Desalination and Water Treatment
www.deswater.com

doi:10.5004/dwt.2019.24397

164 (2019) 206–214
October

Start-up and community analysis of simultaneous partial nitrification and anammox (SNAP) process by immobilization

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Received 24 December 2018; Accepted 13 May 2019

ABSTRACT

In this study, partial-nitrification and anammox biomass were co-immobilized by polyvinyl alcohol and sodium alginate (PVA-SA) to treat the ammonium-rich wastewater. The enriched partial nitrification and anammox sludge entrapped in the beads. The beads were round in shape with 4–5 mm in diameter and not agglomerated with each other. The ammonium removal efficiency of 80% achieved within ten days. The nitrogen loading rate varied from the start (37 mg/L/d) to the end of experiment (75 mg/L/d). The enriched sludge,suitable environment inside of the beads for growth of bacteria, and low diffusion constraints were the possible reasons for the fast start-up of PN-anammox system. The necrosis/apoptosis analysis confirmed that there was no observed death phenomena inside of the gel beads in a normal operation of the reactor. The microbial community analysis showed that the dominance of phyla *Proteobacteria* (35.35%) while the abundance of phyla *Planctomycetes* was 2.42%. Likewise, at the genus level, *Candidatus Brocadia* accounts for 1.33% while *Nitrosomonas* was 0.89%, but the highest shared observed from the genus *Burkholderia*. The use of immobilization technology in the application of the SNAP process may represent a valuable alternative to other technologies such as granulation, suspended cell growth and biofilm system.

Keywords: Candidatus brocadia; Immobilization; *Nitrosomonas*; *PVA-SA*; SNAP

1. Introduction

Ammonium in the wastewater from different industrial operations is one of the major concern of the environment. Traditionally, nitrogen removal was carried out with nitrification-denitrification (N/DN) from the past decades. The conventional N/DN route increased the cost of treatment for the wastewater with lower carbon to nitrogen ratio due to the need of external carbon sources and aeration. So, decreasing the cost of treatment facility needs more attention from the wastewater treatment engineers. The last decades of 20th century revolutionized in the removal of nitrogen from wastewater with the discovery of anaerobic ammonium oxidation (anammox) [1]. Compared to conventional nitrogen removal process, anammox free from oxygen and organic matter requirements could reduce the cost of wastewater treatment [2]. The combined partial nitrification and anammox in a single stage process reduce the requirement of oxygen about 60% and 100% of organic matter, produce about 90% less sludge compared to conventional N/DN [3,4]. Furthermore, the anammox process removed ammonia with an amount of nitrite as an essential substrate-from wastewater in the form of nitrogen gas, and produce a little amount of nitrate [5]. Anammox bacteria activated the genes system when detect ammonium nitrogen in the environment by a unique structure know as ammonium antenna [6]. The nitrite is firstly oxidize to NO in a unique compartment of anammox bacteria known as anammoxosome, then NO condensed with and converted into N_2H_2 by hydrazine synthase enzyme and the pathway complete by conversion of N_2H_2 to N_2 by hydrazine dehydrogenase enzymes [7].

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Predominantly, anammox bacteria belonged to the planctomycetes group of microorganisms which has low growth characteristics [5]. Typically, reported doubling time of anammox bacteria varied from 2.1 to 16 d [8–10]. Thus, low biomass yield and long start-up period of the anammox [11] need more research. Besides, anammox need an amount of nitrite as an electron acceptor. Ammonium rich wastewater should be pretreated for the balanced nitrite concentration before feeding into the anammox reactor [12]. The combination of partial nitrification (PN) and anammox seems to be a good alternative to treat the ammonium rich wastewater. However, the different growth environment of both bacteria could cause a problem in the real application. The PN bacteria need an amount of oxygen while oxygen-free environment is essential for anammox growth. The diverse approaches have been reported including PN-anammox process (SNAP) in a single reactor system [13] as well as two-reactor system [14]. The two-reactor system increases the cost of the treatment facility and higher nitrogen concentration rises the process instability [15]. While, one reactor system need strict control of DO and pH [16].

Similarly, in a single reactor system, PN bacteria need oxygen for carrying their activity, but anammox bacteria are sensitive to oxygen concentration. The different strategies including the formation of granules [17,18] and the development of biofilm on the different surfaces [19–21] have been applied from the discovery of anammox. But, even the effectiveness of granular sludge, long-term process stability is still a problem due to the formation of gas pockets inside the granules which ultimately cause the floatation of granules [2,22,23] and lead toward the washout of sludge from the system. Similarly, biofilm formation also has some demerits for long term stability of the process. The thick biofilm causes the reduction of water head which lead to the failure of the treatment system [24]. In that case, further efforts must be made to control the biofilm thickness by providing enough shear-stress.

So, there is a need to address the problems of biomass washout. The immobilization of anammox and nitrifying bacteria was well reported in the past decades. The different gel material has been applied for the co-immobilization of anammox and partial nitrification bacteria as well. The reported material used for the immobilization of anammox included polyethylene glycol gel [25], waterborne polyurethane [26], polyvinyl alcohol [27], sodium alginate and the combined polyvinyl alcohol-sodium alginate [28]. Immobilization of bacterial cell within the PVA-SA gel material was achieved by two methods. The main difference between these two methods was the second step of immobilization, which included the hardening process of gel beads or cubes. The reinforcing of beads or cubes was carried out by chemical [28] as well as by freezing methods [29]. The suitable oxygen concentration and provision of moderate nitrite in the co-immobilized system of AOB and anammox were also studied. The primary consideration behind the used of gel co-immobilized PN-Anammox system was that the PN bacteria grow on the outer layer of the beads and provide the protection to anammox growing in the inner layers by consuming oxygen and provided nitrite in the vicinity of anammox [30]. But, the practical evidence of nitrifying and anammox bacterial activity within the beads was missing. Moreover, there were not reported studies about the lethal phenomena inside of the gel beads. The novelty of this research is the practical evidence of bacterial performance by scannng electron microscopy (SEM) and apoptosis/necrosic anlysis.

So, this research aimed to investigate the nitrogen removal and startup of the co-immobilized PN-Anammox system in the single reactor system and to verify the activity of immobilized biomass inside the gel beads. The lethal phenomena for AOB and anammox inside the gel beads and the microbial characteristics of the SNAP system were also investigated.

2. Methods

2.1. The operation of the system and synthetic wastewater

The serum bottle with the working volume of 1.0 L was used as a reactor. PN-anammox gel carriers with a volume of 180 ml were placed in the reactor (18% filling ratio). The hydraulic retraction time (HRT) of 32 h was controlled by adjusting the peristaltic pump (Model No. BT100-2J) speed (about 0.7 rpm). The continuous mixing of the beads was carried out by a magnetic stirrer. The reaction temperature was maintained between 33–35°C by using a thermostatic water bath. The influent tank was flushed with oxygen to control the DO in the reactor. The synthetic medium was applied as a source of influent wastewater. All materials were analytically graded and buy from Sino-pharm chemical reagent co. Ltd. Composition of synthetic medium (g/L) included NH_4HCO_3 (0.282), KH_2PO_4 (0.81), $CaCl_2$ (0.20), $MgSO_4{\cdot}7H_2O$ (0.03) with 1 ml trace element solution. Trace elements solution (g/L) consisted of $MnCl_2{\cdot}4H_2O$ (0.2), $NaMoO_4{\cdot}2H_2O$ (0.11), $CoCl_2{\cdot}6H_2O$ (0.2), $ZnSO_4{\cdot}7H_2O$ (0.1), $NiCl_2{\cdot}6H_2O$ (0.04), and $FeCl_3{\cdot}6H_2O$ (0.24).

2.2. Preparation of microbial immobilized gel beads

PN sludge was cultivated in the lab under the same conditions as reported [31]. Mature anammox granular sludge was taken from the lab-scale working reactor [32]. Granular sludge of anammox was crushed by using a pestle-type tissue homogenizer to get sludge flocs with a size of less than 100 µm. The mature PN and ANAMMOX sludge were entrapped in polyvinyl alcohol-sodium alginate by a similar process as reported [33]. The biomass ratio of PN and ANAMMOX was 1:2 (wet weight of sludge).

2.3. Scanning electron microscopy (SEM)

The surface morphology, inner structure, and profile of immobilized bacteria were studied by SEM. Gel beads were taken out from the reactor and washed with phosphate buffer solution. The samples were fixed in 2.5% glutaraldehyde for 12 h, then cleaned by deionized water three times for 10 minutes each. The cleaned beads were dehydrated in ascending order of 50%, 70%, 80%, 90%, 95% and 100% ethanol solutions for 10 min each. Following that, samples were washed three times with t-butanol for 10 min each time, and then freeze-dried. Finally, the samples were observed by SEM (S-570, Japan) coupled with EDX (Oxford INCA X, Japan).

2.4. Cell apoptosis analysis

The beads were cleaned by deionized water and then placed in a conical flask with phosphate buffer solution mixing with a magnetic stirrer until agglomerated. Next, the supernatant was removed after centrifugation, and the precipitate was washed by D-Hanks balance solution for 2–3 times, then it was filtered through a cell filter with a pore size of 40 μm for three times to obtain a cell suspension. The detection of apoptosis was followed by apoptotic and necrotic assay kit (Apoptosis and Necrosis Assay Kit, Beyotime Biotechnology, China).

2.5. Chemical analysis

Ammonium, nitrite, and nitrate were measured according to the standard methods (APHA-2009). The measurement of pH was carried out using a digital, portable pH meter.

2.6. DNA extraction and analysis of microbial community (Illumina MiSeq sequencing)

The separation of the sludge from the gel beads was the same as in the cell apoptosis process. The DNA extraction kit of MO BIO (USA) was selected for the extraction of sludge sample, and the specific operation procedure depends on the method of the manufacturer's instructions.

The DNA sample was amplified, and the quality was detected by agarose gel electrophoresis (genomic DNA: gel concentration 1%, voltage 100 V, electrophoresis time 40 min before sequencing; PCR products: gel concentration 2%, voltage 80 V, electrophoresis time 40 min). The concentration, purity, fragment size and completeness of the sample genome and amplified products were detected. The results showed that the bands were single and apparent. Afterwards, Illumina MiSeq sequencing was carried out by NuoheZhiyuan Technology Co. Ltd (Beijing, China) to detect the microbial structure and abundance.

3. Results and discussion

3.1. Characterization of microbial immobilized gel beads

The shape and size of the beads are important parameters for the stable SNAP process. The agglomeration of the beads is another factor affecting the process efficiency and life of the beads. The morphology of beads (Fig. 1a) is round in shape and not clustered with each other. The steady performance of the process also depends on the presence and distribution of the microorganisms inside of the beads. Fig. 1b shows the presence and equal distribution of sludge in the section of gel bead. The diffusion of the substrate inside of the beads also needs to be considered for the successful PN-anammox process. Fig. 1c is the SEM photograph of the whole beads while Fig. 1d depicts the section of the beads showing the network structure of the beads. The networking of the PVA-SA material created a micro whole to facilitate the transport of substrate deep inside of the beads.

Two kinds of bacteria (anammox and AOB) were distinguished based on their sizes and shapes. As it was confirmed that anammox cell size varied from 0.8 to 11.0 μm in diameter with distinct round and oval shape [34]. At the same time, AOB bacteria were divided into five genera on the base of their shapes as well as intracytoplasmic membrane arrangement [35]. Furthermore, the micro-units of anammox was developed when they tightly integrated [36]. In this study, the dissemination of AOB and anammox were carried out microscopically on day 1. The scanning electronic microphotograph of the beads on day one was taken to characterize the AOB and anammox bacteria (Figs. 1e and 1f). Fig. 1e and 1f showed the inside profile of the beads and presence of the microbial biomass. Partial nitrifying bacteria and anammox bacteria morphologically characterized inside of the beads. The results of SEM analysis were in parallel with the result obtained by Qiao et al. [30].

3.2. The performance of SNAP by immobilization

The nitrogen removal process was carried out with gel immobilized SNAP biomass. Fig. 2 shows the influent and effluent concentrations of nitrogenous compounds (ammonium, nitrite, and nitrate). The experiment was carried out for about 57 d to check the inhibitory effects of the cross-link solution and the start-up period of the SNAP process. The influent ammonium concentration gradually increased from 50 to 100 mg/L (NLR varied from 37 mg/L/d to 75 mg/L/d). Average ammonium removal efficiency reached about 54%, and the accumulation of nitrite was not observed during the start of the experiment. Our result was well correlated with the previous study, which reported about 56% removal efficiency of ammonia with no nitrite in the effluent [37]. It was reported that anammox activity was not observed within 20 d due to the lag phase period with polyethylene glycol gel immobilized anammox [25]. There was no lag period observed in this study. From day 10, influent ammonium concentration increased to 75 mg/L. The ammonium removal efficiency of about 80%was achieved. The higher ammonium removal efficiency may attribute to the higher anammox affinity for nitrite over NOB bacteria. Furthermore, it was reported that the change in the influent ammonium concentration decreased the removal efficiency owing to the abrupt higher ammonium loading rate [30]. But in our study this phenomenon was not observed. So, the method with gradually increased ammonium concentration is suggested for the stable nitrogen removal efficiency. The nitrate concentration was lower than the detection limit which satisfied that the growth of NOB was suppressed. From day 20, the concentration of influent ammonium was increased to 100 mg/L. The effluent ammonium concentration increased sharply with the accumulation of nitrite. Then, effluent ammonium showed a downward trend. The effluent nitrite concentration was below the detection limit from day 30. The nitrate concentration started to increase from day 20, and the values were stabilized between 15–16 mg/L. The nitrate concentration with reference to ammonium removal should be 0.11 [38]. But the values in this study were a little higher than reported which indicated that the NOB started their growth. A study revealed the same result that some NOB also consumed nitrite [37]. So, it was assumed that at the beginning of the experiment the co-immobilized PN-anammox process combined well but on the later stage, a little number of NOB became active and

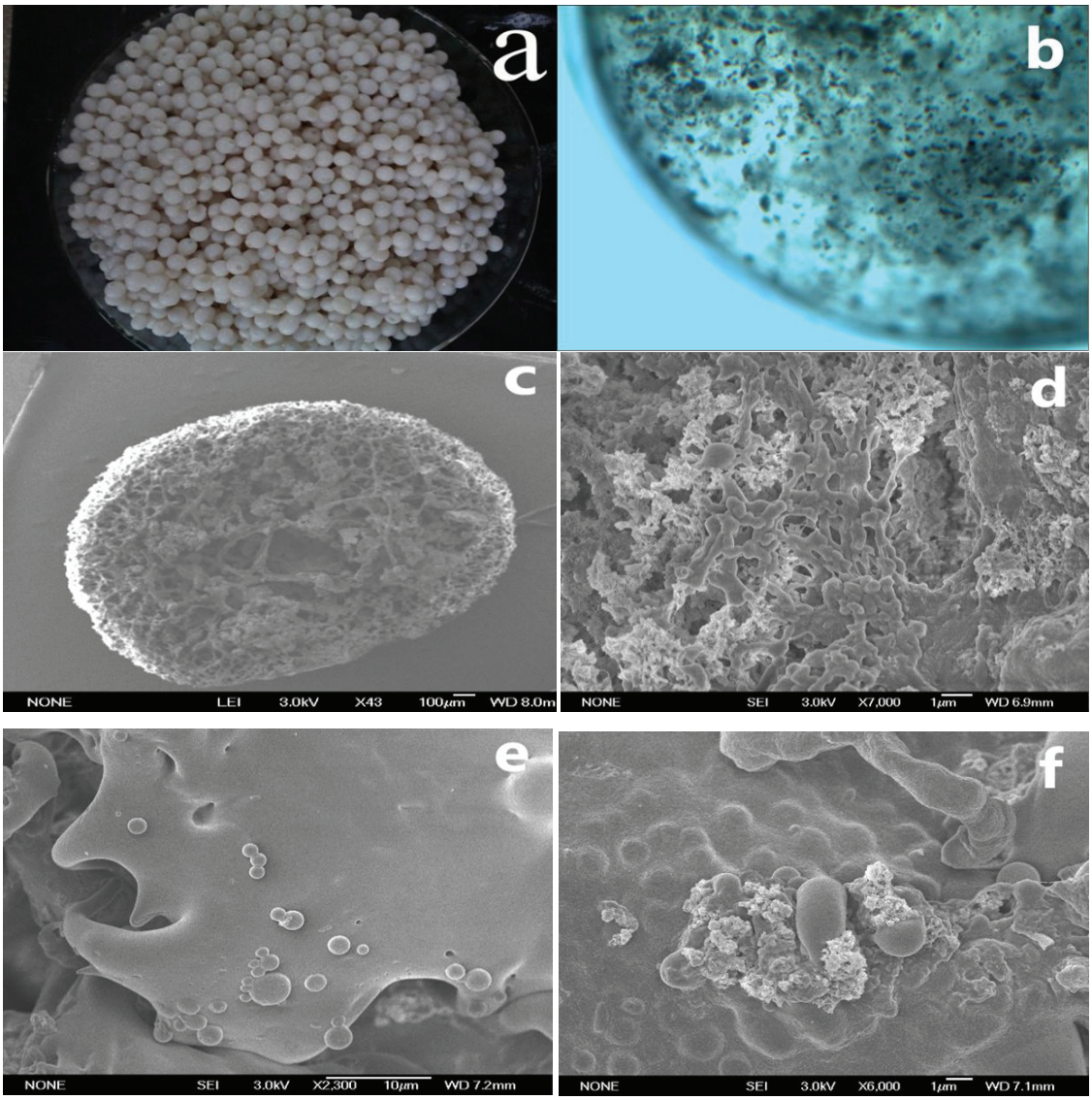

Fig. 1. The photograph of the PVA-SA gel balls in different forms (a); the SEM image of the appearance of sludge in the section of gel balls (b); the SEM image of the whole gel ball (c); the SEM photograph of the internal networking of the PVA-SA gel beads (d); morphology of immobilized bacteria (e and f).

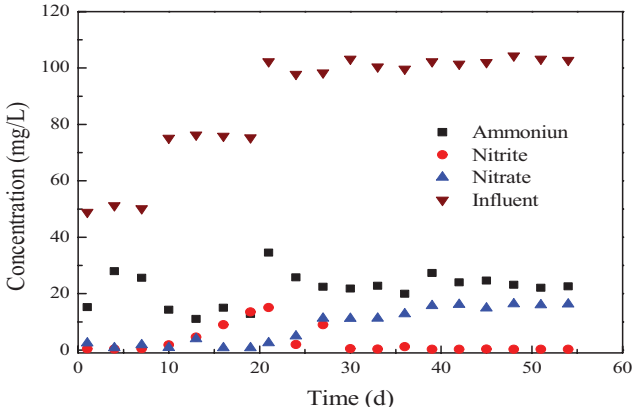

Fig. 2. The time courses of nitrogen removal.

consumed an amount of nitrite. The gel immobilization of PN-anammox sludge exhibited a better alternative to other techniques if the growth of NOB suppressed properly. The different strategies should be adopted to suppress the NOB growth in the latter stage. The heat-shock treatment was applied to suppress the growth of NOB, and some studies reported that heat-shock treatment had more detrimental effects over NOB as compared to AOB with a temperature above 60°C [39,40]. It was suggested that after the appearance of NOB activity heat-shock treatment should be verified on the PN-anammox process. Furthermore, the combined effects of free ammonia (FA), dissolved oxygen (DO), and heat-shock for immobilized biomass should also be verified in further research.

Overall, the combined process is more suitable for immobilized biomass. Previously, the establishment of PN/

anammox microorganisms took more than one and half months [41]. Further, different reactor configurations have been applied for the combined process of nitritation and anammox in singles reactor system. The major configurations included the sequencing batch reactor (SBR), upflow granular system, moving bed biofilm reactor (MBBR). The nitrogen removal rate of 0.4 kg·N/m^{-3}·d was achieved in a SBR system [42]. However, SBR system associated with two issues; firstly, the loss of biomass due to poor settling, and secondly, the aggregation of sludge may increase the floc size which affect the mixing operation [43]. In an up flow granular sludge reactor, a good removal efficiency was realized [42]. However, up flow granular sludge system associated with serious drawbacks. The system may cause the poor substrate transfer under lower up flow velocities [44]. At the same time insufficient mixing can also deteriorate the quality of effluent [15]. The gel immobilization is advantageous because of low substrate diffusion constraints inside of the beads, no development of gas pocket inside the gel balls, and better settlement performance with mild stirring [25]. However, the NLR in our study was lower than the previous study and the period of study was also short. The future study is recommended for a longtime period (at least one year) to upgrade this technology in the full-scale nitrogen removal process.

3.3. Microbial metabolic activity

It was reported that PN bacteria grew only on the surface of biofilm due to easy access to the dissolved oxygen and ammonium [45]. The diffusion of ammonium and nitrite inside the gel beads may affect the activity of the immobilized cell. Moreover, the AOB on the surface of gel beads consumed oxygen to produce nitrite and provided the protection to anammox from oxygen inhibition, and subsequently, anammox consumed nitrite in the proximity area and avoided the inhibition of nitrite on anammox as well as AOB [30]. So, to investigate the metabolic activity of co-immobilized PN-Anammox sludge, the gel beads from the reactor were taken out, washed with deionized water then cut along the middle line position of the beads. Then, the bead slice was placed in the culture medium (synthetic wastewater) under the optical microscope to observe the profile of the metabolic activity of the combined process. After 15 min observation, bubbles appeared from the middle as well as from the surface of the beads (Fig. 3). The appearance of the gas bubbles indicated that the ammonium could be partially oxidized to nitrite by AOB and at the same time anammox utilized produced nitrite with remaining ammonium to produce nitrogen gas. The results confirmed that the diffusion limitation of the substrate inside the beads should be overlooked. The FISH analysis was performed to identify the presence of anammox in the core of the beads [25]. But there is not report about the activity of PN-anammox inside the gel beads. The microscopic image of the gas bubbles formation satisfied the simultaneous function of AOB and anammox inside the core as well as the surface of the beads. It is the first time to experimentally prove the activity of immobilized PN-Anammox sludge inside the beads.

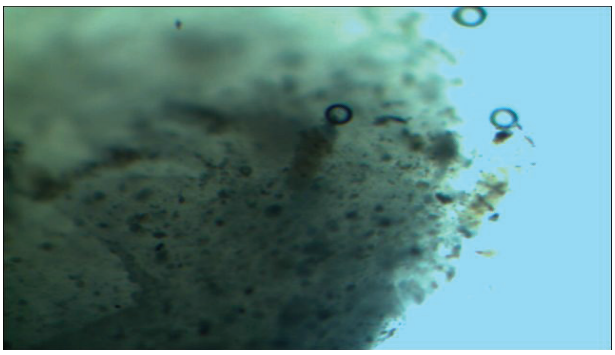

Fig. 3. The release of generated N_2 from immobilized gel balls.

3.4. Cell apoptosis analysis

Generally, cell undergoes death by two ways, i.e. necrosis caused by some external forces and apoptosis (also called programmed cell death) due to some cellular phenomena. A study showed that the *Trichodesmium* spp., marine cyanobacteria experienced the programmed cell death under the phosphorus and nitrogen starvation [46]. In order to verify the existence and activity of nitrifying bacteria and anaerobic ammonium oxide bacteria in a large number of gel particles, the detection of immobilized sludge cells was carried out with apoptosis and necrosis assay kit in this experiment. The gel particles in the reactor were taken out and observed. The figure divided into live cell region and death cell region. The Q2-3 depicted the live cell region while Q3-2 showed death cell region. The cluster of the cell in both region characterized if there is any death phenomena inside of the beads. There were distinct clusters of cells in the active cell region of Q2-2 (Fig. 4a), but there was no distinct cluster in Q2-3, indicating that there is no obvious death phenomenon inside of the gel beads and the cells could carry out their life activities in the immobilized reagent. The bacteria developed strategies were helpful for bacteria to persist in the unfavorable environment for a long time [47]. To confirm that either the above description is true or not, the particles from the reactor were cultured in the non-nutritious environment. After three days the detection of the cell was carried out as shown in Fig. 4b. There were apparent clusters of cell in the live cell section Q4-3 as well as death cell region Q4-2. Fig. 4b confirms that the substrate was easily diffused inside of the beads. Apoptosis was observed under the non-nutritious environment. So, there was no environmental stress on the bacteria and bacteria could carry out the removal of nitrogen efficiently in the presence of substrate. If there were any substrate constrains in the core of the beads, the apoptosis analysis of the fresh beads would show the cluster in the death cell region. The distinct difference between clusters of bacteria in the active cell region as well death cell region before and after the starvation treatment confirmed that bacteria were active in the core of the beads in the presence of essential substrate. The events similar to apoptosis were perceived in *Staphylococcus pneumoniae* when treated with HAMLET (a complex of milk lipid and protein) [48]. This is another confirmation of the immobilized cell activity within the gel beads in a normal treatment process with immobilized bacteria, and there was not any nutritious limitation within the gel balls.

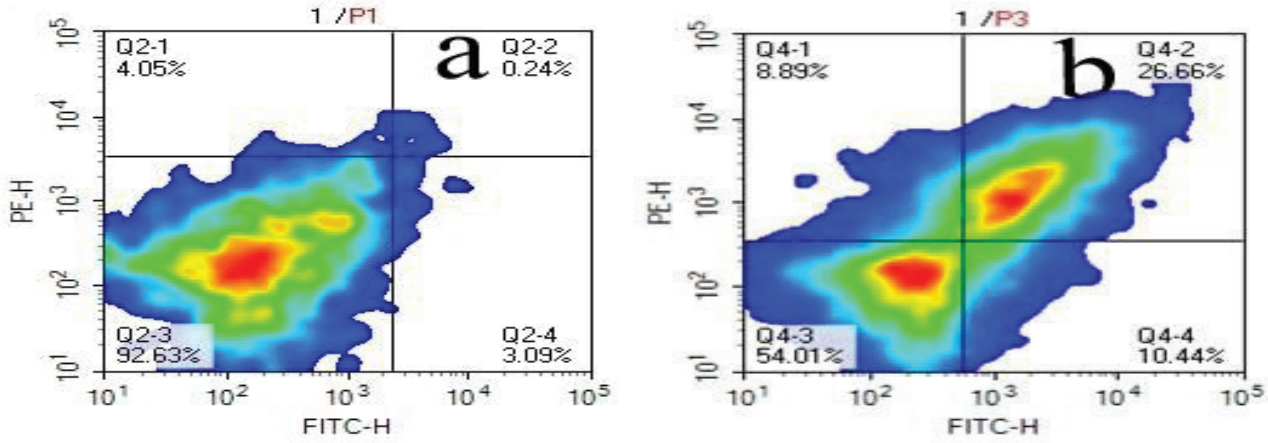

Fig. 4. The apoptosis of immobilized bacteria before (a) and after (b) starvation treatment.

3.5. Analysis of microbial community

In order to investigate the microbial community in the SNAP system, 16SrRNA-based pyrosequencing was used to evaluate the community composition and specific content of microbes. The microbial sample was collected at the end of the experiment (day 54). The results of high-throughput sequencing analysis at three different levels phylum, class, and genus are depicted in Fig. 5 representing the microbial structure of the SNAP process. At phylum level (Fig. 5a), the dominant phyla were in the order of *Proteobacteria, Chlorobi, Chloroflexi, Firmicutes, Acidobacteria, Planctomycetes, Bacteroidetes, Nitrospirae,* and *Actinobacteria*, accounting for about 94.44%. Among them, the phylum *Proteobacteria* occupied the proportion of 35.35%, and *Planctomycetes* shared about 2.42%. Another study showed that bacteria involved in the SNAP reactor commonly consisted of above reported phylum [49]. *Proteobacteria* was the most abundant phylum in the anaerobic reactor fed with pretreated wastewater [50]. The phylum *Chlorobi* and *Chloroflexi* were also reported in the partial nitritation and anammox system [37,51]. The phylum *Chloroflexi* consumed the soluble microbial product and decayed material of autotrophs [52,53]. Moreover, Fig. 5b showed the microbial distribution of the system at the class level. The class *Ignavi bacteria* (18.94%) belonging to the phylum *Chlorobi* and the class belonging to the phylum *Proteobacteria* including *Betproteo bacteria* (18.94%) were the most abundant classes followed by *Anaerolineae* (13.97%) and the abundance of other classes belong to the phylum *Proteobacteria* including *Gammaproteo bacteria* (8.07%), *Deltaproteo bacteria* (4.18%) and *Alphaproteo bacteria* (4.07%). The abundance of *Betproteo bacteria* was more compared to the previous study [54] and less than that reported by [55]. The class *Planctomycetacia* is belonging to the phylum *Planctomycetes* which includes anammox also presented with the abundance of 1.71%.Furthermore, the microbial community distribution of microorganisms at the genus level was shown in Fig. 5c. The relative abundance of *Nitrosomonas* (AOB) was 0.89% which was lower than that of *Burkholderia* (3.91%) and *Candidatus Brocadia* (1.33%). The lower abundance of AOB can provide required amount of nitrite for simultaneously anammox process, and was not limited step [56]. While to maintain the balance between AOB and anammox percentage is also problematic in suspended growth as well as biofilm system. Moreover, three more genera of anammox bacteria were also identified including *Candidatus Kuenenia, Candidatus Jettenia,* and *Candidatus Nostocoida*. In sequencing batch reactor, the anammox strain *Candidatus Brocadia* and *nitrosomonas* also reported in combined PN/Anammox process [57]. *Candidatus Brocadia* and *Candidatus Kuenenia* were spotted as the principal anammox genera in the laboratory scale reactor fed with synthetic wastewater [58]. A higher proportion of *Candidatus Brocadia* compared to other anammox species may attribute to the higher growth rate of *Candidatus Brocadia* than other anammox species [59].

4. Conclusion

The study proved that immobilization of PN-anammox is suitable to decrease the startup period compared to granulation and suspended growth system. The PN process made sure the availability of nitrite for the anammox in the vicinity area. Anammox consumed nitrite from the PN process with remaining ammonia and released the nitrogen gas in the form of bubbles with the production of nitrate.

The apoptosis analysis is a good approach to know the activity of bacteria, providing information about the death and life of the bacteria. This approach can be applied in the granular system to know the behavior of granules in the system. Here, the apoptosis analysis proved that there was not any death phenomenon inside the beads which guaranteed the suitable environment and diffusion of the substrate inside of the beads for the growth of anammox. The phylum *Proteobacteria* (35.35%) was the most abundant among all other detected phyla while at genus level *Candidatus Brocadia* and *Nitrosomonas* were the primary anammox and partial nitrifying bacteria. Moreover, three more genera of anammox bacteria were also identified including *Candidatus Kuenenia, Candidatus Jettenia,* and *Candidatus Nostocoida*. The *Candidatus Brocadia* have advantage of higher growth rate as compared

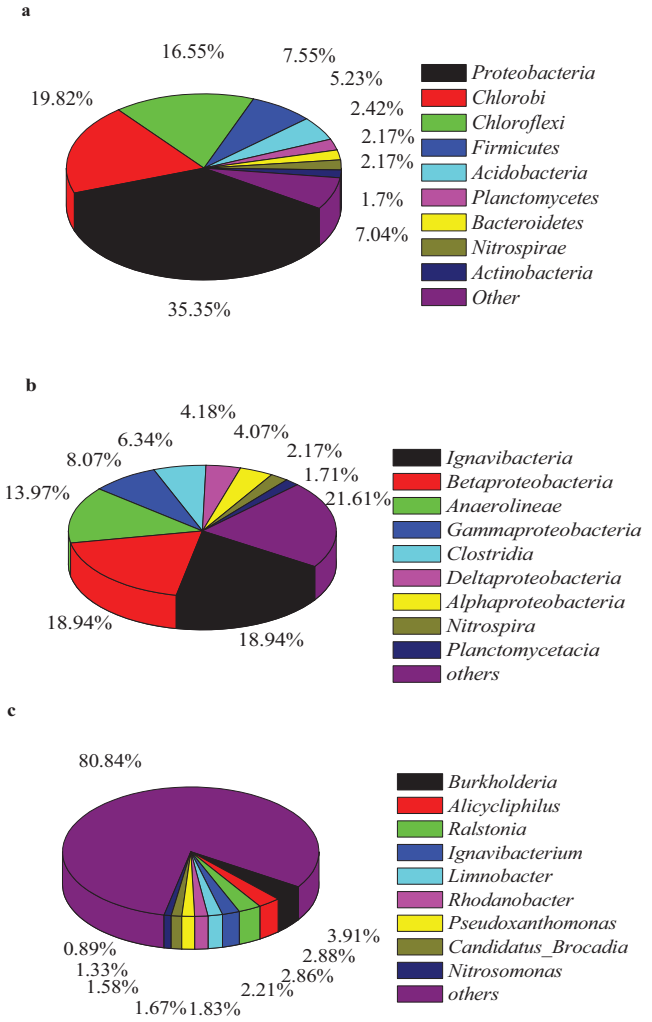

Fig. 5. Relative abundance of different communities at phylum level (a) class level (b) and genus level (c).

to other genera of anammox, which could increase the abundance of *Candidatus Brocadia* in the SNAP system.

Author contributions

Xueyou Liang performs the research; Shou-Qing Ni and Zhaojie Cui design the research; Hafiz Adeel Ahmad writes the paper; Haochen Ni revises the paper. All authors mutually agree for the submission of the manuscript.

Competing of interest

There is no competing interest.

Funding

This work was supported by the Shandong Provincial Water Conservancy Research and Technology Promotion Project (SDSLKY201802) and Shenzhen Science and Technology Research and Development Funds (JCYJ20170307092918052).

Acknowledgements

The authors acknowledge the efforts of Cuina Bu and Chenghao Gefor proof reading.

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