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Effects of phosphate deficiency on the removal of anammox nitrogen and sludge characteristics

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ABSTRACT

In this work, anaerobic ammonium oxidation (anammox) reactor was run for 320 d to study the effect of phosphate deficiency on the removal efficiency of anammox's nitrogen and sludge characteristics. The results showed that long-term phosphate deficiency would lead to the death of anammox bacteria, whereas the nitrogen removal efficiency of anammox deteriorated slowly and continuously. After 28 d of operation, the nitrogen removal rate of the reactor decreased from 23.23 to 10.36 kg N/m^3/d. The addition of phosphate could not prevent the decrease in the removal efficiency of nitrogen. However, the removal efficiency of nitrogen of the reactor could be recovered slowly by reducing the nitrogen loads and adding exogenous phosphate at the same time. The reduction of exogenous phosphate led to the slow release of endogenous phosphate in the sludge system. Phosphate deficiency caused the surface of granular sludge to be coated with calcium carbonate. The volatile suspended solid/suspended solid value decreased, and the extracellular polymeric substances concentration of sludge decreased significantly. The lack of phosphate led to a decrease in the diversity of the microbial communities in the sludge. In short, in order to maintain the long-term stable operation of anammox, the presence of sufficient phosphorus in the system must be ensured.

Keywords: Anammox; *Candidatus Kuenenia*; Granular sludge; Nitrogen removal; Phosphate deficiency

1. Introduction

In 1995, Mulder et al. [1] found that a large amount of ammonia nitrogen disappeared in a fluidized bed during anaerobic denitrification. Based upon their experiments, it was found that 4 mol N_2 was produced when 5 mol ammonia-nitrogen and 3 mol nitrate–nitrogen disappeared under anaerobic conditions. Therefore, the process was named "anaerobic ammonium oxidation (anammox)". With the in-depth study of anammox, the generally accepted reaction equation of the anammox process is given by Eq. (1) [2].

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 0.066CH_2O_{0.5}N_{0.15} + 0.26NO_3^- + 1.02N_2 + 2.03H_2O \quad (1)$$

The microorganisms that can undergo anammox reaction are called the anammox bacteria (AnAOB), whereas the discovered AnAOB belonged to the family Anammoxaceae in the *Phylum planctomycetes* [3]. So far, six species of anammox bacteria have been found by the study of Nikolaev et al. [4], namely the *Anammoxoglobus*, *Anammoximicrobium*, *Brocadia*, *Jettenia*, *Kuenenia* and *Scalindua*, which are divided into 24 species.

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The anaerobic ammonia oxidation process does not require dissolved oxygen and organic carbon sources, whereas the remaining sludge yield is low. Meanwhile, the cost of the anaerobic ammonia oxidation process to treat nitrogen-containing wastewater is far lower than that of the traditional nitrogen removal methods [5], making it a hot research topic in recent years [6]. However, anammox has not yet been widely used. The activity of AnAOB is easily affected by various environmental factors (such as dissolved oxygen (DO), organic carbon, matrix nitrogen, and phosphate), which is one of the limiting factors for its widespread practical application [7,8].

Phosphoric acid (PO_4^{3-}) is one of the essential elements for the formation of phospholipids, nucleic acids and phosphate esters, and plays an important role in the generation and conversion of metabolic energy in the form of Adenosine Triphosphate (ATP) [9,10]. The first report on the effect of phosphate on anammox was conducted by van de Graaf et al. [11], who found that phosphorus was an obvious inhibitor of anammox. Approximately 155 mg-P/L resulted in the complete loss of the activity of anammox, while below 31 mg-P/L had no effect on anammox. Other studies reported different results. Egli et al. [12] found that 620 mg-P/L of phosphate had no effect on the denitrification efficiency of anammox. Pynaert et al. [13] found that 55.8 mg-P/L reduced the anammox activity to 63%. Carvajal found that 310–1,550 mg-P/L enhanced the activity of anammox, whereas the granular sludge increased by 60% [14]. Zhang et al. [15] found that 31–155 mg-P/L could slightly enhance the activity of anammox granular sludge in batch experiments. In the continuous experiments, it was found that the phosphate concentration at 2–500 mg-P/L had no significant effect on the removal efficiency of anammox for nitrogen.

Phosphate is very important for biological cells. Phosphate starvation affects the DNA synthesis of *Thiobacillus ferrooxidans* cells, causing the cells to cease to divide and grow longitudinally [16]. The above-mentioned studies have focused on the inhibition of anammox reaction due to the high concentration of phosphate, and there are only a handful of reports focusing on phosphate deficiency. Xue et al. [17] investigated the effect of phosphate concentration on the anammox-HAP process and reported that for a phosphate concentration of less than 5.7 mg-P/L, a floatation of sludge occurred that caused the deterioration of the overall process. The focus was on the bulking stage and floating of the granular sludge under different phosphate concentrations, without paying attention to the changes in the nitrogen concentration of the reactor's influent. Additionally, the variation in the concentration of phosphate concentration in the influent water was less in the work of Xue et al. [17]. The great tolerance of anammox bacteria to substrate concentration is one of the advantages of anammox [18], because it means that it can withstand higher nitrogen removal efficiency, as compared with the traditional biological nitrogen removal process. Therefore, it is necessary to study high-concentration substrate for anammox activity under phosphate deficiency.

Anammox has been studied to treat a variety of wastewaters with high ammonia nitrogen. Not only high ammonia nitrogen concentration, some special wastewaters, but also own low phosphate content [19], such as waste leachate, coking wastewater, etc. For this kind of wastewater, Liang and Liu [20] conducted a study on the denitrification performance of waste leachate through a combined process of PN-anammox fixed-bed biofilm reactor and land treatment system; Li et al. [21] used a combined denitrification-partial nitrification-anammox process and a dual circulation system to remove ammonia nitrogen from mature waste leachate; Toh and Ashbolt [22] explored the anammox treatment of coking wastewater and successfully initiated the anaerobic ammonia oxidation process. However, these reports hardly considered the effect of long-term phosphate deficiency on anammox and could not systematically demonstrate the reliability of anammox for high ammonia nitrogen and low phosphate wastewater treatment, so it is necessary to study the effect of phosphate deficiency on the anammox process.

The purpose of the current paper is to analyze the effects of long-term phosphate deficiency on denitrification efficiency, the surface structure of granular sludge, and the structure and abundance of microbial population in the sludge of anammox under high-concentration substrate conditions. The significance of this study is to provide a theoretical reference for the application of anammox in engineering, especially in the treatment of wastewater containing low phosphorus and high ammonia nitrogen, and the combination of anammox combined with the phosphorus removal process.

2. Materials and methods

2.1. Synthetic wastewater and inoculums

Anammox seed granules harvested from a laboratory-scale up-flow anaerobic sludge blanket (UASB) reactor were used for batch and continuous-flow experiments. This parent reactor was fed with synthetic wastewater as summarized in Table 1, and the pH of the influent was about 7.5 ± 0.3. Concentrations of total nitrogen and P in influent water at each stage are shown in Table 2. It has

Table 1
Composition of the synthetic wastewater

Composition	Composition
NH_4Cl	Add as required[a]
$NaNO_2$	Add as required[a]
KH_2PO_4[b]	Add as required
$NaHCO_3$	1,000 mg/L
$MgSO_4{\cdot}7H_2O$	300 mg/L
$CaCl_2{\cdot}2H_2O$	56 mg/L
Trace element I[c]	1.0 mL/L[e]
Trace element II[d]	1.0 mL/L[e]

[a]Molar concentration ratio of NH_4^+–N and NO_2^-–N was 1:1.2.
[b]KH_2PO_4 was the only source of phosphate.
[c]Trace element solution I was composed of 5.00 g/L EDTA, 5.00 g/L $FeSO_4$.
[d]Trace element solution II was composed of 15 g/L EDTA, 0.014 g/L H_3BO_4, 0.99 g/L $MnCl_2{\cdot}4H_2O$, 0.25 g/L $CuSO_4{\cdot}5H_2O$, 0.43 g/L $ZnSO_4{\cdot}7H_2O$, 0.21 g/L $NiCl_2{\cdot}6H_2O$, 0.22 g/L $NaMoO_4{\cdot}2H_2O$ and 0.24 g/L $CoCl_2{\cdot}6H_2O$.
[e]1.0 mL of trace element solutions I and II were added per liter of wastewater.

been operating stably for more than six months, and the nitrogen removal rate (NRR) is about 4.32 kg N/m^3/d. The granules had a mean diameter of 2.4 ± 1.5 mm.

2.2. Anammox reactors and operational strategy

Fig. 1 shows the schematic of the UASB reactor used in the current work. The reactor was made up of plexiglass material and wrapped in a black cloth to reduce the transmission of light. The effective volume of the reactor was 5.0 L. The influent water was injected into the reactor from the bottom of the reactor using a metering pump. A three-phase separator was used to intercept the floating sludge and discharge the gas and effluent produced by the reaction. The hydraulic retention time was maintained at 1.5 h. The outer layer of the reaction zone was the water bath zone, while the continuous circulation of hot water in the water bath zone controlled the temperature of the reaction zone at 32°C ± 1°C. The entire experimental process was divided into three operating phases.

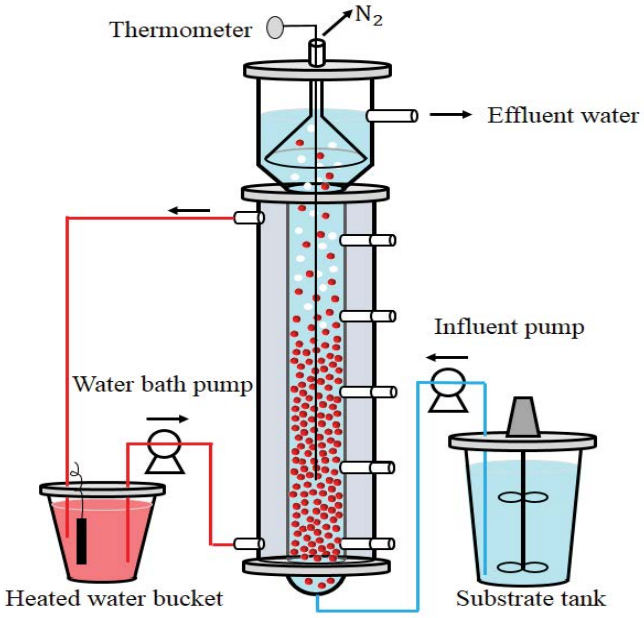

Fig. 1. Schematic of the up-flow anaerobic sludge bed used during the experiments.

2.3. Extracellular polymeric substance extraction and testing

Extracellular polymeric substances (EPS) were extracted by the ultrasound method [23]: 40 mL mixed liquors were taken from each reactor and washed three times using PBS buffer and the supernatant was discarded after sedimentation. Next, the granules were crushed (mortar and pestle) and resuspended in PBS for sonication (20 kHz and 5.59 W/mL for 1 min); Finally, the suspension was centrifuged (20 min and 20,000 g), and the supernatant was collected after filtering by 0.45 μm polytetrafluoroethylene filters. The content of proteins and polysaccharides was measured according to Badireddy et al. [24].

2.4. DNA extraction and Illumina high-throughput sequencing

Sludge samples were collected from the UASB reactor on day 18, 244 and 295. Total community genomic DNA extraction was performed using E.Z.N.A. Soil DNA Kit (Omega, USA), following the manufacturer's instructions. To ensure that adequate amounts of high-quality genomic DNA had been extracted, the concentration of the DNA measured by a Qubit 2.0 (life, USA) The V3-V4 hypervariable region of the 16S rRNA gene was amplified using standard protocols. Sequencing was performed using the Illumina MiSeq system (Illumina MiSeq, USA) by Sangon Biotech Company (Shanghai, China).

2.5. Scanning electron microscope and energy-dispersive spectrometer

In order to observe the morphology of the granular sludge and analyze the element composition, the granular sludge was taken out of the reactor on the 268th day of the experiment to make observation samples, which were observed and analyzed by scanning electron microscope (SEM)and energy-dispersive X-ray spectrometer (EDS) (Carl Zeiss ΣIGMA500, Germany). The main steps in the sample processing process were to (1) Washed several times with Tris-HCl buffer solution with pH 7.0; (2) Placed the sample in a 2.5% glutaraldehyde solution (pH = 7.0) and fix it for 3 h at a temperature of 4°C; (3) Rinsed with Tris-HCl buffer solution of pH 7.0 for three times and each time for 10 min; (4) Dehydrated with 30%, 50%, 70%, 90% tert-butanol (dissolved in absolute ethanol) for 7 min each time, and then dehydrated with 100% tert-butanol 3 times for 5 min each time, and finally, after immersing the sample in tert-butyl

Table 2
Concentrations of total nitrogen and P in influent water at each stage

Phase 1			Phase 2			Phase 3		
Time (d)	Influent total nitrogen (mg/L)	Influent P (mg/L)	Time (d)	Influent total nitrogen (mg/L)	Influent P (mg/L)	Time (d)	Influent total nitrogen (mg/L)	Influent P (mg/L)
0–28	500	2.3	120–150	500	0	270–340	500	2.30
30–58	500	1.5	152–178	800	0	–	–	–
60–88	500	1	180–208	1,000	0	–	–	–
90–118	500	0.5	210–238	1,300	0	–	–	–
–	–	–	240–268	1,600	0	–	–	–

alcohol, freeze in the refrigerator at 4°C for 1 h; 5. The sample wrapped with solidified tert-butyl alcohol was freeze-dried in a vacuum for 3 h and gold was sprayed before the test.

3. Results and discussion

3.1. Effect of phosphate deficiency on anammox nitrogen removal efficiency

In Phase 1 (0–118 d), the concentration of the influent substrate was maintained at 500 mg-N/L, while the nitrogen loading rate (NLR) of the reactor was maintained at approximately 8.044 kg-N/m^3/d. When the influent phosphate concentration was gradually decreased from 2.3 to 0.5 mg-P/L, the reactor operated stably, and the effluent NH_4^+–N, NO_2^-–N and NO_3^-–N concentrations were maintained at about 0.75, 0.85, and 57.45 mg-N/L, respectively (Fig. 2). The mutual ratio of NH_4^+–N removal, NO_2^-–N removal, and NO_3^-–N production was 1:1.203:0.252, respectively, which was close to the theoretical value of anammox reaction (1:1.32:0.26, respectively) [2], indicating that the main denitrification reaction in the reactor at this stage was anammox.

In Phase 2 (120–268 d), the influent phosphate concentration was maintained at 0 mg-P/L, while the substrate concentration was gradually increased. When the substrate concentration was gradually increased from 500 to 1,300 mg-N/L, the nitrogen removal efficiency of the reactor did not change significantly. The effluent NH_4^+–N and NO_2^-–N concentrations were maintained at about 1.65 and 1.87 mg-N/L, whereas the removal rate was maintained at about 99.64%. With the increase of NLR, the NO_3^-–N produced by the anammox reaction also increased gradually. The effluent NO_3^-–N concentration gradually increased from 52.76 to 96.07 mg-N/L, while the ratio of NH_4^+–N removal, NO_2^-–N removal, and NO_3^-–N production was found to be 1:1.205:0.163, respectively. During the increase of substrate, the production of NO_3^-–N decreased, which may be due to the death of some microorganisms caused by the deficiency of phosphate. After dissolving, the dead bacteria released organic matter to supply a small amount of denitrifying bacteria in the reactor for denitrification to convert part of the NO_3^-–N produced by anammox reaction into nitrogen [25]. In this stage, with the gradual increase of NLR, the NRR of the reactor also increased gradually. On the 120th to 238th days, the NLR increased from 8.06 to 21.00 kg-N/m^3/d, whereas the NRR increased from 7.16 to 19.44 kg-N/m^3/d (Fig. 3). The increase in NRR was not prevented by the deficiency of phosphate. Furthermore, the P uptake in many microbes occurs through the P-specific transport (Pst) system during P-limiting conditions, or through the P-inorganic transport (Pit) system under P-replete conditions [26]. It has been reported that lipid accumulation is elevated by phosphorus starvation [27] and that the microorganisms respond to phosphorus starvation by replacing amounts of phospholipids for glycolipids to allow them to support other phosphate-requiring processes [28].

Maintaining the influent without the addition of phosphate, and increasing the influent substrate concentration

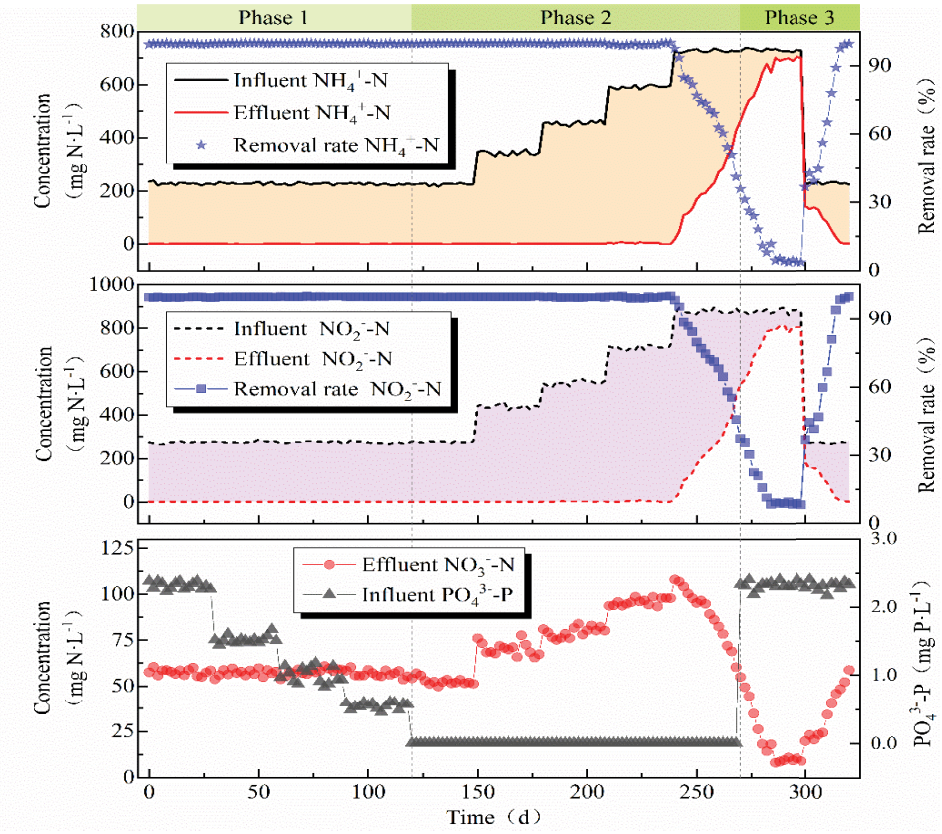

Fig. 2. Nitrogen removal during the whole test period (0–320 d) in the up-flow anaerobic sludge blanket reactor.

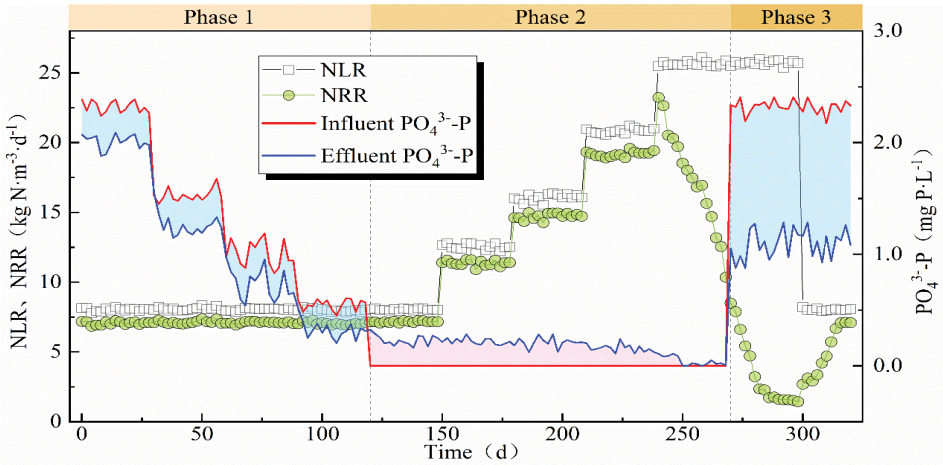

Fig. 3. Changes in NLR, NRR, concentration of PO_4^{3-} in influent and concentration of PO_4^{3-} in effluent of the reactor.

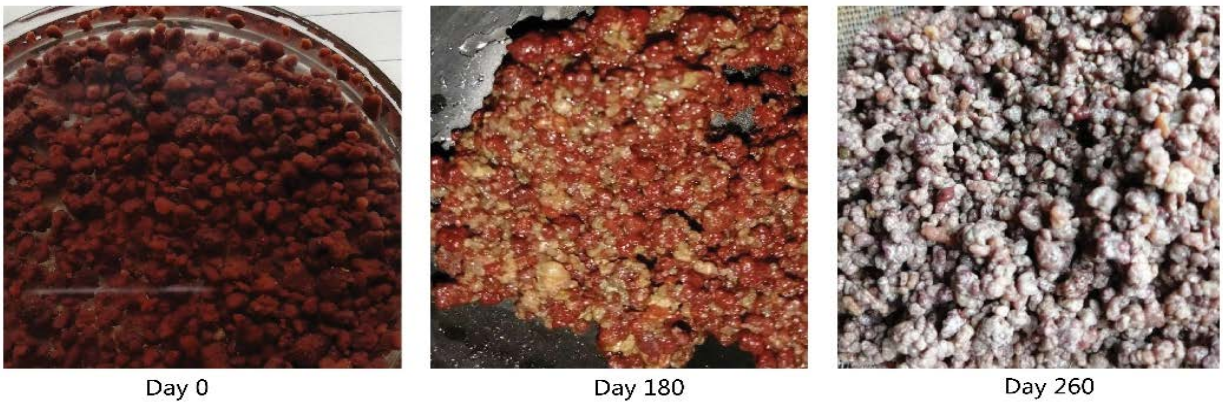

Fig. 4. Photos of the granular sludge at different time periods.

from 1,300 to 1,600 mg-N/L (NLR reached the value of 25.66 kg-N/m^3/d) on the 240th day, the nitrogen removal efficiency of the reactor began to slowly decrease. By the 268th day (after 28 d) the concentration of NH_4^+–N and NO_2^-–N in the effluent and their removal rates reached the values of 425.69 and 484.355 mg-N/L, and 41.27% and 45.77%, respectively, whereas the NRR was reduced by 55.59% to 10.36 kg-N/m^3/d (compared to that on the 240th day; NRR = 23.23 kg-N/m^3/d) (Figs. 2 and 3). After the substrate concentration in the control group reached 1,600 mg-N/L, the denitrification efficiency did not deteriorate, and the removal rates of ammonia nitrogen and nitrite nitrogen remained more than 98%. The experiment was not continued to study the highest substrate concentration that the anaerobic ammoxidation could withstand. In addition, unlike the slow reduction of nitrogen removal efficiency in this experiment, the inhibitory effect of the substrate was intense. Generally, when the substrate concentration exceeds the tolerance range of AnAOB, the nitrogen removal efficiency decreases by more than 50% within a week [18,29,30]. With this backdrop, it can be inferred that the reason for the deterioration of nitrogen removal efficiency in the current experiments was related to the phosphate deficiency rather than the inhibition of the substrate. Markou [31] reported that phosphorus deficiency led to an increase in carbohydrates and lipids in addition to the decrease in proteins.

In order to prevent further deterioration of nitrogen removal efficiency, the nitrogen removal efficiency was restored by increasing the concentration of influent phosphate and reducing the substrate concentration in Phase 3 (270–320 d). Anammox nitrogen removal efficiency kept on decreasing even after increasing the influent phosphate concentration to 2.3 mg-P/L and maintaining the substrate concentration at 1,600 mg-N/L on the 270th day. By the 290th day of the experiment, the concentration and removal rate of NH_4^+–N and NO_2^-–N in the effluent had values of 695.57 and 815.04 mg-N/L, and 4.07% and 8.92%, respectively, whereas the NRR decreased to 1.6 kg-N/m^3/d (compared to the values for the 270th day). It is speculated that the phosphate deficiency caused the death of anammox bacteria, due to which, the anammox activity could not be restored by the addition of phosphate.

In the next few days, the nitrogen removal efficiency remained at this level and did not deteriorate, reaching a stable state of inhibition. On the 300th day, the influent substrate concentration was reduced to 500 mg-N/L,

and the NLR was reduced to 8.07 kg-N/m^3/d. The nitrogen removal efficiency began to recover, and the effluent quality gradually improved. By the 316th day, the concentrations of NH_4^+–N, NO_2^-–N, and NO_3^-–N were 5.63, 4.63 and 48.16 mg-N/L, respectively. The removal rates of NH_4^+–N and NO_2^-–N increased to 97.52% and 98.32%, respectively, while the NRR increased to 7.10 kg-N/m^3/d. These results indicate that the lower NLR is beneficial to the recovery of anammox nitrogen removal efficiency.

3.2. Effect of phosphate deficiency on phosphate concentration in the effluent

In Phase 1 (0–118 d), due to the absorption and metabolism of microorganisms or the formation of insoluble hydroxyapatite with calcium and deposits, the concentration of phosphate in the influent was always higher than that in the effluent. When the influent phosphate concentration was 2.3 mg-P/L, the difference between the influent and effluent phosphate concentrations was 0.31 mg-P/L. However, when the influent phosphate concentration was reduced to 0.5 mg-P/L, the difference between the influent and effluent phosphate concentrations was 0.25 mg-P/L. The results showed that, with the decrease of influent phosphate concentration, the effluent phosphate concentration decreased slightly. This may be due to the gradual release of endogenous phosphate in the sludge system.

In Phase 2 (120–268 d), the concentration of phosphate in the influent was 0 mg-P/L. However, there was still a certain concentration of phosphate in the effluent. When the substrate concentration was 500–1,000 mg-N/L (120–208 d), the effluent phosphate concentration did not change and remained to be 0.22 ± 0.04 mg-P/L. It may be due to the decomposition of some dead microorganisms that released a large amount of organic phosphorus and some phosphate in the environment that had phosphate deficiency and high concentrations of ammonia and nitrite nitrogen. Some previous studies have shown that high ammonium loading can increase alkaline phosphatase activity and promote the release of sediment phosphorus [32]. When the substrate concentration was increased to 1,300 mg-N/L (210–238 d), the effluent phosphate concentration decreased to 0.16 ± 0.04 mg-P/L. The decrease in effluent phosphate concentration can be attributed to two aspects. On the one hand, the nitrogen removal efficiency was increased by AnAOB that required more energy and protein, so the demand for phosphate may have increased. On the other hand, it may be due to the limited amount of endogenous phosphate in sludge and the decrease of phosphate releasing. When the substrate concentration was increased to 1,600 mg-N/L (240–268 d), the effluent phosphate concentration significantly reduced to 0.04 mg-P/L, indicating that the phosphate in the entire system was severely deficient at this stage. Due to this reason, the nitrogen removal efficiency of the reactor decreased.

After the influent phosphate concentration was increased to 2.3 mg-P/L in Phase 3 (270–320 d), the effluent phosphate concentration quickly recovered to about 1.1 mg-P/L. Meanwhile, the influent phosphate concentration was higher than that of effluent, and the difference of phosphate concentrations in the influent and effluent was large. The results show that, after culturing under phosphate deficiency, the sludge system can quickly and massively absorb phosphate in the environment [33].

3.3. Effect of phosphate deficiency on the form and elemental composition of anammox granular sludge

3.3.1. Macroscopic and microscopic appearance of granular sludge

During the experiment, with the increase in the cultivated time without the exogenous phosphorus, the surface of granular sludge gradually became wrapped by white sediments (Fig. 4). The sediment on the surface of the granular sludge hindered the exchange of material between the granular sludge and the external environment. This exchange may result in a decrease in the activity of AnAOB.

Fig. 5 shows a photograph of the inside of the particles after cutting it open. It is obvious that the outside of the particles was almost completely surrounded by white sediments, while the interior of the particles was still dark red. As there are a lot of *heme c* in AnAOB cells [34], AnAOB was red. The color of AnAOB mixed culture was determined by the content of *heme c* and directly related to the activity of anammox bacteria [35]. Although the nitrogen removal efficiency was almost completely inhibited at that time, it could be seen that there was still a large number of anammox bacteria in the granular sludge.

The specific microscopic morphology of the surface of granular sludge could be observed using SEM. The surface of the granular sludge was covered with thorn-like whiskers (Fig. 6a). The whiskers were short fibers of micro-to-nano size and grew from high-purity single crystals [36]. There is no spherical or oval substance similar to anammox bacteria in the SEM diagram. The SEM images of the particles after being cut (Fig. 6b) clearly showed that there were spherical and oblate anammox cells, and the whisker-like material was significantly less than that seen in Fig. 6a. Yu et al. believed that a large number of crystals blocked the material exchange pores on the granular sludge, resulting in the inactivation of the anaerobic granular sludge [37].

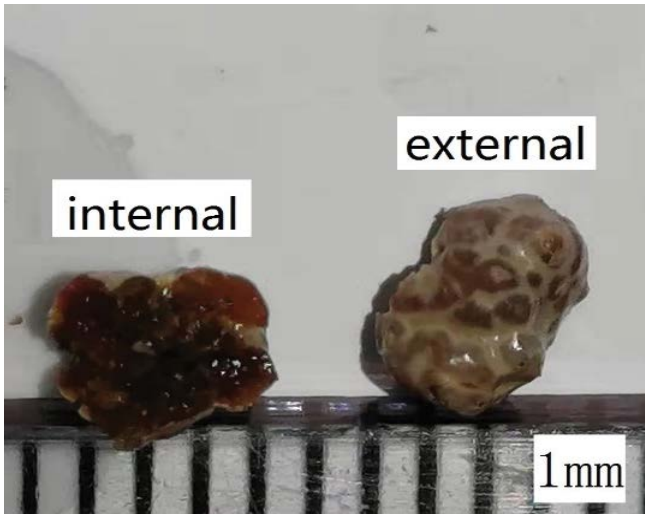

Fig. 5. Internal and external photographs of the granular sludge.

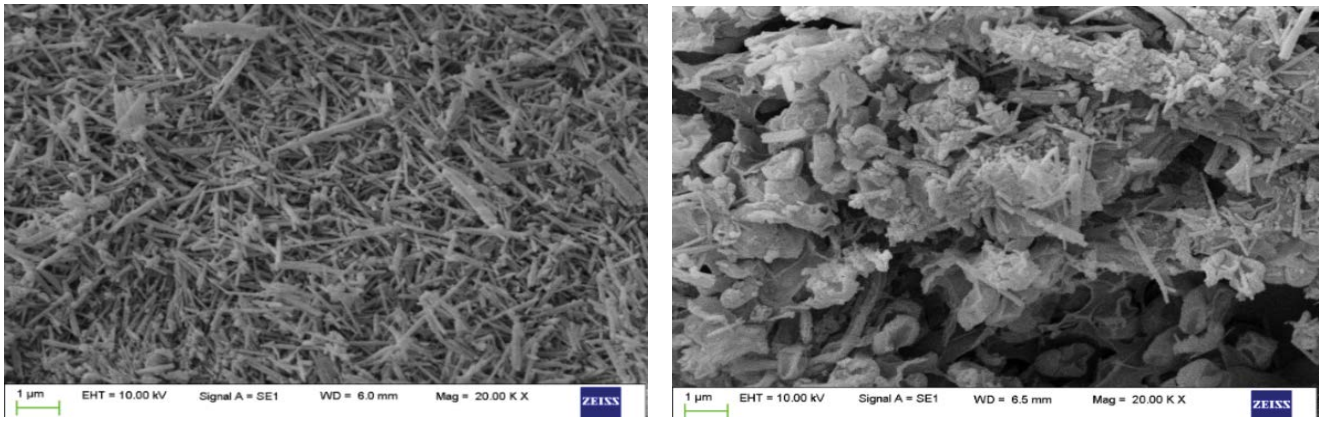

Fig. 6. SEM of the granular sludge (20,000 times magnified) (a) external SEM of the granular sludge and (b) internal SEM of the granular sludge.

3.3.2. Elemental analysis of the particle surface material

The results of EDS analysis showed (Fig. 7) that there were mainly three elements in this region. The normalized proportions of oxygen, calcium and carbon, and their atomic quantities were found to be 58.55%, 22.08% and 19.37%, respectively, while the contents of other elements were relatively low. The mutual ratio of calcium, carbon, and oxygen was 1:0.877:2.652, which was close to the mutual ratio of calcium, carbon and oxygen (calcium carbonate) (Ca:C:O = 1:1:3). Calcium carbonate generally exists in the aerobic granular sludge as a condensation core, which can make the granular sludge become more rigid in structure and of a higher strength [38]. The accumulation of calcium carbonate in the sludge was due to the microbially-induced calcite precipitation (MICP). Moreover, MICP is very common in various natural and artificial environments around the world, such as soil and caves [39]. The main related microorganisms of MICP formed the accumulation of calcium carbonate through denitrification, sulfate reduction, and urea hydrolysis. On the one hand, magnesium sulfate was added to the simulated wastewater in this study. Magnesium sulfate not only provided Mg^{2+}, but also released SO_4^{2-} into the water, which provided the substrate conditions for the existence of sulfate-reducing bacteria. On the other hand, there was a small amount of denitrifying bacteria in the anammox reactor. Although nitrite was added without the addition of organic carbon, the metabolism of microorganisms in the sludge system would release a certain

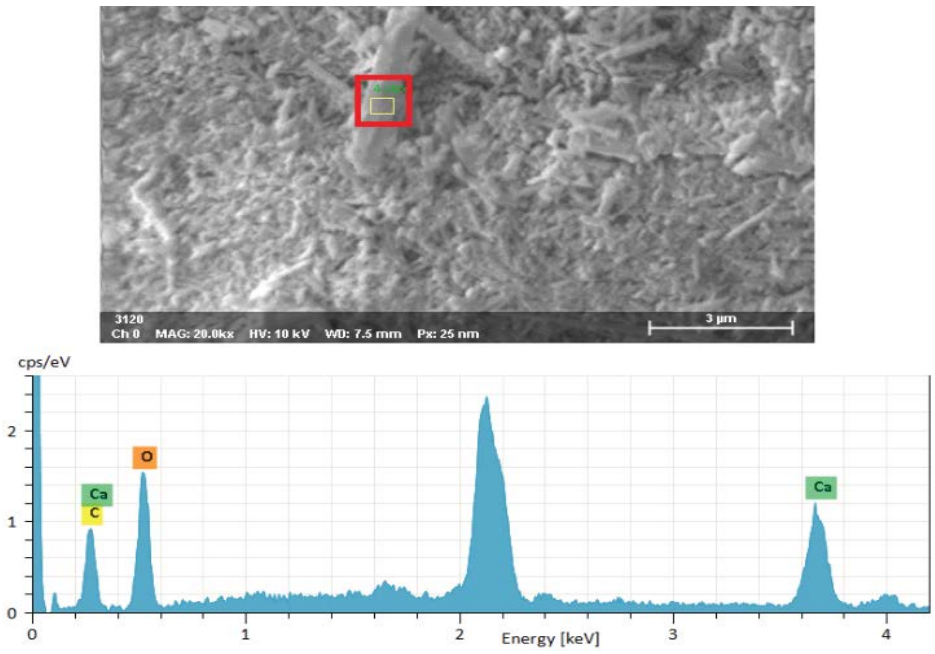

Fig. 7. EDS analysis of the outer surface of the granular sludge.

concentration of organic carbon. In addition, the good anaerobic environment in the reactor provided certain conditions for the proliferation and growth of denitrifying bacteria. The results of macro-genomic sequencing of the sludge in the reactor on the 148th day showed (Fig. 8) that there was a certain number of denitrifying bacteria, such as *Defluviimonas* (0.44%) and *Pseudomonas* (0.16%) in the sludge system, which further indicated that the crystallization of calcium carbonate was caused by MICP.

3.3.3. Effect of forest deficiency on suspended solids and volatile suspended solid of sludge in the reactor

The variations in suspended solids (SS) and volatile suspended solids (VSS) were measured at the end of the first stage, the second stage, the third stage and the blank group (Fig. 9). In Phase 2, SS increased from 31.19 to 41.42 g/L, whereas VSS concentration decreased from 22.32 to 17.66 g/L. The SS and VSS concentrations of the control group (phosphate concentration 2.3 mg/L; substrate concentration increased) increased from 34.57 and 24.4–36.85 and 25.58 g/L, respectively, indicating small increases in both. The VSS/SS ratio showed a downward trend during Phase 2, and decreased from 71.6% to 42.68%. The deposition of calcium carbonate on the surface of granular sludge increased the content of non-volatile substances in the sludge, which led to the decrease of VSS/SS value. However, there was no calcium carbonate deposition on the sludge of blank group, due to which, the VSS/SS ratio remained basically unchanged. After the recovery of Phase 3, the concentrations of SS and VSS of sludge increased to 36.82 and 19.53 g/L, respectively, whereas the ratio of VSS/SS also increased slightly.

3.3.4. Effect of phosphate deficiency on EPS in the sludge

The EPS of the sludge were extracted using the method reported in previous work [42] at different time periods (Fig. 10). EPS ubiquitously exist inside and on the surface of the activated sludge flocs and has important physiological functions. Its main components include protein (PN) and polysaccharides (PS). In Phase 2, the EPS content dropped significantly from 147.53 to 57.75 mg/g VSS, whereas the PN/PS ratio increased from 0.93 to 1.24. However, the EPS concentration of sludge in the blank group did not decrease. Instead, the EPS concentration slightly increased, which may be due to the operation of high nitrogen load. The changes in EPS indicated that the phosphate deficiency led to the decrease of EPS concentration, whereas the release of phosphorus in EPS contributed towards fulfilling the phosphorus demand of the system. The increase in the PN/PS ratio was consistent with the results reported by Xue et al. [17]. In the study of Xue et al. [17], phosphorus deficiency caused loose sludge particles to float up. This phenomenon did not appear throughout the current study, which may be due to the reason that calcium carbonate deposited on the surface of the sludge increased the density of the sludge and "trapped" the sludge inside the particles.

3.4. Effect of the absence of exogenous phosphate on the structure of microbial community and its abundance in the sludge system

On the 148th and 268th days of the experiment, appropriate amounts of granular sludge samples were taken from the reactor for metagenomic classification and sequencing (Fig. 11).

From the 148th day to the 268th day, the Shannon index of the sludge decreased from 4.00 to 3.43. The Shannon index indicates the complexity of the composition of the biological community. The larger the value, more is the complexity of the community [43], indicating that the phosphate deficiency led to a reduction in the complexity of the community in the reactor, and the structure of the community in the sludge tended to become singular. The larger Simpson index indicates that the dominant flora accounted for a larger proportion of the overall biological flora [43]. The Simpson index increased from 0.05 to 0.14 (Table 3), and the proportion of dominant bacteria in the reactor

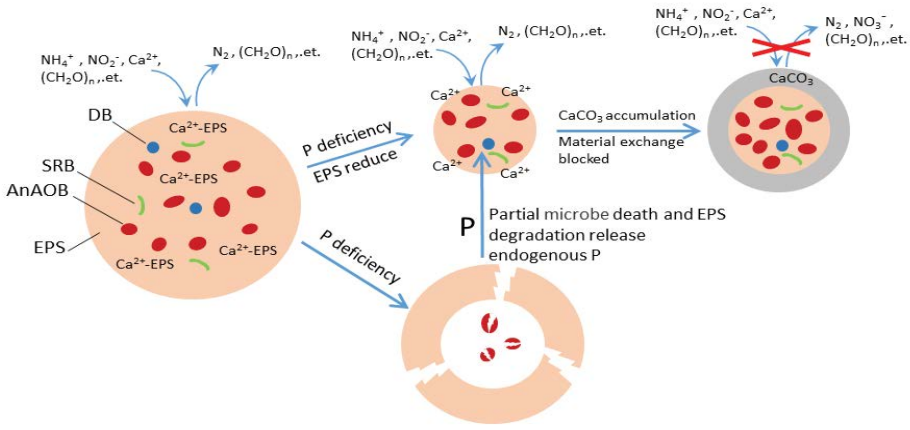

Fig. 8. Schematic of the influence of phosphate deficiency on anammox. Microbial EPS is composed of acidic organic compounds, which can bind to divalent metal cations in the surrounding environment, such as Ca^{2+} and Mg^{2+} [40]. When EPS is degraded, it will release the bound Ca^{2+}, so that the local Ca^{2+} will reach a high concentration, which is conducive to the deposition of calcium carbonate [41].

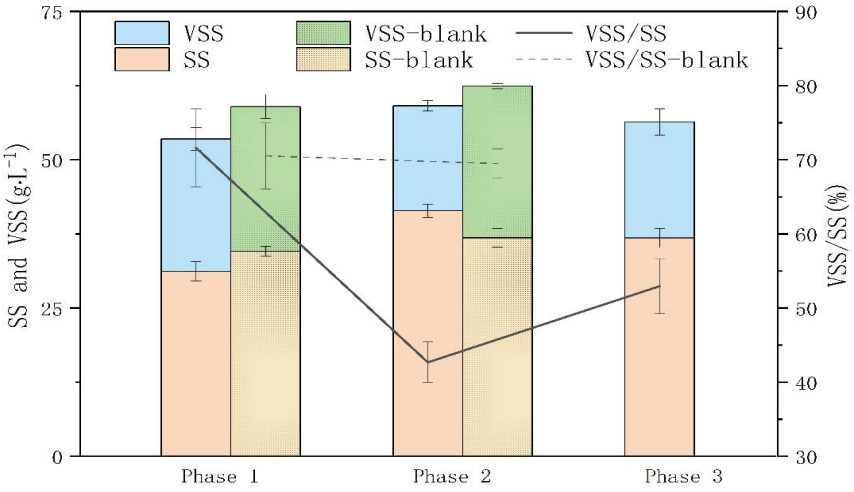

Fig. 9. Effect of phosphate concentration on VSS, SS and VSS/SS ratio.

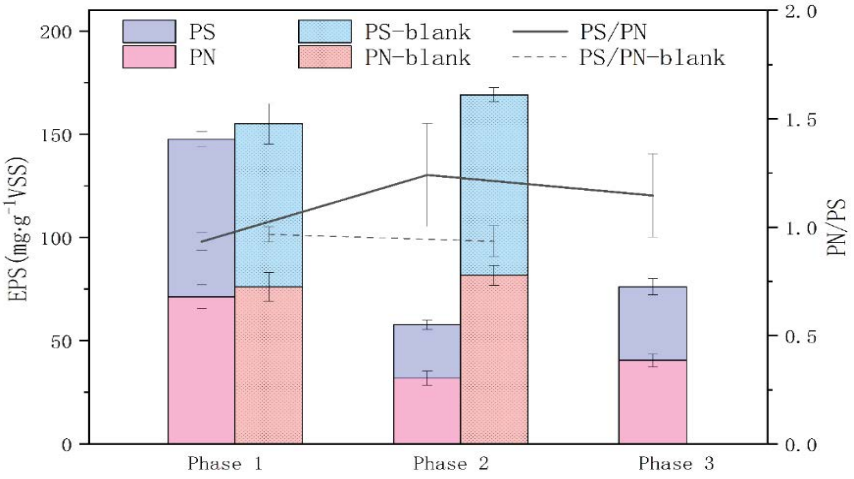

Fig. 10. Changes of EPS, PS, PN and PS/PN ratio at different time periods.

Table 3
Values of Shannon index, Simpson index and coverage of the sludge on 148th and 268th days

Sample	Shannon index	Simpson index	Coverage
Day 148	4.00	0.05	0.98
Day 268	3.43	0.14	0.98

increased significantly. This may be due to the phosphate deficiency and the increase in the substrate concentration in the water, which eliminated the weaker microorganisms. Previous studies have shown that AnAOB had better resistance to phosphate deficiency and high substrate concentration, and could survive in similar environments.

On the 148th day of the experiment, the AnAOB in the reactor mainly consisted of *Candidatus Kuenenia* (16.49%) and *Candidatus Anammoxoglobus* (4.37%). However, *Candidatus Kuenenia* was the most abundant dominant genus in the system. The AnAOB in the reactor was mainly *Candidatus Kuenenia* (35.92%). The system still maintained the status of the dominant genus on the 268th day. Previous studies have shown that only one species of AnAOB is dominant in a particular environment [44]. The results showed that, although the nitrogen removal efficiency was very low on the 268th day, the abundance of AnAOB in the sludge did not decrease, but rather increased from 20.86% to 36.08%. This was mainly due to the increase in the abundance of *Candidatus Kuenenia* from 16.49% to 35.82%. Fu et al. found that AnAOB could also be enriched and complete the start of anammox by inoculating the ordinary activated sludge in an environment without exogenous phosphate [45]. It is speculated that *Candidatus Kuenenia* can survive under the conditions of phosphate deficiency, however the function of removing nitrogen cannot be performed normally. It is worth point out that the current work did not conduct in-depth research on the interaction of different bacteria in the sludge system under phosphate deficiency.

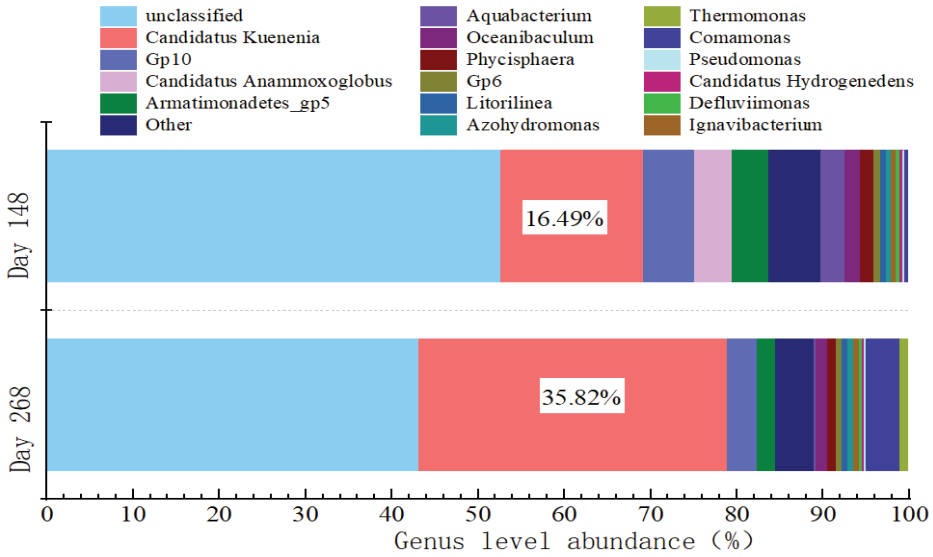

Fig. 11. Species composition and abundance at genus level of the sludge on 148th and 268th days.

4. Conclusions

Short-term phosphate deficiency in influent did not affect the nitrogen removal efficiency of anammox. However, the long-term phosphate deficiency led to a slow deterioration of nitrogen removal efficiency of high-load anammox. After 28 d, the NRR decreased from 23.23 to 10.36 kg-N/m^3/d. The nitrogen removal efficiency could not be restored by simply adding exogenous phosphate. However, the method of reducing the NLR accompanied by the addition of exogenous phosphate could slowly restore the nitrogen removal efficiency. The exogenous phosphate deficiency led to the slow release of endogenous phosphate in the sludge system, and the effluent phosphate concentration was higher than that in the influent. The VSS/SS value of sludge decreased from 71.6% to 42.68%, whereas the EPS content decreased from 147.53 to 57.75 mg/g VSS. Furthermore, PN/PS ratio increased from 0.93 to 1.24, and the diversity of microbial community decreased. Meanwhile, *Candida Kuenenia* remained dominant and the abundance increased from 16.49% to 35.82%.

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