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Granulation of Simultaneous Partial Nitrification and Anammox Biomass in One Single SBR System

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Abstract The granulation of simultaneous partial nitrification and anaerobic ammonium oxidation (Anammox) was investigated in a single, oxygen-limited, sequencing batch reactor. In this research, the reactor was started anaerobically and fed using the synthetic medium described by Van de Graaf et al. to cultivate Anammox biomass after inoculation with methanogenic granular sludge. Subsequently, mixture gas (air and nitrogen gas) was supplied to the reactor and a nitrifying population developed. Research results indicated

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that autotrophic granules was cultivated successfully by controlling the dissolved oxygen in the reactor between 0.3 and 0.5 mg/L, and a total inorganic nitrogen removal efficiency of 63.7% was obtained with a higher nitrogen load increased by reducing HRT to 3 days. It was also seen that the Ca and P concentrations of the feeding medium are important factors that influence the autotrophic granules from process running. When the Ca and P concentrations were exceeded the necessary quantity, salt precipitation was observed, interfered with microbial activity, and caused a decrease of the nitrogen removal rate of the system. After diminishing adequately the Ca and P concentrations, salt precipitation was avoided and the activity of the system restored quickly. Moreover, visual indication and scanning election microscopy observation revealed the process of sludge evolution and inner structure of the granules.

Keywords Partial nitrification · Anammox · SBR · Granules · Salt precipitation

Introduction

The presence of nitrogenous or nitrogen-containing wastes in the final effluents from sludge digesters or several industries [1–3] can adversely impact or pollute aquatic life and cause dissolved oxygen depletion, eutrophication, and methemoglobinemia in receiving water. For this reason, greater efforts have been exerted on improving and discovering techniques and strategies to reduce the amount of nitrogen in wastewater. Nitrogen is mostly present in wastewater as ammonium and is removed by physicochemical and biological processes. Biological treatment to remove nitrogen from wastewater is less expensive and more effective than physicochemical treatment and thus has been used more often to achieve nitrogen removal from domestic and industrial wastewaters [4].

Conventional biological removal of nitrogen from municipal and industrial wastewaters has been widely studied. It consists of two biological steps: the nitrification, the conversion of ammonium to nitrite or nitrate, and the denitrification—the transformation of nitrite or nitrate to nitrogen gas. But, in many wastewaters, the low level of organic compounds (COD) is hardly for complete heterotrophic denitrification, and addition of an external organic matter source, such as methanol, is often necessary to achieve complete denitrification [5–7]. This increases the operating costs in the wastewater treatment plant (WWTP) due to the cost of the chemicals added and the treatment of the additional sludge that is generated.

Recently, anaerobic ammonium oxidation (Anammox) as a new and promising biological alternative to conventional nitrogen removal from wastewater has received special attention since its discovery. Under completely anaerobic conditions, ammonium is oxidized to nitrogen gas using nitrite as the election acceptor, and carbon dioxide is used for growth of the Anammox microorganisms involved [8, 9]. The Anammox process was first discovered in a WWTP in Delf, The Netherlands [10], and today, Anammox reactions have been reported from several other treatment plants [11–13]. Furthermore, the process has also been shown to occur in nature in marine sediments and anoxic water columns [14, 15].

The Anammox process could be combined with previous partial nitrification step, the so-called SHARON process [16], during which around 50% of ammonium could be converted to nitrite. In this way, a completely autotrophic nitrogen removal is achieved in two separate reactors. Alternatively, a one-reactor ammonium removal process with partial nitrification and Anammox combined in a biofilm reactor, seems competitive with respect to the investment (engineering, construction, and materials) costs [17]. This process was called CANON (completely autotrophic N-removal over nitrite). Some similar processes also have been studied, such as: OLAND (oxygen-limited autotrophic nitrification–denitrification; [18, 19]) and aerobic deammonification [20, 21] and SNAP (single-stage nitrogen removal using Anammox and partial nitrification; [22]) and the recent developments SNAD (simultaneous partial nitrification, ANAMMOX, and denitrification [23, 24]), etc. In such cases, ammonium would be converted partly to nitrite (Eq. 1) by oxygen-limited aerobic ammonium oxidizers, such as *Nitrosomonas* and *Nitrosospira*, and subsequently, the residue ammonium was directly oxidized to nitrogen gas with nitrite as electron acceptor by Anammox bacteria (Eq. 2) [25]. The combination of the above two reactions makes the removal of nitrogen successful. In comparison to traditional nitrification–denitrification process, such combinations have some potential benefits, e.g., low oxygen demand, no requirement for external carbon sources, minimized surplus sludge, and reduced CO_2 emissions [26].

Partial nitrification:

$$NH_4{}^+ + 1.5O_2 \rightarrow NO_2{}^- + H_2O + 2H^+ \tag{1}$$

Anammox:

$$\begin{aligned} & NH_4{}^+ + 1.32NO_2{}^- + 0.066HCO_3{}^- + 0.13H^+ \\ & \quad \rightarrow 1.02N_2 + 0.26NO_3{}^- + 0.066CH_2O_{0.5}N_{0.15} + 2.03H_2O \end{aligned} \tag{2}$$

However, the practical application of these simultaneous partial nitrification and Anammox processes can be limited by its inhibition by certain compounds. One of the most important inhibitors is dissolved oxygen (DO), which reversibly inhibits Anammox bacteria. The other one is substrate nitrite which negatively impact Anammox bacteria when its concentrations reach a certain level. Apart from the two major external compounds inhibition, the extremely low growth rate (0.0027 h^{-1}, doubling time 11 days) of these Anammox bacteria as an inner inhibitory factor also limit these microbial processes. Moreover, since aerobic ammonium oxidizers are aerobes while Anammox bacteria are strictly anaerobes, which are very difficult to be cultured, especially in one single reactor. For this reason, an efficient system and operation strategy in order to achieve high biomass retention is required, especially in one single reactor.

Theoretically, biofilm systems and aerobic granulation processes could be employed for this purpose as a result of a nature gradient distribution of dissolved oxygen in those systems, causing aerobic and anaerobic conditions coexisted for microorganisms in one single system and leading to partial nitrification and Anammox processes occurred simultaneously.

Actually, most previous studies have reported, but mainly focused on, the biofilm systems which were described to be suited to slow growing cultures such as Anammox bacteria, enhancing the start-up of the process, while less effort has been made for exploring the feasibility of developing granules for the combined partial nitrification–Anammox. Meanwhile, there is still a limited knowledge concerning the behavior of inoculation bacteria during the start-up and the factors that would impact the process, or the kind of aggregates formed by various biomass. Therefore, the main objective of this study is to present a start-up of simultaneous partial nitrification and Anammox obtained in sequencing batch reactor (SBR) and to investigate the feasibilities of developing granules.

Materials and Methods

SBR System

A lab-scale SBR with a working volume of 7.0 L and a height-to-diameter ratio of 7 was used (Fig. 1). The plexiglass cylindrical reactor was operated at a fixed temperature of (35±1)°C by means of an external water jacket, which was referred to be the optimum temperature for aerobic ammonia-oxidizing bacteria and Anammox bacteria [27]. A black vinyl sheet enclosure was used to keep the bacteria away from the light. The synthetic wastewater was flushed with nitrogen gas for at least 0.5 h to deoxygenate. Before feeding, the sludge in SBR was allowed to settle for 1 h. About 5 L of the supernatant was removed, and 5 L of the freshly prepared synthetic wastewater was fed; feeding time lasted 0.5 h. The pH was adjusted in the range of 7.5–8.3 with HCl (1 M) and NaOH (1 M) stock solution. Effluent was discharged from the middle part of the column reactor. Nitrogen gas was sparged from the bottom of the reactor at a maximum gas flow of 40 mL/min controlled by a mass-flow controller for the fluidization of the biomass and the maintenance of anaerobic conditions of the reactor.

Origin of Biomass

Anaerobic methanogenic granular sludge with high COD removal activity, which came from an internal circulation reactor in a WWTP, was used as the source for inoculation. The main characteristics were mixed liquor suspended solids (MLSS) 50.43 g/L, mixed liquor volatile suspended solids (MLVSS) 21.30 g/L, MLVSS/MLSS 0.42.

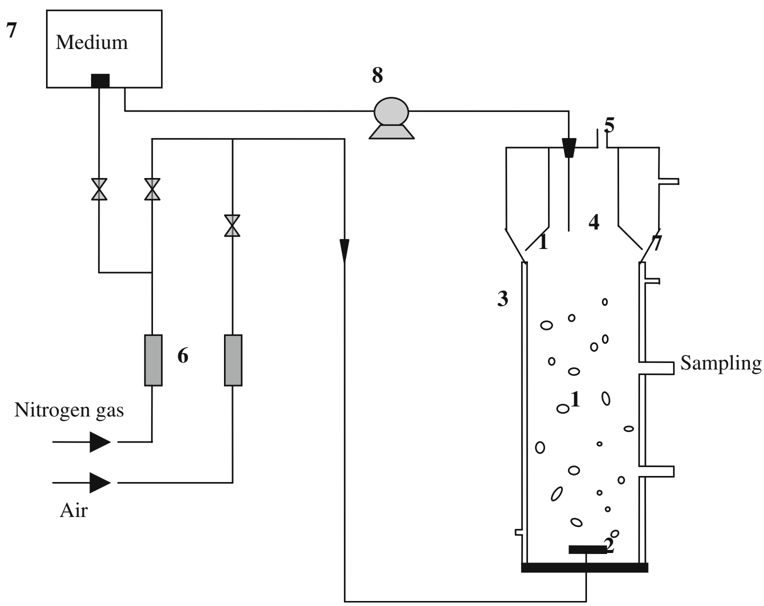

Fig. 1 Sequential batch reactor (SBR) layout. (*1*) reaction zoom, (*2*) blow system, (*3*) water jacket, (*4*) three-phase separator, (*5*) vent-pipe, (*6*) flow controller, (*7*) water tank, (*8*) peristaltic pump of the feeding media

Analytical Methods

For monitoring the performance of the reactor, samples were obtained from the influent and effluent at regular intervals and then analyzed for the concentrations of NH_4^+-N, NO_2^--N and NO_3^--N after filtering with filter paper. COD, NH_4^+-N, NO_2^--N, and NO_3^--N were determined spectrophotometrically according to APHA [28]. Total inorganic nitrogen (TIN) was calculated as: $TIN = [NH_4^+ - N] + [NO_2^- - N] + [NO_3^- - N]$. The pH was determined potentiometrically with a digital, portable pH meter. The DO level was measured with a digital, portable DO meter (YSI, Model 55, USA). Volatile suspended solids was determined by weight method.

Feeding Media

Synthetic wastewater was composed as described in Table 1. Synthetic wastewater A was the one described by van de Graaf et al. [9] which is, at the moment, the most used synthetic medium to operate the Anammox process [18, 29–31]. This wastewater contained mainly nitrite and ammonia to support Anammox activity, and it was used for experiments under anoxic conditions. Synthetic wastewater B contained no nitrite, but did contain ammonium to establish aerobic ammonium oxidation and, due to oxygen limitation, only part of the ammonium was converted to nitrite. The resulting mixture of ammonium and nitrite supports subsequently Anammox. Synthetic wastewater C contained lower concentrations of calcium salt and phosphorus to increase organic ingredients of granular sludge. The composition of trace element solution (grams per liter):

EDTA 15, $ZnSO_4{\cdot}7H_2O$ 0.43, $CuSO_4{\cdot}5H_2O$ 0.25, $CoCl_2{\cdot}6H_2O$ 0.24, $FeSO_4{\cdot}7H_2O$ 6.25, $MnCl_2{\cdot}4H_2O$ 0.99, $NaMoO_4{\cdot}2H_2O$ 0.22, $NiCl_2{\cdot}6H_2O$ 0.19, H_3BO_4 0.014, and $NaWO_4{\cdot}2H_2O$ 0.054.

Operational Strategy

The methodology for the enrichment of aerobic ammonia-oxidizing bacteria and Anammox organism was conducted in three main operating periods: (1) start-up; (2) partial nitrification and Anammox simultaneously; and (3) high nitrogen load applied, and the loading rate was increased gradually by reducing the hydraulic retention time (HRT) from 22 to 3 days. During the start-up period (from operating day 0 till day 55), synthetic wastewater A was used as influent medium to support Anammox activity under strictly

Table 1 Composition of the synthetic wastewater used in this study

Substance	Synthetic waste A	Synthetic waste B	Synthetic waste C
$(NH_4)_2SO_4$	0.31	0.31	0.31
$NaNO_2$	0.345	0	0
$KHCO_3$	1.258	1.258	1.258
NaH_2PO_4	0.05	0.05	0.005
$MgSO_4{\cdot}7H_2O$	0.20	0.20	0.20
$CaCl_2{\cdot}2H_2O$	0.30	0.30	0.005
Trace element solution	1.0 mL/L	1.0 mL/L	1.0 mL/L

Values are in grams per liter

anaerobic conditions. The HRT was controlled by a peristaltic pump. From operating day 56 till day 155 (stage 2), during the period of partial nitrification and Anammox simultaneously, the reactor was fed with synthetic wastewater B and conducted under controlling DO at the range of 0.5–0.8 mg/L by sparging mixture gas (air and nitrogen gas). During stage 3 (from operating day 156 to the end of the experiment), synthetic wastewater C was used as feeding media.

Results and Discussion

The simultaneous partial nitrification and Anammox process was operated in SBR for nearly 200 days, feeding synthetic wastewater without any biodegradable organic matters.

Stage I: Start-up of the Anammox Reactor

The SBR for the Anammox process was started-up during the first stage, which was composed of four periods. To accumulate the low growth rate of Anammox bacteria, a 22-day HRT was maintained at the first period and then gradually shortened to a 10-day at the fourth period to increase the loading rate. Figure 2 shows the evolution of the concentration of nitrogenous compounds (ammonium, nitrite, and nitrate). NH_4^+-N and NO_2^--N in the influent for the reactor were both at 80 mg/L. Under strictly anaerobic conditions, the process was operated under the same operational conditions of pH and temperature controlled at 7.5–8.3 and (35±1)°C, respectively.

During the first operating period, after inoculation, the system showed almost no reduction of ammonium and only rapid decrease of nitrite. Nitrite decreased rapidly in the initial 10 days, and average nitrite removal rate of 21.7 mg/L per day was obtained during this period. According to McCarty's et al. [32] research, nitrite removal was caused mainly by heterotrophic denitrification and denitrification as the dominant process competed nitrite with Anammox in the reactor. Between day 36 and day 45 in third cycle, the simultaneous removal of NH_4^+ and NO_2^- was clearly observed, indicating occurrence of Anammox

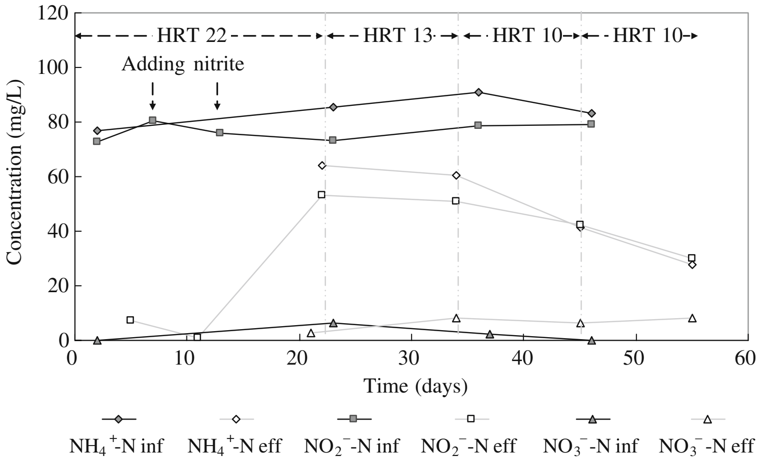

Fig. 2 Performance of SBR during stage 1

reaction. By the end of stage 1, on day 55, the removal efficiencies of NH_4^+-N and NO_2^--N reached 66.7% and 62.2%, respectively, total nitrogen removal efficiency reached 59.4%. Therefore, the Anammox process was fully operative in the SBR, which provided good foundation for the following experiments.

Stage II: Simultaneous Partial Nitrification and Anammox

To start up the process of combined partial nitrification and Anammox, the influent gas of the same reactor was changed from nitrogen gas to mixture gas (air and nitrogen gas), and at the same time, synthetic wastewater B containing only NH_4^+-N was supplied to the reactor. For the enhancement of loading rate, the initial HRT was controlled at 5 days during first 45 days, then reduced to 3 days. DO in the reactor was controlled at about 0.5–0.8 mg/L by adjusting the gas flow speed, while keeping the other operating parameters unchanged. Figure 3 shows the results of operation stage of partial nitrification and Anammox simultaneously.

Influent NH_4^+-N concentration was maintained at about 80 mg/L during this stage. After adding air to the reactor, the concentration of dissolve oxygen increased immediately from 0 to 1.2 mg/L and then fluctuated at 0.8 mg/L within 5 days (first cycle). Also, by the end of this cycle, the nitrite concentration increased to 30.8 mg/L, and the ammonium concentration only decreased 32 mg/L; the amount of conversion of ammonium was almost equal to generation of nitrite, which indicated that there is almost no presence of Anammox reaction. According to the study [33], DO higher than necessary could cause nitrite accumulation and at the same time inactivate the Anammox process. Due to this fact, the presence of inhibitory concentration of oxygen was considered to be the reason of loss of activity of Anammox. In order to prevent the impact of oxygen on the system, DO in the reactor was reduced to 0.3–0.5 mg/L. After that, a small improvement of the Anammox activity was observed in the second cycle. About 2 weeks after the impact of oxygen on the system, Anammox activity was recovered rapidly with the high total nitrogen removal efficiency of 51.8%.

Nitrogen removal performances maintained at a stable stage in the next 25 days (five cycles), the interspecific competition of aerobic ammonia-oxidizing bacteria and Anammox

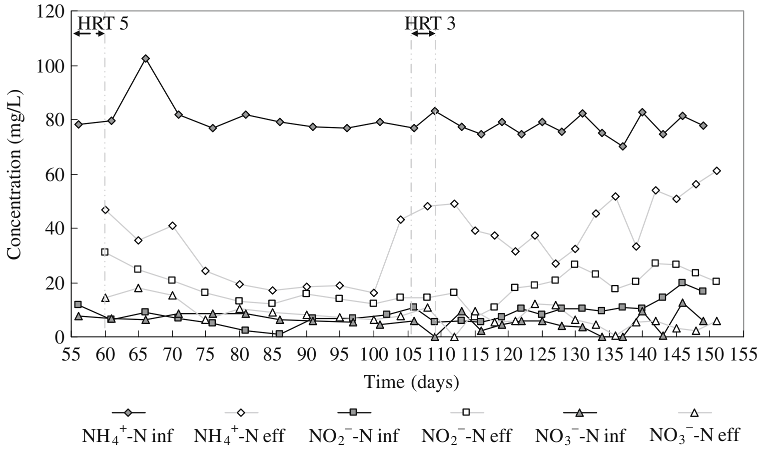

Fig. 3 Performance of SBR during stage 2

bacteria reached a balance that could remove NH_4^+-N efficiently and stably. However, after operating day 52, when the HRT was shorten to 3 days, the ammonium conversion and total nitrogen removal efficiency started to decrease. During the next 40 days, biomass color gradually changed to gray–white color. Calcium phosphate precipitation was considered as a feasible reason of loss of biomass activity in the reactor. By the end of this stage, total nitrogen removal efficiency dropped to only 12.2%.

Stage III: Enrichment at High Applied Nitrogen Loading

In order to recover the activity of the SBR, calcium and phosphorus concentrations in the feeding medium were diminished at the beginning of this period. The concentration of calcium salt was lowered 60 times (from 300 to 5 mg/L), and the phosphorus salt was lowered ten times (from 50 to 5 mg/L). DO in the reactor was slightly dropped to 0.3–0.5 mg/L to strengthen Anammox activity. From Fig. 4, we can see that few days after reducing the concentration of calcium and phosphorus in the feeding medium both the activity and the nitrogen uptake of the system increased quickly. During the first 30 operating days of this stage, the NH_4^+-N removal efficiency was increased from 37.4% to 61.8%. Since no external organic compounds except EDTA was supplied to the reactor, these bacteria (probably heterotrophs) could only utilize a very small amount of organic compounds derived from dead cells as the electron donor for denitrification, which means that the effect of heterotrophic denitrifiers could be neglected in this process. Under oxygen-limiting conditions, the aerobic ammonia-oxidizing bacteria oxidized ammonia to nitrite, consumed dissolved oxygen, and so created an anaerobic microenvironment for the inner zone of granule, where the Anammox mainly occurred while the aerobic ammonia-oxidizing bacteria were apparently not involved. These results conjectured that both partial nitrification in the aerobic zone of granule and Anammox in the anaerobic interior took place dominantly within the same granule. During the last 15 days, the removal efficiency of NH_4^+-N increased steadily from 61.8% to 74.5% and total nitrogen removal rate reached 19.2 mgN/L per day. In brief, the operation of the SBR was successful during the last stage.

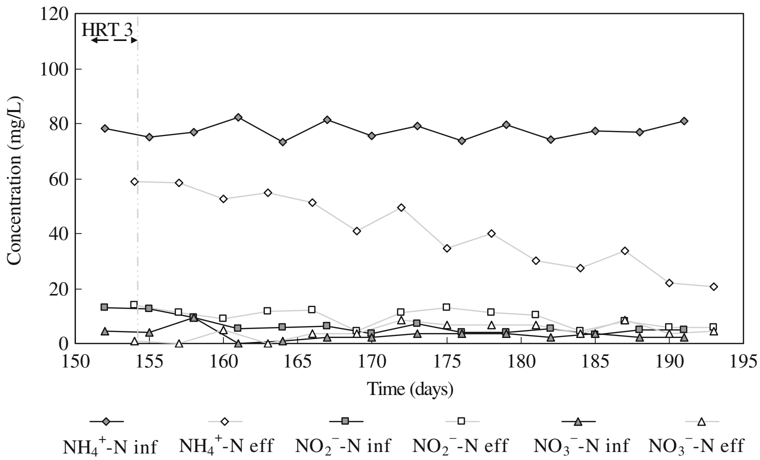

Fig. 4 Performance of SBR during stage 3

Sludge Evolution

The experiment had been operated for over 6 months to cultivate autotrophic granules. Some characteristics of granule sludge, such as concentration, settling characteristics, shape, and color, were changed extremely with the development of SBR performance.

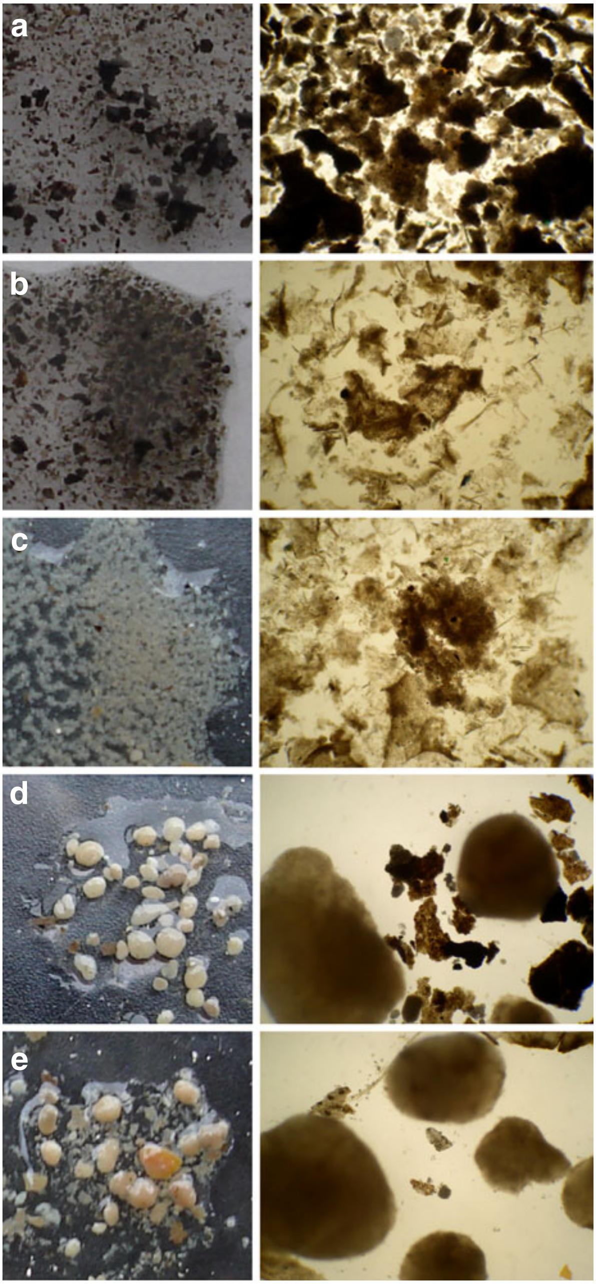

Fig. 5 Micrographs of granules in SBR during different stages (*left*: digital picture (original); *right*: micrograph (×40)), **a** initial sludge, **b** sludge at day 22, **c** sludge at day 105, **d** sludge at day 152, **e** sludge at day 190

The initial sludge concentration in the reactor was 7.2 g/L after inoculation with anaerobic granular sludge. During the next days, there was a gradual decrease of the biomass concentration in the system due to the decay of the original biomass. By the end of the first cycle (t=21 day), the concentration reduced to 1.4 g/L, and at the same time, initial black granular sludge (Fig. 5a) started to break up into dust-black granules (Fig. 5b). On the operating day 52, when biomass concentration further drops to 5.224 g/L, the Anammox process was fully operative in the reactor. The operating system performed very well; however, it experienced significant decreases in both NH_4^+-N conversion rate and TIN removal efficiency from day 107 to day 112 (Fig. 3), which coincided with the presence of white floc sludge in SBR (Fig. 5c). It supposes that the low concentration of oxygen in the reactor was the possible reason leading the loss of activity according to all appearances. The amount of NO_2^--N produced by aerobic ammonia-oxidizing bacteria was not enough to support the growth of Anammox bacteria, when the concentration of oxygen was very low; consequently, the growth of these bacteria was in famine phase during this period. In order to avoid the decline of system activity, more air was fluxed into the reactor and concentration of DO in the reactor was increased from the range of 0.3–0.5 to 0.5–0.8 mg/L. Toxic nitrite accumulation was observed from day 115 to day 153, causing TIN removal efficiency to drop to only 12.2%; meanwhile, the previous floc sludge transformed into white granules with a mean diameter 2.7±0.7 mm (Fig. 5d). Taking these observations into account,

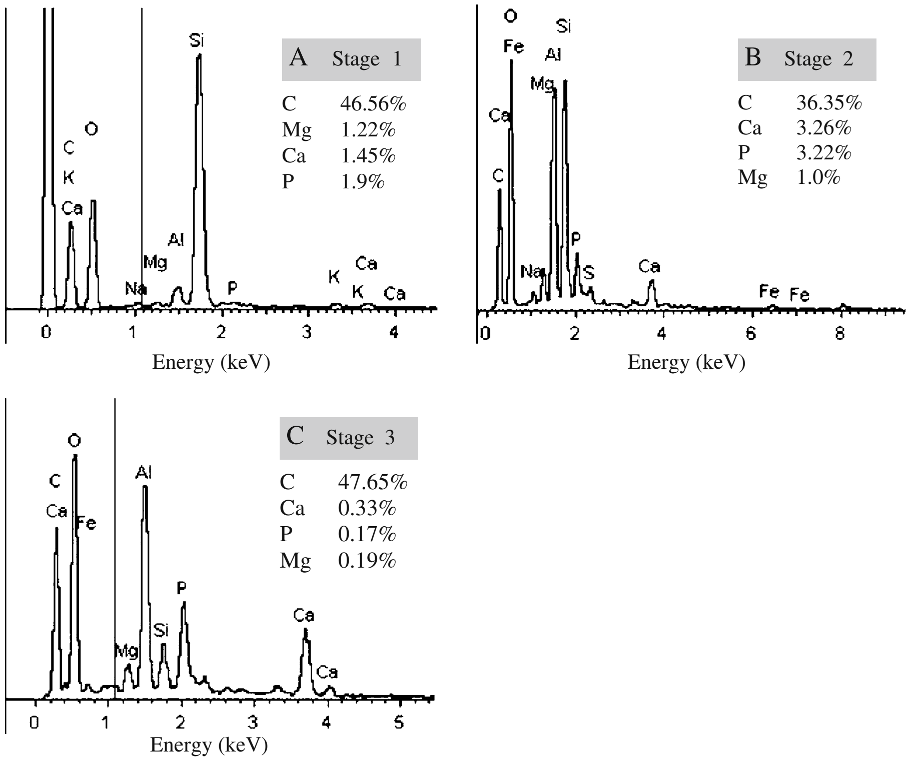

Fig. 6 Elemental analysis carried out with the SEM system, indicating the percentage composition by mass of the most abundant elements of the surface of biomass samples during stage 1 at the operating day 0 (*A*), during stage 2 at the operating day 112 (*B*), and during stage 3 at the operating day 190 (*C*), respectively

it was verified that the precipitation was present in the system, which indicated that the selected phosphorus and calcium concentrations in feeding medium were not the adequate ones. For this reason, an elemental analysis of the surface and the microscopically observation of the surface of biomass was carried out by means of scanning election microscopy (SEM; Fig. 6). By comparison with elemental analysis of seed sludge, elemental analysis of white floc sludge manifest enormous variations after 107 days cultivation, mass percentage of carbon went down from 46.56% (day 0) to 36.35% (day 107), moreover, mass percentage of calcium and phosphorous was increased from 1.45% and 1.9% (day 0) to 3.26% and 3.22% (day 107), respectively. These facts illustrated that the system lost its activity owing to the accumulation of precipitation of calcium phosphate salts in granules. Thus, from day 152 on, synthetic wastewater C containing diminished phosphorus and calcium concentrations was used as influent medium. The concentration of calcium salt ($CaCl_2{\cdot}2H_2O$) was reduced from 300 to 5 mg/L (Table 1). After 12-cycle (days 152–190) acclimation, the activity of the system increased quickly, and TIN removal efficiency of 65.1% was obtained on day 190. It was possible to avoid the precipitation of calcium phosphate due to the reduction of the concentration of calcium. This fact was also demonstrated by analyzing chemically the surface of the biomass. Fig. 6c shows clearly the increase of C percentage, reached to 47.65%, which means an improvement of organic ingredient, and the reduction of P and Ca percentage in the granule surface with regard to those observed during period 2. Additionally, granules color varied from white to brownish red color (Fig. 5e) because the Anammox

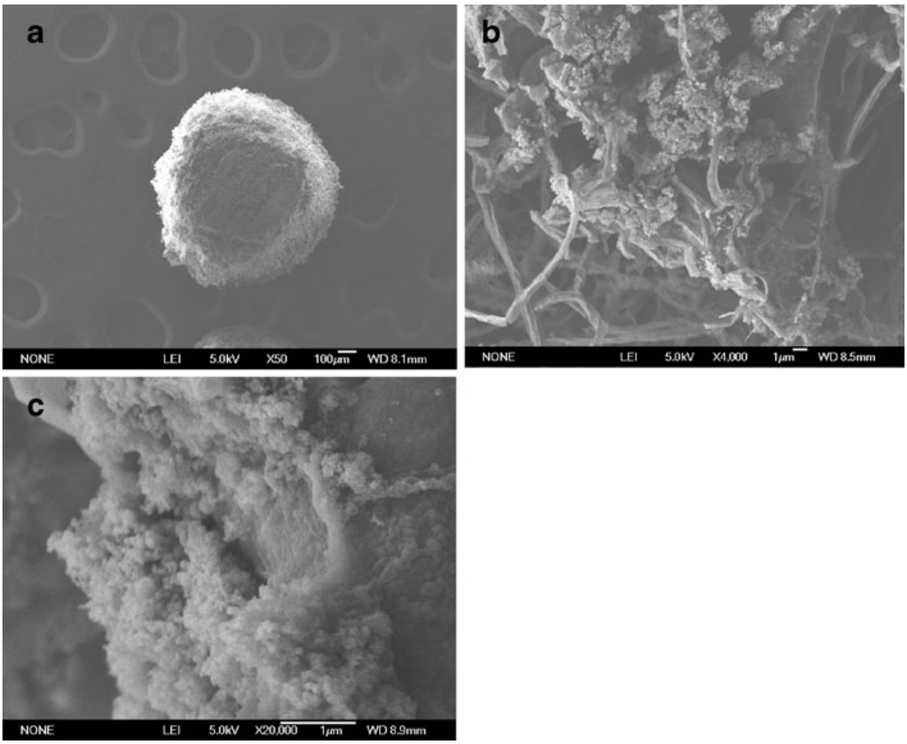

Fig. 7 Scanning electron micrographs of mature granular sludge (**a** at ×50 magnification; **b** at ×4,000 magnification; **c** at ×20,000 magnification)

bacteria contain plenty of cytochrome c, so red is specific for them. The change of biomass color could also indicate the operating course of the bioreactor.

The morphology and inner structure of the granules observed in more detail with photographs and SEM are shown in Fig. 7. The SEM images of the granules sampled from SBR on day 190 display that the granules developed in SBR had compact and round structure with a clear outer shape. It is also found that structure of the granules in the two regions (inner zone and outer layer) appeared to have an obvious difference because of the different situation of dissolved oxygen. In the outer layer of a granule, biomass was mostly micrococcus, presumably aerobic ammonia-oxidizing bacteria. On the other hand, in the inner layer of a granule, biomass was typical cauliflower-like aggregates, presumably Anammox organisms. In addition, lots of cavities were present in the granules, which can enhance substrate transfer from the bulk to granules and intermediate or by-product transfer from inside granules to the bulk. The observations together with system performance results revealed the co-existence of partial nitrification and Anammox.

Conclusions

Some concluding remarks can be made from the present study as follows:

1. Under oxygen-limiting conditions, granules capable of simultaneous partial nitrification and Anammox could be developed successfully in one sequencing batch reactor by reducing HRT stepwise.
2. The experimental results of stage 1 clearly showed that the methanogenic granular sludge was a suitable inoculum and initial longer HRT (22 day) as an important limiting factor was in favor of the growth of Anammox bacteria.
3. The inadequate Ca and P concentrations of the feeding medium which led to the accumulation of salts precipitation on the biomass surface negatively affected system performance and caused a decline of organic ingredient in granules during operating stage 2. Modification of the Ca and P concentrations of this medium were necessary to avoid precipitation. Finally, the system maintained a good and stable activity during stage 3 by avoiding salt precipitation.

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