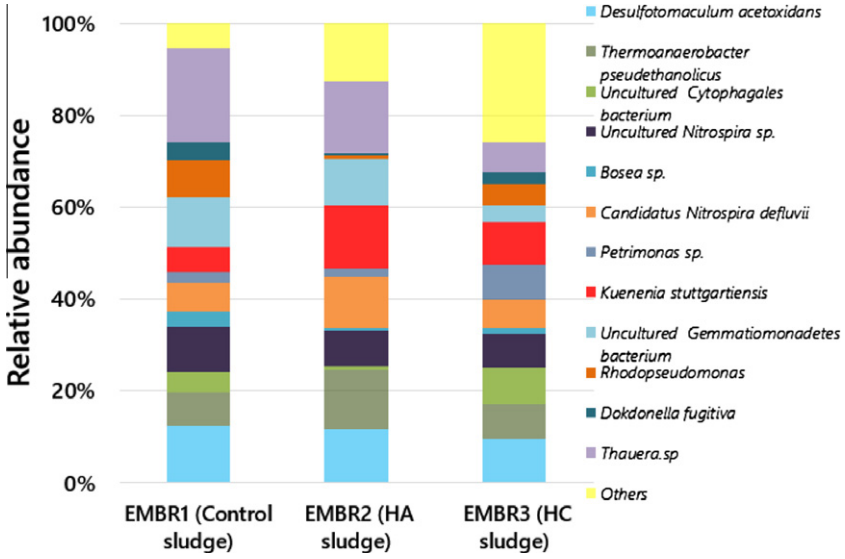

Fig. 2. Relative abundance of major genera (species) in each EMBR after pre-inoculation. The relative abundance was calculated based on the percentage of each peak height out of the total height for a given restriction fragment that was measured by terminal restriction fragment length polymorphism (T-RFLP) analysis.

Premix Ex Taq (TaKaRa Biotechnology, Dalian, China), 200 nM of each primer and 1 μL of DNA template. Amplification was applied in an ABI 7500 instrument (Foster City, CA) under the following conditions: 95 °C for 30 min, followed by 40 cycles of 30 s at 95 °C, 30 s at 94 °C, annealing for 1 min at 55 °C and elongation for 1 min at 72 °C. The standard curves for anammox *hzo* gene copies were constructed from a series of 10-fold dilutions (from 1.9×10^1 to 1.9×10^9 copies) of plasmid DNA (ordered from Beijing Microread Gene Technology Co., Ltd.). Melt curve analysis was performed, confirming that primer dimers did not interfere with signal detection and primer binding was specific. Each sample was run in triplicate wells and all samples were run on the same 96-well plate.

2.6. Anammox activity batch tests

A 100-mL serum bottle was inoculated with about 0.8 g VSS biomass after washing with phosphate buffer (pH 7.8) for three times. The medium had the same content to the EMBR medium except that the concentrations of ammonium and nitrite were fixed (5 mM for each). All the bottles were previously sparged with argon gas for 15 min to obtain an anaerobic condition (dissolved oxygen (DO) was below 0.05 mg L^{-1}). Considering that the serum bottles were completely airtight, as well AOB and NOB consumed the extremely limited DO very rapidly, it can be expected that the anaerobic condition was well kept during the whole tests. The bottles were incubated in an orbital shaker (rotating at 70 rpm) under 33 ± 1 °C. All the tests were performed in triplicate. Every cycle lasted 24 h and liquid samples were taken for ammonium and nitrite analyses. The anammox activity was calculated based on the concurrent depletion of ammonium and nitrite.

2.7. Other analysis

The concentrations of ammonia, nitrite, nitrate and sludge parameters (e.g. mixed liquor suspended solids (MLSS), sludge volumetric index (SVI) and VSS) were measured according to the standard methods (APHA, 1998). DO and pH were tested using a Handheld Multi-Parameter Instrument (pH/Oxi 340i, WTW, Germany). The total organic carbon (TOC) was determined using a Shimadzu TOC-VCPN-6000 analyzer (Shimadzu Scientific Instruments, Kyoto, Japan). Shannon diversity index was calculated based on T-RFLP results. The following equation was used: $H' = -(\mathrm{ni}/N)(\log \mathrm{ni}/N)$, where ni/$N$ is the proportion of community that is made up by species i (the height of each base pair). The Gini coefficient of the sludge microbial community was calculated and plotted based on the T-RFLP data. The normalized area between a Pareto–Lorenz evenness distribution curve and 45° diagonal (the theoretical perfect evenness line) stands for the Gini coefficient of a given community.

3. Results and discussion

3.1. Pre-inoculation community and reactor performance after inoculation

Heterotrophs could be potential competitors of anammox bacteria fighting for nitrite. The 16S rDNA sequencing results proved that at least four heterotrophic bacteria were dominating the microbial community of control sludge (WWTP AS), including *Rhodopseudomonas*, *Thermoanaerobacter pseudethanolicus*, *Desulfotomaculum acetoxidans* and *Bosea* sp. *GSM-187* (Fig. 2). This could possibly be because of the long-term acclimation in an organic-matter-available environment (anoxic sink of a WWTP). To HA sludge, the relative abundance of heterotrophs decreased dramatically (Fig. 2) as a result that heterotrophs were largely eliminated, meanwhile, the biomass concentration of EMBR2 dropped by nearly 40% (Fig. 1D). The manipulation to EMBR2 established a special microbial community that had a concentration of anammox bacteria close to the control sludge, but twice higher in the relative abundance (Fig. 1B). To HC sludge, the anammox bacteria concentration in the HC sludge (1.06×10^6 gene copies mL^{-1}) is about 42 times higher than the control sludge (2.52×10^4 gene copies mL^{-1}) (Fig. 1B). Although the mixing of two types of sludge introduced other heterotrophic populations such as *Petrimonas* sp., *Lachnospiraceae* (Fig. 2), the relative abundance of anammox bacteria in the HC sludge (about 6.7%) was still close to the one in control sludge (5.6%).

In order to supply with adequate substrate stress to anammox bacteria, the total nitrogen loading rates (NLR, $NH_4^+ - N$ plus $NO_2^- - N$) of each EMBR were gradually increased from 0.05 to 0.64 kg N m^{-3} d^{-1} (Fig. 3). During the initial three weeks, the total nitrogen (TN) removal rates of all the EMBRs were low, even below zero for several days. This is probably due to (1) the extremely low activity and abundance of anammox bacteria, and (2) the release of organic nitrogen as a result of cell lysis (Wang et al., 2009). The HC sludge EMBR recovered to normal nitrogen removal much faster than the other two and had the best TN removal capacity. Although the HA sludge EMBR performed better than the control sludge EMBR during the first month, the control gained higher increasing rate after day 50. Both HC sludge EMBR and control sludge reached to a high TN removal rate (over 70%) after day 80, but the HA sludge MBR only gained a half TN removal capacity. Therefore the HC sludge EMBR performed better than the control sludge EMBR, and the HA sludge MBR had the weakest performance.

3.2. Enriching process of anammox bacteria

The biomass for activity analysis and qPCR test were sampled at day 0 (inoculum), day 28, day 65, day 127 and day 175, respectively, representing different stages of start-up. The anammox activity was tested by a series of batch tests. The results showed that the anammox activity of each sludge type presented an increasing trend but at different rate (Fig. 4). The control sludge revealed an exponential increase ($y = 0.0265e^{0.0232x}$, $R^2 = 0.96$), making its activity very close to the HC sludge by the end of the experiment despite of extremely low starting activity (Fig. 4). Both HA sludge and HC sludge had a linear increase but with different coefficients (0.0024 for HA sludge with $R^2 = 0.99$ and 0.0076 for HC sludge with $R^2 = 0.98$). The anammox activity of HA sludge kept low since start and was only one third of the highest one (HC sludge) at the end of the experiment.

The qPCR data show the similar trend to the batch test results that the control sludge had faster growing rate of anammox bacteria concentration than HA sludge and HC sludge (Fig. 4), which is not surprising since a climbing activity was often linked to an increasing number of the functional microorganism. The initial anammox concentration of the control sludge was quite close to the HA sludge and they were only one percent of the HC sludge. However, it reached to the same order of magnitude as HC sludge (10^9 copies mL^{-1}), which was about 60 times higher than HA sludge. The anammox stoichiometry of Control sludge (EMBR1) in the ending period was 1.00:1.41:0.37 (consumed ammonium: consumed nitrite: generated nitrate), which was quite close to the most accepted ratios (1.00:1.32:0.26) raised by Strous et al. (1998). The stoichiometric ratios of HA sludge (EMBR2) and HC sludge (EMBR3) during the robust period were 1.00:1.62:0.44 and 1.00:1.37:0.26, respectively. The FISH test captured the dominating process of anammox bacteria in each EMBR. The start of anammox dominance in EMBR1 and EMBR3 can be observed since day 65 and the complete of anammox dominance was captured on

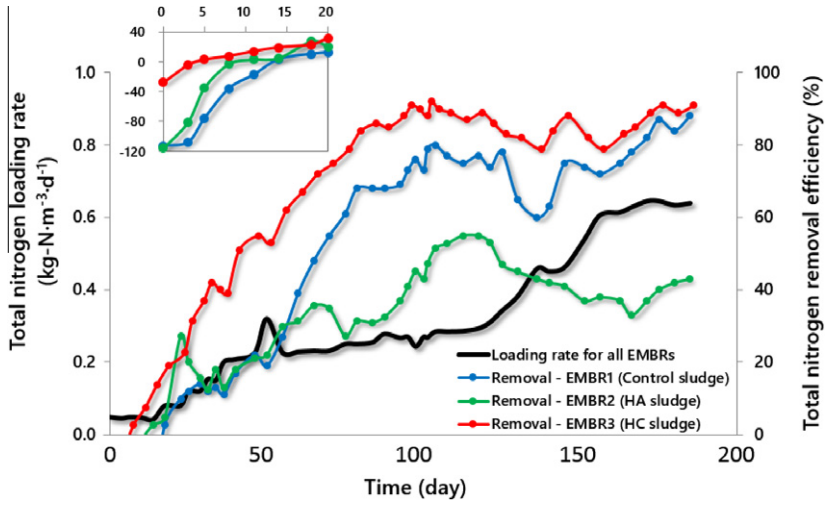

Fig. 3. Total nitrogen (TN) loading rate and removal efficiency of each EMBR. The TN removal efficiency during initial stage (from day 0 to day 20) is shown in the side window because the values were below zero because of ammonium generation from organic nitrogen due to cell lysis.

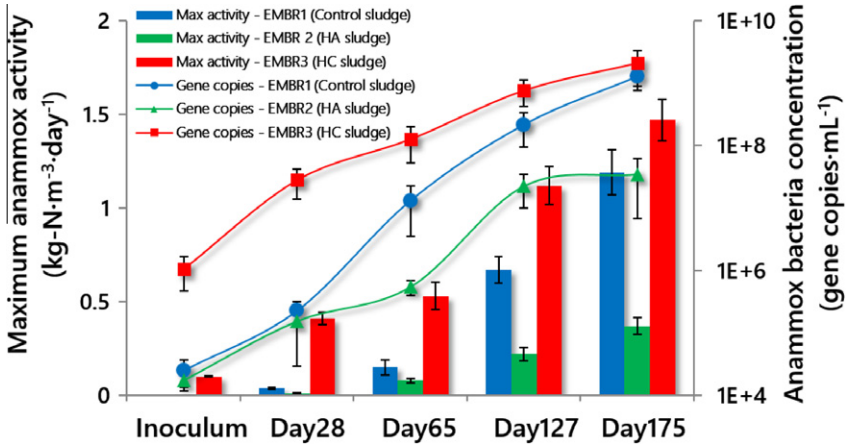

Fig. 4. Maximum anammox activities based on batch tests and anammox bacteria concentration based on qPCR analysis. The maximum anammox activity data (bars) were based on the batch tests of the fresh biomass sampled from three EMBRs. The initial activity of control sludge and HA sludge (inoculum) were below zero (−0.19 and −0.2 kg N m^{-3} d^{-1}, respectively) possibly due to the release of organic nitrogen as a consequence of cell lysis. The anammox bacteria concentration data (circle – EMBR1, triangle – EMBR2, and square – EMBR3) were collected from real-time qPCR tests. Error bars indicate the range of data from the triplicated tests.

day 175. These results were in well accordance to the activity and qPCR data. The concentration of anammox bacteria of all the EMBRs were 10^7–10^9 copies mL^{-1} at the end of the experiment, similar to previously reported values (Hu et al., 2010; Shen et al., 2011).

3.3. The role of initial diversity

The T-RFLP results proved the concurrent existence of anammox bacteria with AOB, NOB, denitrifying bacteria and other heterotrophs in each type of seeding sludge (Fig. 2), which was a normal consequence of an availability of oxygen and organic matter in their reservoir. All the seeding sludge had high microbial diversity (Shannon index > 1.25) and evenly distributed community (Gini coefficient < 0.25). The manipulation for HA sludge obviously worked out, ending with a slightly decrease of Shannon index (1.18) and almost 2.5 times increase of Gini coefficient (0.52), which indicates a limited impact on the number of populations but great strike to their distribution (from an even to an uneven distribution).

It is intriguing to find that the pre-inoculated sludge that had uneven community did not trigger a prompt start-up; in contrast, it led to the weakest performance in anammox bacteria enrichment (Fig. 4). This result indicates that the initial diversity of the seeding sludge plays a very important role on anammox start-up, i.e. a community with high diversity and even distribution is prone to gain a faster dominance under appropriate selective stresses (e.g. extremely low availability of organic carbon and oxygen in this study). Both theoretical and experimental studies have indicated the importance of a diverse community to the stability of a microbial community (Litchman, 2010; van Elsas et al., 2012), even though these studies focus more on the resistance to invasion of a mature community. For an initial community without the threat of invasion, environmental stress could also have huge impact on the community. A recent study demonstrates that species evenness has the same importance as richness in the rejuvenation of ecosystem function (Crowder et al., 2010), and another systematic experiment proves that a microbial community with high initial evenness would favor their functionality under selective stress, by other words, an extremely dominant initial community (highly uneven) would have very weak resistant to stress (Wittebolle et al., 2009). This might be the reason why HA sludge (with highest Gini coefficient, or most uneven) gained the least anammox activity under anammox-cultivation environment. Also we should note that

the initial anammox concentration of HA sludge was also low (2.52×10^4 gene copies mL^{-1}) and this probably is a reason to slow anammox increasing rate.

3.4. The role of initial concentration

Besides of initial diversity, our results also prove that the initial concentration of anammox bacteria in seeding sludge plays an important role. The introduction of anammox biomass into control sludge (i.e. HC sludge) favored the consequent enrichment of anammox bacteria and helped to a successful start-up in less than 50 days (Fig. 4). Previous studies have also demonstrated that the start-up of anammox in EMBRs was very rapid if anammox biomass was inoculated (Trigo et al., 2006). Interestingly, initiated from granular anammox sludge, van der Star et al. (2008) harvested an enrichment of suspended-culture anammox bacteria with the purity over 97% in an MBR. Not only that the engineering anammox inoculum favors anammox reactors, Kindaichi et al. (2011) enriched marine anammox bacteria from natural source (sediment of Hiroshima Bay) within 2 months. Although many studies have shown the possibility of anammox cultivation from conventional sludge, they generally long time, from 4 months to 1 year (Araujo et al., 2011; Chamchoi and Nitisoravut, 2007; Shen et al., 2011). It is important to notice that all the above examples are lab-scale experiments and it could cost much longer for a full-scale anammox reactor due to more difficulties of control and uncertainties involved in scaling up (van der Star et al., 2007).

3.5. Partners versus competitors

It is a challenging proposition that the seeding sludge with diverse community is easier to gain a dominant anammox culture than the one with low diversity, considering the fact that higher diversity also means more competitors needed to wash out. This study pushed us to recognize the role of *partners* and *competitors* from another perspective in case of washing out the right and keeping the wrong. The main competitors to anammox bacteria are AOB, NOB (for electron donors) and denitrifiers (for electron acceptors). Because the EMBRs were anaerobic and heterotrophs could also compete with AOB and NOB by rapidly utilizing the accidentally penetrating oxygen, they can hardly win the competition over anammox species. However, it is more difficult to control denitrifiers, who compete with anammox bacteria for nitrite/nitrate, since (1) they both can live in anoxic environment; (2) anammox is thermodynamically less feasible than denitrification; and (3) the cellular synthesis of denitrifiers is almost five times higher (Shen et al., 2011). Thus, the dissolved organic matter in anammox bulk should be maintained low concentration (Chamchoi et al., 2008; Molinuevo et al., 2009) in case it would indirectly hinder the growth of anammox bacteria by contributing to denitrifiers (Shen et al., 2011). It is also interesting to find that other heterotrophs play a critical role in sponsoring anammox bacteria in the competition to denitrifiers by mineralizing organic compound faster than denitrifiers, which has already been proved in both oceanic environment (Woebken et al., 2007) and bioreactors (Dekas et al., 2009; Meulepas et al., 2009a).

3.6. Indications to engineering start-up

Most of the current full-scale anammox reactors in operation have granular or biofilm biomass, and an MBR is yet proved to be more suitable in actual applications either in previous study or this one. However, as a promising tool enriching slow growing species, MBR is useful in exploring the microbial and ecological characteristics of anammox bacteria (van der Star et al., 2008). Previous studies have put much focus on differentiating the best seeding sludge for anammox, which are of great engineering importance. But the essential property (e.g. population distribution) of a favorable type and the mechanism of the triggering process are less investigated. This study, although warrants more support from full-scale evidences, presents important suggestions that in order to start an anammox reactor in short period, selecting ecologically appropriate seeding sludge is more important than caring for the type of sludge in general. To be specific, the best seeding sludge for anammox would be the one with both high relative abundance and high concentration of anammox bacteria. If it is unavailable, CAS could also be used and no other manipulation or starting strategy is necessary. For the reactors that have already been successfully started up, it is still salutary to realize that high initial diversity can benefit to anammox systems, especially when facing some environmental fluctuations caused by accidents such as power failure or introduction of unwanted oxygen.

4. Conclusions

(1) The initial evenness and bacterial abundance both play critical roles in anammox start-up. An evenly distributed community and a high initial anammox concentration benefit the start-up with less time and higher activity.
(2) The definition of characterizing a partner or competitor to anammox bacteria is sometimes vague and it is important to carefully apply a start-up strategy in case of washing out the right and keep the wrong.
(3) It is necessary to select a seeding sludge that is ecologically appropriate rather than only caring for the type of sludge in general.

Acknowledgements

This research was supported by National Natural Science Foundation of China (No. 21177033) and the Research Fund for the Doctoral Program of Higher Education, Ministry of Education of PR China (20092302110059).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10. 1016/j.biortech.2013.01.147.

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