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Nitrogen removal characteristics and microbial community structure analysis of anammox-bacteria immobilized using polyethylene glycol diacrylate

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ABSTRACT

In the present study, anaerobic ammonium-oxidizing (anammox) bacteria is immobilized in carrier materials of polyethylene glycol diacrylate (PEGDA) using N,N,N,′N′-tetramethylethylenediamine (TEMED) as the promoter and potassium persulfate (KPS) as the initiator, respectively. Based on orthogonal experiments, the optimal conditions of immobilizing is found to be 10% PEGDA monomer, 0.25% KPS, and 0.5% TEMED for polymerizing at 20°C for approximately 5 min, with bacteria to gel ratio of 1:1. In a continuous-flow experiment, the average ammonium and nitrite removal rate of immobilized anammox granules with 8 h hydraulic detention time (HRT) gradually increases after a short period of activity recovery, and finally reaches 91% for ammonium and 83% for nitrate. The removal rate of ammonium and nitrite is still over 50% (63% and 55%, respectively) as the HRT decreases to 4 h, indicating that the immobilized granules have a strong resistance to nitrogen shock-loading. Additionally, no granule crushing or suspended solids are observed in the effluent during the 100 d operation, indicative of the strong mechanical properties of the anammox immobilized granules. Notably, the content of heme *c* in the anammox immobilized granules rises with a corresponding increase of nitrogen removal rate, and reduces due to the nitrogen loading shock. The formation of channels inside the PEGDA granules and a large number of anammox bacteria filling the channels is observed by scanning electron microscopy. Microbial community analysis using high-throughput sequencing reveals that Candidatus Kuenenia, the primary anammox bacteria inside the PEGDA granules, increases from 6.58% to 9.8% after the 100 d run, indicative of superb biocompatibility and substrate transferring performance of PEGDA as a carrier.

Keywords: Anammox; Polyethylene glycol diacrylate; Immobilization; Microbial community structure

1. Introduction

Anaerobic ammonia oxidation (anammox) refers to the process where a type of Planctomycetes bacteria uses nitrite as an electron acceptor to convert ammonium into N_2 and produces a small amount of nitrate under anaerobic or anoxic conditions [1–3]. In 1977, Broda [4] predicted that there were autotrophic bacteria in nature that could conduct the ammonia oxidation reaction using nitrate or nitrite as oxidizer. Strous [5] discovered such autotrophic bacteria hidden in nature at the end of the 1980s, and subsequently, anammox processes were developed by the Kluyver Biotechnology Laboratory in 1990. Compared with traditional biological wastewater treatment technology, the anammox process has many advantages including low energy consumption, occupying less area, creating less excess activated sludge,

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and has no need for an additional organic carbon source [6]. However, there are also many issues with this process including easy loss of biomass, slow granule formation, and more sensitivity to environmental factors in the nitrogen removal process for both flocculent and granule anammox sludge [7,8]. Therefore, reducing or preventing the loss of anammox bacteria and maintaining biomass in the reactors have become the focus of research and development of the anammox technique [9].

Microbial immobilized technology is a novel biological nitrogen removal technology developed in the modern bioengineering field which has been in practical application since its initial introduction into the field of water treatment in the mid 1970s. By fixing free bacterial cells or enzymes in a constrained area through specific methods, microbial immobilized technology can reduce or even prevent the loss of anammox bacteria, further improving the utilization rate of functional bacteria, simplifying the treatment process, and enhancing the efficiency of the application [10]. Immobilizing technology usually entraps the microbial strains within semi-permeable polymer gel or membrane by specific materials to maintain the biomass [11,12]. However, existing immobilizing materials such as carrageenan, sodium alginate (SA), and Polyvinyl alcohol (PVA), or SA-PVA, which are used for preparing anammox embedded granules, generally exhibit a series of problems such as weak mechanical strength, low biological activity, and lack of stability long-term [13–15]. The bioactivity and nitrogen degradation efficiency of immobilized granules cannot be obviously strengthened even through several enhancing methods [16,17]. It is difficult to immobilize anammox bacteria by embedding materials due to its long generation cycle, low cell yield, and sensitivity to environmental conditions [18,19].

Polyethylene glycol diacrylate (PEGDA) is reported to have superb mechanical stability and good mass transfer capability as a new type of immobilization material. In previous studies, PEGDA immobilized granules have been predominantly used in denitrification or nitrification, with satisfying nitrogen removal efficiency. Li et al., [20] reported that the nitrogen degradation rate of nitrifying granules reactor immobilized by PEGDA reached up to 39 mg N/ (L·h) after 30 d of operation, which was higher than 25 mg N/ (L·h) of nitrifying biofilm reactor filled with plastic elastic fillers. Isaka et al., [21] applied the PEGDA immobilized denitrifying granules to treat nitrate synthetic waste water, finding that the nitrogen removal rate in the denitrification process was as high as 4.4 kg N/(m^3·d) after 16 d operation. Thus far however, the nitrogen removal characteristics and microbial community structure of PEGDA immobilized anammox granules in long-term operation are still unclear. Additionally, heme *c*, which is a vital cofactor of both hydroxylamine oxidoreductase and hydrazine oxidoreductase in anammox bacteria, is reported to contribute to the red color of anammox sludge [22]. The content of heme *c* is closely related to the specific anammox activity (SAA) and nitrogen removal rate (NRR). In this study, the immobilized experiment was carried out on anammox bacteria using PEGDA as immobilized materials. The orthogonal experiments were designed to optimize immobilizing conditions for superb mechanical properties and nitrogen degradation efficiency of anammox immobilized granules. Along with nitrogen removal efficiency, the variation of heme *c* content and microbial community structure in the long stable operation was investigated to provide theoretical basis for the application of immobilizing techniques in wastewater treatment.

2. Materials and methods

2.1. Experimental materials and instruments

The anammox sludge used in the present study was taken from an up-flow anaerobic sludge blanket (UASB) reactor with stable operation of four years in the authors' laboratory [23]. Experimental agents including PEGDA, tetramethylethylenediamine (TEMED), potassium persulphate (KPS), $NaH_2PO_4{\cdot}2H_2O$, $Na_2HPO_4.12H_2O$ and KCl were all AR grade. The PEGDA was purchased from the Sigma official website (https://www.sigmaaldrich.com).

Synthetic wastewater was also used in the experiments. The main components of wastewater are presented in Table 1. Trace elements I and II refer to [23]; its pH ranged from 7.5 to 8.0. Trace element I consisted of 5 g·L^{-1} of $FeSO_4$ and 5 g·L^{-1} of EDTA. Trace element II was made up of EDTA 15 g·L^{-1}, $CuSO_4{\cdot}5H_2O$ 0.2 g·L^{-1}, $ZnSO_4{\cdot}7H_2O$ 0.43 g·L^{-1}, $CoCl_2{\cdot}6H_2O$ 0.24 g·L^{-1}, $MnCl_2{\cdot}4H_2O$ 0.99 g·L^{-1}, $NaMoO_4{\cdot}2H_2O$ 0.22 g·L^{-1}, $NiCl_2{\cdot}6H_2O$ 0.19 g·L^{-1}, $NaSeO_4$ 0.11 g·L^{-1}, and H_3BO_3 0.014 g·L^{-1}.

2.2. Preparation of PEGDA-immobilized granules

Preparation of Sludge Concentrate, The anammox granule sludge taken out of the UASB reactor was broken and then washed 2 to 3 times by phosphate buffered saline (PBS, 0.1 M and pH = 7.4) to remove residual substrate on the sludge surface. Sludge concentrate was then obtained after centrifuging for 10 min at 4,000 rpm.

Immobilization procedure, Varying qualities of PEGDA polymers and TEMED as promoter for orthogonal experiments were dissolved in phosphate buffer solution. The pH value was adjusted to 7.0 and the prepared solution and sludge concentrate of anammox bacteria was mixed homogeneously. KPS, as initiator, was added immediately to initiate polymerization, and the solution was rapidly stirred by magnetic stirrer with constant temperature. After polymerization for 30 min under temperature from 20°C to 30°C, the mixture solution became solid and formed a gel which was cut into 3 × 3 × 3 mm cubes to obtain the PEGDA immobilized granules. The prepared immobilized granules were rinsed thoroughly using deionized water to wash out the non-cross-linked PEGDA monomer and unfixed anammox bacteria.

Table 1
Compositions of artificial wastewater

Main components	Mass concentrations
KH_2PO_4	25 mg/L
$CaCl_2$	120 mg/L
$MgSO_4{\cdot}7H_2O$	260 mg/L
$KHCO_3$	753 mg/L
Trace element I	1 ml/L
Trace element II	1 ml/L

2.3. Measurement of mechanical stability of the immobilized granules

Thirty granules of similar size were selected and added to the serum bottle reactor, as shown in Fig. 1. Next, 400 mL of deionized water was added, and the proportion of the number of intact immobilized granules to the total number of granules was observed after 48 h of 600 rpm magnetic stirring [7].

2.4. Orthogonal experimental designs

There are many factors that affect the bioactivity of anammox bacteria and the treatment efficiency of synthetic wastewater by immobilized granules, including the concentrations of PEGDA polymer, promoter (TEMED), initiator (KPS), polymeric temperature, and the ratio of bacteria and gel. These five factors were used in the orthogonal experimental designs to optimize polymeric conditions. Two orthogonal tests were designed to investigate the influence of these individual elements on the relative bioactivity and mechanical stability of the immobilized granules. The experimental conditions used in the orthogonal polymerization tests are illustrated in Tables 2 and 3.

2.5. Determination of anammox performance

Measurement of the anammox performance of immobilized granules was conducted using the device pictured in Fig. 1. First, 40 ml of activated immobilizing granules were put into a 500 ml serum bottle which was covered by black shading material. Subsequently, 400 ml of synthetic wastewater was added, and the mixture was put into a constant temperature magnetic stirrer. High-purity N_2 was passed through the air intake for about 30 min to blow out dissolved oxygen in the water, and guarantee an anaerobic environment. The pH was adjusted by 1 mol/L HCl/NaOH. The magnetic agitator rotated at the speed of 150 rpm, and the temperature was kept at 30°C. Samples were taken from the air inlet with a syringe at time intervals. Each experiment was undertaken in three groups to obtain an average.

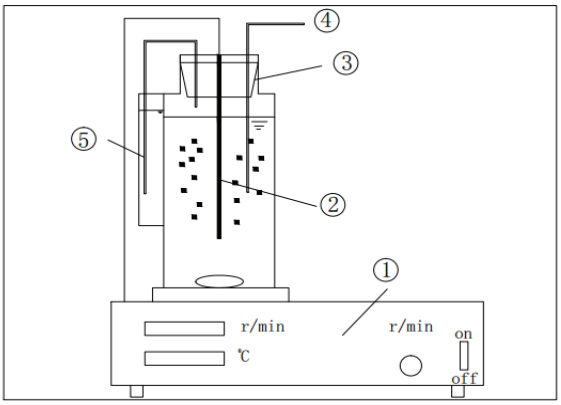

Fig. 1. ANAMMOX performance measurement device of immobilized granules: (1) constant temperature magnetic stirrer, (2) temperature probe, (3) serum bottle, (4) air inlet, and (5) Air outlet.

Table 2
Factors and levels of the first orthogonal experiment

Levels	A	B	C	D
	PEGDA (%)	TEMED (%)	KPS (%)	Temperature (°C)
1	8	0.25	0.25	20
2	10	0.5	0.5	25
3	12	1	1	30

2.6. Long-term continuous flow experiment

To further investigate the nitrogen removal efficiency of anammox immobilized granules, a long-term continuous flow reactor was set up. An up-flow UASB reactor with the effective volume of 6 L was used as the operation device, and the external surface was covered with a shading insulation layer. The operating temperature was stabilized at around 32°C by water bath. Next, the immobilized granules were homogeneously distributed in the reactor through a flow-separated ball with volume filling rate of 10%. Synthetic wastewater was used in the experiment, and the concentrations of NH_4^+-N and NO_2^--N were about 35 and 45 mg/L respectively. The concentration of other components was prepared according to Table 1.

2.7. Determination of heme c content during the long-term operation

The anammox sludge without immobilizing and PEGDA immobilized granules cultivated in the continuous flow reactor at 0, 10th, 41st and 100th d was taken out and broken by mortar. The fragments of immobilized granules were washed three times by PBS to remove residual substrate on the sludge surface. The volume of washed sediments was set at 20 mL by PBS, and 200 μL lysozyme solutions with concentration of 25 mg/mL was added. Subsequently, the mixture reacted at 37°C for 30 min and was stored at 20°C overnight. Next day, the anammox fragments were unfrozen and broken by ultrasonic for 10 min at 225 W and 4°C. Finally, the supernatant was obtained by centrifugation at 12,000 rpm for 20 min at 4°C, to store at the same temperature and be used for the determination of protein and heme *c* content. The concentration of heme *c* was determined according to the method described by Tang et al., [19]. Protein content was measured by the modified Bradford protein assay kit (Sangon Biotech, China) according to the manufacturer's instructions.

Table 3
Factors and levels of the second orthogonal experiment

Levels	A	B	C	D
	PEGDA (%)	TEMED (%)	KPS (%)	The ratio of bacteria and gel
1	8	0.25	0.25	0.5:1
2	10	0.5	0.5	1:1
3	12	1	1	1:2

2.8. Scanning electron microscopy and microbial community analysis

2.8.1. Morphological properties of immobilized granules

Scanning electron microscopy (SEM) analysis was used to observe the morphological structure of immobilized granules. The bacterial immobilized granules were taken out from the reactor, cleaned with PBS, and fixed by 2.5% pentanediol for 1.5 h. Next, the granules were washed with PBS buffer three times. Subsequently, gradient dehydration of granules was conducted using ethanol with the volume fraction of 50%, 70%, 80%, 90%, and 100% for 10 to 15 min at each time. Lastly, isoamyl acetate was used for displacement. A 1,500 nm thick layer of metal membrane was coated on the sample surface after freeze drying for 24 h, and a Hitachi S-4300 SEM was used for observation.

2.8.2. DNA extraction, PCR and high-throughput sequencing

To investigate the effect of immobilized materials on anammox bacteria and the variation of community structures and species diversity in the continuous-flow operation, two granule samples were collected to be analyzed and sequenced using the technique of Illumina high-throughput sequencing. In the microbial analysis experiment, initially immobilized anammox granules and the granules cultivated for 100 d were named sample S1 and S2, respectively. The E.Z.N.A™ Mag-Bind Soil DNA Kit (OMEGA) was applied to extract the total genomic DNA on the basis of the manufacturer instructions. The integrity and concentration of DNA was checked using 1% agarose gel and a NanoDrop ND-1000 (NanoDrop Technologies, USA). Illumina bridge polymerase chain reaction (PCR) was conducted by two cycles of amplification using the compatible primers. The V3–V4 regions of bacterial 16S rDNA gene were amplified by PCR using the primers 341F: CCTACGGGNGGCWGCAG/ and 805R: GACTACHVGGGTATCYAAYCC. The PCR reaction mixture (30 μL) consisted of 15 μL 2×Taq master Mix, 1 μL primer F (10 μM), 1 μL primer R (10 μM), 10–20 ng genomic DNA, and added ddH_2O of 30 μL. The reaction conditions for PCR amplification were 3 min of denaturation at 94°C, 5 cycles of 30 s at 94°C, 20 s at 45°C, 30 s at 65°C, 20 cycles of 20 s at 94°C, 20 s at 55°C, 30 s at 72°C, and 10 min at 72°C. The second amplification was carried out using PCR product of the first round as a template. The components of PCR mixture (30 μL) included 15 μL 2×Taq master Mix, 1 μL primer F (10 μM), 1 μL primer R (10 μM), 20 ng PCR products, and supplied ddH_2O to 30 μL. The PCR reaction conditions were 3 min of denaturation at 95°C, 5 cycles of 20 s at 94°C, 20 s at 55°C, 30 s at 72°C, and 5 min at 72°C. The DNA produced in the PCR was then purified and quantified using the kit. Finally, the DNA of both sample S1 and S2, with the concentrations of 20 pmol, were sequenced by Illumina Miseq™ in Shanghai Sangon Biotech, China.

2.9. Analytical methods and statistical analysis

The concentrations of NH_4^+-N, NO_2^--N, and NO_3^--N were measured using standard methods [24]. Temperature and pH were determined by WTW/Multi 3420 multiparameter. All data were expressed as the mean ± standard deviation. One-way ANOVA was carried out in SPSS software for statistical analysis. Significant differences were assessed using a post hoc least significant difference test. Differences between samples were considered significant at $p < 0.05$.

3. Results and discussion

3.1. Optimization of immobilizing conditions by orthogonal design experiments

According to the single factor experiments, the formation time of the immobilized gel was closely related to the temperature of the polymerization process (the specific data was not listed). The higher reaction temperature was usually accompanied by a shorter gel forming time when the concentrations of PEGDA polymer, promoter (TEMED) and initiator (KPS) remained constant. It is worth noting that the reaction of gel polymerization struggled to occur when the temperature was below 15°C. The polymerization time of gel shortened sharply with the increase of temperature in the range of 20°C–30°C, and the formation of carrier took only five min when the polymerization temperature was 20°C. However, in overly high polymerization temperatures, the reaction rate will be too rapid, which can lead to inhomogeneous distribution of cross-linking product. This could also cause the water gel to become muddy and with uneven hardness. In the present study, considering the inactivated effect of high temperature on microorganisms and controlling the appropriate rate of polymerization, the temperature was maintained between 20°C–30°C. The other parameters were set as follows: PEGDA polymer concentrations were within the range of 8%–12%, the promoter (TEMED) concentrations were within the range of 0.25%–1%, initiator (KPS) concentrations were within the range of 0.25%–1%, the ratio of bacteria and gel were within the range of 0.5–2, and polymeric time was 5 min.

The effect of the various factors on the relative bioactivity of immobilized granules obtained by orthogonal test is shown in Table 4. Based on the results of the orthogonal test,

Table 4
Results of the first orthogonal experiment

Experimental number	A	B	C	D	Relative activity (%)
1	8	0.25	0.25	20	65±1.21
2	8	0.5	0.5	25	50±2.11
3	8	1	1	30	30±0.89
4	10	0.25	0.5	30	39±1.01
5	10	0.5	1	20	53±3.10
6	10	1	0.25	25	43±0.55
7	12	0.25	1	25	42±1.19
8	12	0.5	0.25	30	29±0.61
9	12	1	0.5	20	40±2.15
K1	48.33	48.67	45.67	52.67	
K2	45	44	43	45	
K3	37	37.67	41.67	32.67	
R	11.33	11	4	20	

the optimal combination of the four factors was $A_1B_1C_1D_1$, that is, 8% PEGDA polymer, 0.25% promotor (TEMED), 0.25% initiator (KPS) and 20°C reaction temperature. The influencing degree of each factor followed the order: temperature > PEGDA polymer concentration > promotor (TEMED) concentration > initiator (KPS) concentration. The result of the optimization experiment revealed that polymerization temperature was the main factor influencing the bioactivity of immobilized granules which was consistent with the aforementioned single factor experiments. It was clear that a smaller dosage of reagents and lower temperature (in the range of 20°C–30°C) could lead to higher bioactivity of immobilized granules. The promoter TEMED produced biological toxicity on anammox bacteria. Thus, it is advisable to reduce the dosage of TEMED to the greatest degree on the basis of ensuring successful polymerization and preventing it from causing irreversible effects on the activity of immobilized granules.

The influence of different factors on the mechanical stability of immobilized granules is described in Table 5 which indicates that the mechanical strength of all the experimental groups reached over 80%. Chen at al., [7] demonstrated that the mechanical strength of SA, PVA, and SA-PVA-immobilized granules was 47%, 73%, and 60%, respectively, after 48 h of high-speed rotation. The various immobilized granules have different mechanical stability due to the diverse properties of the immobilization materials. The SA is a natural polymer, which could form the network structure with Ca^{2+} in the cross linking agent. This network structure was easily damaged by the Mg^{2+} and phosphates in the water, causing the relatively low mechanical strength. The PVA is a synthetic polymer, which contributed to the good mechanical strength and swelling properties of the immobilized granules. However, many hydrophilic hydroxyl groups in PVA molecules cause the PVA immobilized granules to conglutinate. The auto-condensing properties of PVA decrease the specific surface area, which significantly affects the mass transfer properties [25]. Some researchers introduced SA into PVA to prepare PVA-SA immobilized granules. The –OH bonds on the molecular chains of PVA can form hydrogen bonds with the $–COO^-$ in SA, which could improve the conglutination ability of immobilized granules. Meanwhile, the macro-porous network structure of SA molecules could promote the mass transfer property of PVA. However, the introduction of SA will cause the immobilized granules to become soft, swollen, and easily crushed during long-term operation. As a synthetic polymer, PEGDA demonstrated good mass transfer ability and mechanical strength as immobilized material. The PEGDA-immobilized granules demonstrate satisfying nitrogen removal performance [20,21]. In this orthogonal test, the results of experimental group number 2 and 6 reached 93.33% and 96.67%, respectively, exhibiting superb mechanical stability. Factors that affect the mechanical stability were found to follow the order: PEGDA polymer concentration > ratio of bacteria and gel > initiator (KPS) concentration > promotor (TEMED) concentration. The optimal combined level of four factors is as follows: 10% PEGDA polymer, 1% promotor (TEMED), 0.25% initiator (KPS) and the ratio of bacteria and gel at 1:1. Results obtained from Tables 4 and 5 show 0.5% TEMED is able to meet the requirements of the mechanical stability of immobilized granules. Therefore, the optimal conditions of immobilization were selected as follow: 10% PEGDA, 0.5% TEMED, 0.5% KPS, and the ratio of bacteria and gel at 1:1.

Table 5
Results of the second orthogonal experiment

Experimental number	A	B	C	E	Mechanical stability (%)
1	8	0.25	0.25	0.5:1	86.67±0.89
2	8	0.5	0.5	1:01	93.33±1.21
3	8	1	1	2:01	80±2.05
4	10	0.25	0.5	2:01	83.33±1.15
5	10	0.5	1	0.5:1	90±3.22
6	10	1	0.25	1:01	96.67±0.99
7	12	0.25	1	1:01	80±1.54
8	12	0.5	0.25	2:01	80±1.22
9	12	1	0.5	0.5:1	80±0.71
K1	86.67	83.33	87.78	85.56	
K2	90	87.78	85.55	90	
K3	80	85.56	83.33	81.11	
R	10	2.22	4.45	8.89	

3.2. Long-term continuous flow operation

To further investigate the nitrogen degradation characteristics and mechanical stability of the PEGDA immobilized anammox bacteria in long-term operation, a method in which the hydraulic retention time was regularly decreased in different stages was used for the continuous-flow reactor. Fig. 2 presents the variations of influent and effluent nitrogen of the reactor during the experimental period, which reveals that the previously mentioned polymeric reaction caused slight repression on the bioactivity of anammox bacteria. During a period of 0–10 d, the activity of immobilized granules was gradually recovered with hydraulic detention time (HRT) of 8 h. The degradation rate of NH_4^+–N and NO_2^-–N increased from 24% and 22% initially to 48% and 43%, respectively. On the 40th d, the concentrations of effluent NH_4^+–N was 2.98

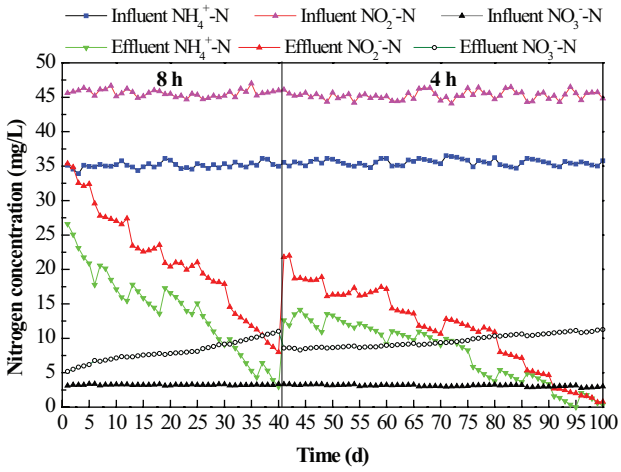

Fig. 2. Variation curves of NH_4^+-N, NO_2^--N, NO_3^--N in influent and effluent of the continuous-flow reactor.

and NO_2^-–N was 7.97 mg/L. Accordingly, the removal rates rose to 91% and 83%, respectively, which indicated the successful start-up of the immobilized anammox reactor. Bae et al., [26] demonstrated that superior enrichment efficiency at a NRR of 0.78 kg N/m^3-d of anammox bacteria immobilized by PVA-SA occurred after 42 d recovery. Additionally, Magrí et al., [16] reported that the enrichment of anammox bacteria entrapped in PVA cryogel showed a longer start-up period of 60 d for a NRR of 0.54 kg N/m^3-d. The HRT was decreased from 8 to 4 h on day 41. Though the effluent NH_4^+–N is increased to 12.61 mg/L and NO_2^-–N is increased to 21.78 mg/L, the degradation rates still remained 63% and 55%, which were all over 50%. As shown in Fig. 2, the nitrogen removal rate became stable in all operating stages, suggesting that the PEGDA immobilized granules exhibited strong ability to resist nitrogen shock loading caused by the variation of HRT. The existence of immobilized materials could weaken the inhibitory impact that occurs with an increase of influent nitrogen loading on the anammox bacteria. In the present experiment, there was a stable period of nitrogen removal rate from day 41 to day 46. After this, the bioactivity of granules continued to increase, which was consistent with the phenomenon reported by Bae et al., [27]. The degradation rate of NH_4^+–N and NO_2^-–N finally reached 99.06% and 98.22% with HRT of 4 h at day 100. Lu et al., [28] reported that the ammonium and nitrite removal efficiency of anammox bacteria immobilized by PVA-SA reached 82.3% and 84.7%, respectively, with 24 h HRT. In addition, no suspended anammox sludge or granules broken in the effluent were observed during the continuous-flow operation, indicating the PEGD immobilized granules had superb mechanical stability and sludge retention capacity.

3.3. Variation of heme c content during the long-term operation

For anammox bacteria, heme *c* had a catalytic effect and could promote electron transfer in the enzymatic reaction, a process to which the unique red color of anammox sludge is attributed [29]. Furthermore, the content of heme *c* tended to increase with the rise of anammox activity and nitrogen degradation efficiency. The heme *c* content of the anammox bacteria differs significantly ($p < 0.05$) in the different phases during the long-term continuous flow experiment. As shown in Fig. 3, heme *c* concentration of the initially immobilized granules, equal to 0.21 ± 0.02 μM/mg protein, was lower than that of the anammox sludge (0.34 ± 0.01 μM/mg protein) due to slight inhibition caused by the immobilized materials. After reactivation, the content of heme *c* continuously increased and reached 0.40 ± 0.01 μM/mg proteins on day 20. Notably, the NRRs and heme *c* content was considerably reduced as a result of nitrogen shock-loading caused by the decrease of HRT on day 41, with the corresponding content of 0.32 ± 0.03 μM/mg protein. Subsequently, heme *c* content rose with the increase of SAAs and NRRs from 41–100 d. This finding was consistent with previous study [30]. The content of heme *c* at day 60 and day 100 was 0.54 ± 0.02 and 0.72 ± 0.01 μM/mg protein, respectively. According to the heme *c* content and high nitrogen removal rate, the anammox bacteria could grow well in PEGDA immobilized material and effectively treat nitrogen-rich wastewater.

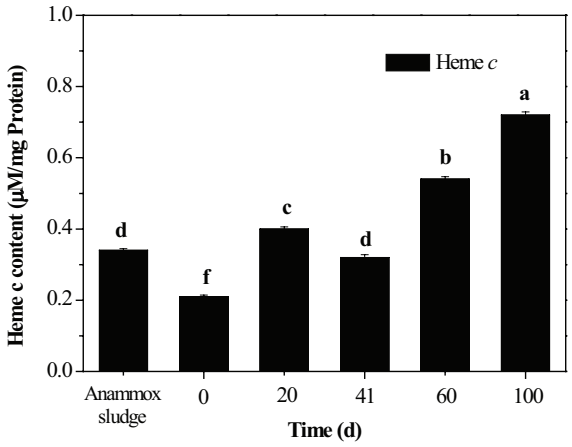

Fig. 3. Heme *c* content of anammox sludge and immobilized granules during the operation. (Data are the means for three independent experiments and presented as the means±SE.).

3.4. Morphological analysis by Scanning electron microscopy

As shown in Fig. 4, the PEGDA immobilized granules were cut into 3 × 3 × 3 mm cubes, differing from the spherical shell of the PVA, SA, and PVA-SA-immobilized granules with poor elasticity, as reported in the previous study [31,32]. The digital image of ANAMMOX-immobilized granules after 100-d continuous (Fig. 4(b)) flow operation exhibited the enrichment of ANAMMOX-bacteria and integrality of granules, suggesting the improvement of ANAMMOX performance and superb mechanical stability. The transparent

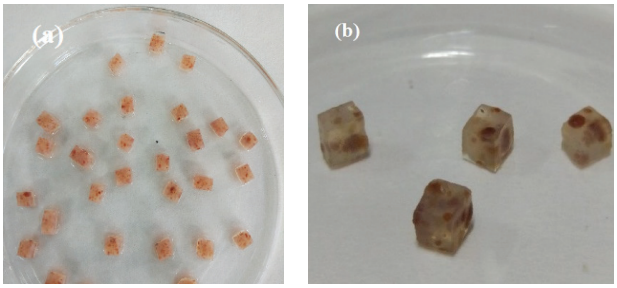

Fig. 4. Digital images of the anammox-immobilized granules, (a) the initial-immobilized granules and (b) the granules after 100-d operation.

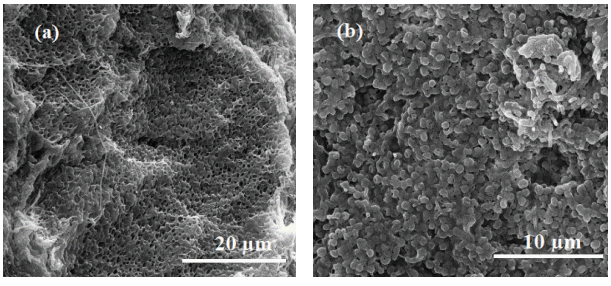

Fig. 5. SEM microscopy analysis of anammox-immobilized granules after 100-d continuous-flow operation, (a) the channel structure on the surface of granules (5000×) and (b) the anammox-bacterium inside the immobilized granules (10000×).

PEGDA immobilized gels exhibited a red color due to the heme *c* produced by anammox bacteria. Fig. 5 shows the surface structure of the immobilized granules and the morphology under SEM after being cut open. As illustrated in Fig. 5(a), there were many channels on the surface of PEGDA immobilized granules (5,000×), indicative of superb mass transfer performance. Fig. 5(b) shows the enrichment of anammox bacteria after 100 d of continuous flow running by the up-flow UASB reactor, where the typical morphology of anammox bacteria is exhibited with spherical shape and volcanic crater-like concaves on the two sides of each bacterium. In summary, it was demonstrated that the immobilized material of PEGDA could provide bacteria a stable environment by weakening the impact of harmful factors. In addition, this immobilized material displayed good mass transfer performance and could maintain the bioactivity of anammox bacteria in the long-term continuous flow operation of the reactor.

3.5. Microbial community analysis by high-throughput sequencing

The comparison of two samples by high-throughput sequencing revealed the succession of the microbial community during long-term continuous flow operation using an up-flow reactor. The statistical analysis indexes of community richness and diversity are provided in Table 6. There were 58,048 and 56,709 sequences obtained from sample S1 and S2, respectively, and effective reads of S1 and S2 were divided into 3,028 and 3,206 OTUs. The results show that the ACE and Chao values of sample S1 were higher than S2, indicating that S1 had more community richness than S2, as reported by Zhao et al., [33]. The coverage values of the constructed sequence libraries in both samples were all over 95%, indicating the high confidence level of the results. The larger Shannon index and the smaller Simpson index revealed a more diverse microbial community. The two Shannon values, 4.54 for S1 and 4.46 for S2, in the present experiment were lower than that of anammox-UASB reactor measured by Du et al., [34] which were equal to 4.94. This result suggests that the more complicated components of real domestic sewage in the research of Du et al., led to the more diverse microbial community. In this study, the diversity of the microbial community decreased and the functional bacteria increased due to the acclimatization caused by nitrogen-loading substrate during long-term operation by the continuous-flow reactor.

Table 6
The analysis of microbial community richness and diversity indices

	S1	S2
Sequences	58,048	56,709
OUT	3028	3206
Shannon	4.54	4.46
ACE	67,369.53	66,074.60
Chao	29,463.57	25,919.52
Coverage	0.96	0.95
Simpson	0.03	0.04

According to Fig. 6, the same seven phyla were identified in the two samples of immobilized anammox granules: Proteobacteria, Planctomycetes, Bacteroidetes, Chloroflexi, Acidobacteria, Ignavibacteriae and Firmicutes, of which the relative abundance was ≥ 1%. However, due to the change of nitrogen-loading, the relative abundance of various bacteria varied with time during the long-term continuous-flow operation. The relative abundance of Proteobacteria decreased from 70.79% of the initial immobilized granules to 48.78% of the granules cultivated for 100 d. The Proteobacteria was still the most dominant phyla in sample S1 and S2, despite a sharp decrease during cultivation which was consistent with the microbial community structure of the anammox reactors described in the previous literature [35]. The phyla of Planctomycetes were the second largest microbial community in the two samples, of which the relative abundance increased from 7.46 to 15.9%, increasing more than two times. The anaerobic ammonia oxidizing bacteria, as the main functional bacteria in the process of anammox, belonged to Planctomycetes at phyla level. Such results reveal the immobilized material of PEGDA is suitable for the growth of anammox bacteria and could promote the enrichment of functional bacteria. The proportion of Planctomycetes measured in this study was higher than that of the anammox sludge granules (3.1%) in the UASB reactor reported by Li et al., [36]. Moreover, the relative abundance of Bacteroidetes and Acidobacteria decreased from 5.48 and 5.25% to 5.25 and 3.48%, respectively, whereas the relative proportion of Firmicutes, Ignavibacteriae, and Chloroflexi increased from 2.91%, 3.61%, and 2.53%, to 11.31%, 4.86%, and 6.08%, respectively.

On a genus level, as shown in Fig. 7, the richest microbial community in the initial immobilized granules (sample S1) was Dokdonella genus, accounting for 9.67%. There were two kinds of typical anammox bacteria, Candidatus Brocadia and Candidatus Kuenenia, identified in the whole process of the continuous-flow reactor, and the relative abundance of Candidatus Kuenenia was far more diverse than that of Candidatus Brocadia genus. This result diverged from the previous study reported by Cao et al., [35], in which Candidatus Brocadia was confirmed to be the most dominant anammox bacteria. The physiological characteristics and

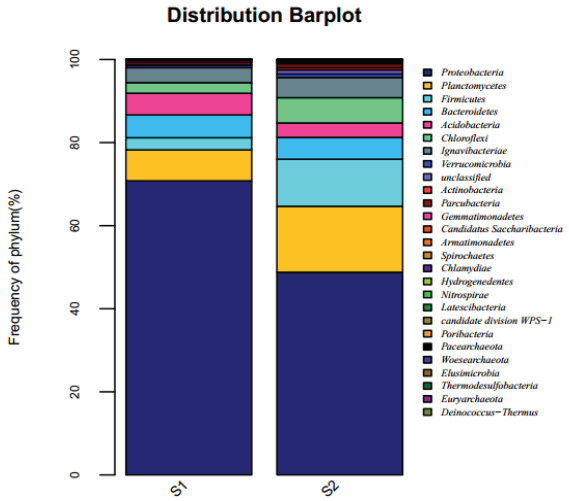

Fig. 6. Relative abundance distribution at phylum level.

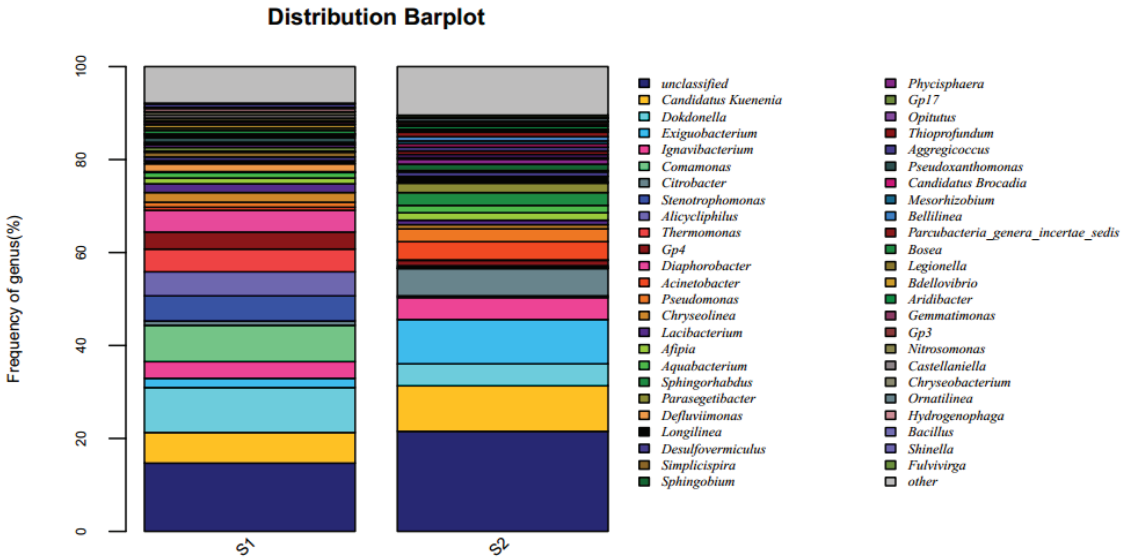

Fig. 7. Relative abundance distribution at genus level.

distribution in anammox reactors of Candidatus Brocadia and Candidatus Kuenenia has been recently reported on. The genus of Candidatus Brocadia, classified as R-strategists with low affinity for substrates and easy ability to adapt to environments consisting of organic carbon, was discovered in an UASB reactor receiving synthetic wastewater containing 50–700 mg/L of COD [37]. Conversely, the genus of Candidatus Kuenenia was identified as K-strategist, surviving at low substrate concentrations by optimizing the substrate affinities [38,39]. The Candidatus Kuenenia, which is widely distributed in freshwater systems and anammox bioreactors, gradually became the most dominant during the continuous-flow operation, increasing from 6.58% to 9.8%. These findings revealed that the PEGDA immobilized material could provide a relatively suitable environment by resisting the shock of harmful substances for anammox bacteria to grow and metabolize, further increasing the biomass. The PEGDA was found to have no negative effect on the anammox bacteria and the microbial community structure as a carrier. Overall, the material of PEGDA was demonstrated as promising for use in practical wastewater treatment by entrapping the functional bacteria in gel granules.

4. Conclusion

In considering the mechanical stability of the immobilized granules, relative bioactivity, and nitrogen removal performance of the anammox bacteria, PEGDA was found to be a superb immobilization material for anammox sludge. For the present study, the optimal polymeric conditions were obtained by orthogonal tests. These are as follows: 10% PEGDA polymer, 0.25% KPS, 0.5% TEMED, with the bacteria to gel ratio of 1:1, at temperature 20°C polymerizing for 5 min. Additionally, the PEGDA immobilized granules exhibited strong shock-loading resistance with excellent functional bacteria retaining ability and good mass transfer performance during the long-term continuous flow operation. The continuous flow reactor maintained stably with the removal rate of NH_4^+–N up to 99.06% and NO_2^-–N up to 98.22% at HRT of 4 h. The heme *c* content increased with the increase of nitrogen degradation efficiency and anammox activity. Based on the microbial community analysis by high-throughput sequencing, the relative abundance of Candidatus Kuenenia, a typical anammox bacteria, increased significantly from 6.58% to 9.8%. The findings revealed that PEGDA used in the study had good biocompatibility and no harmful effect on the growth and metabolism of anammox bacteria.

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