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Fast start-up of the anammox process with addition of reduced graphene oxides

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HIGHLIGHTS

- The start-up time of anammox process was reduced 26.9% with RGO addition (100 mg/L).
- The TN removal rate of R2 with RGO addition was enhanced 27.4% on day 229.
- The HDH activity of R2 enhanced 42% compared with that of control on day 200.
- FISH analysis showed the percentage of anammox bacteria was 62.9% in R2 on day 200.

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ABSTRACT

In this study, two upflow column reactors were built up to investigate the effects of reduced graphene oxide (RGO) on the startup of anammox process. Continuous experiments indicated that the start-up period of anammox process with RGO addition could be shortened from 67 to 49 days. The further investigation proved that the anammox reactor with RGO addition possessed relatively higher anammox bacteria activity and stronger stability even against the high nitrogen loading rate (NLR) impacting. After 220 days cultivation, the nitrogen removal rate of R2 (RGO addition) reached 1.08×10^3 g-N/(m^3 d), which was 27.4% higher than 0.846×10^3 g-N/(m^3 d) of the R1 (the control). The hydrazine dehydrogenase (HDH) activity increased dramatically with RGO addition, which could be the trigger of fast anammox start-up. Compared with the control reactor, the quantity of anammox bacteria in R2 increased during the whole operation period by quantitative PCR and fluorescence in situ hybridization analysis.

1. Introduction

Since the discovery of anaerobic ammonium oxidation (anammox) in a denitrifying fluidized bed reactor in the early 1990s, anammox-related technology has been developing rapidly and become a promising bioprocess for treating high ammonium concentration and low COD content in the last years [1]. Compared with the conventional nitrification–denitrification processes, anammox process offers significant advantages such as no demand for oxygen and organic carbon, low sludge production and reduced CO_2 or N_2O emissions [2]. Therefore, anammox process was considered as a sustainable biological nitrogen removal technique.

Although a number of studies on laboratory-scale, even full-scale anammox processes have been conducted, the rapid enrichment of anammox bacteria still remains challenging owing to its relatively slow growth rate with doubling time ranging from 11 to 20 days [3]. The start-up of the first full-scale anammox reactor took almost 3.5 years [4], indicating an emergent need to seek a faster start-up strategy for anammox process. During the past years, efforts to shorten start-up period generally aimed at selecting suitable reactor types [5,6], trying different kinds of seed sludge, and using different types of carriers [7,8], which were all shown their own drawbacks and merits. Moreover, enhancing the activity of anammox bacteria and further accelerating the cell growth could be another way to shorten the start-up period of anammox process.

Graphene oxides (GOs), which are layered and oxygenated graphene sheets with epoxide, carboxyl, and hydroxyl groups on their basal planes and edges, are attracting extensive attention in microbiology [9]. There are some extraordinary characteristics in these materials, such as large surface area, excellent colloidal property, and low cytotoxicity [10]. More importantly, the electron transfer ability of reduced graphene oxide (RGO) was found to be

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approximately three orders of magnitude higher than that of GO. Ruiz et al. [11] demonstrated that RGO coated on filters can induce the faster growth of *Escherichia coli*. Also, previous investigations have shown that the RGO could be reduced from GO with anammox biomass [12]. Besides, Wang et al. [13] reported that 75 mg/L RGO addition could increase total nitrogen (TN) removal rate of anammox biomass by 10.2%, although the promotion mechanism was not interpreted. Moreover, Yin et al. [12] proved that 17.2% promotion of TN removal rate and 1.5–2 folds enhancement of enzymes activities of anammox biomass were tested with RGO addition. Therefore, it was reasonable to speculate that the anammox cell quantities could increase by continuous cultivation with RGO addition so that the anammox process was started up rapidly. Up to now, studies conducted on start-up of the anammox process from activated sludge with RGO addition have never been reported.

Thus, the main objectives of this study were to: (1) verify the possibility of developing a rapid start-up anammox reactor via appropriate RGO addition (with a dose of 100 mg/L) (2) evaluate the effect of RGO on the quantity of anammox bacteria and enzymes activities during the entire operation.

2. Materials and methods

2.1. Microorganisms and feed media

The activated sludge from Lingshui Sewage Treatment Plant (Dalian, China) was inoculated in two upflow column reactors. The seeding sludge had a final mixed liquor volatile suspended solids (MLVSS) concentration of about 4960 mg/L in two reactors. The composition of the trace mineral medium was described by van der Graaf [14]. The anammox nutrient mediums consisted of $(NH_4)_2SO_4$, $NaNO_2$, $KHCO_3$ (500 mg/L), KH_2PO_4 (27 mg/L), $MgSO_4{\cdot}2H_2O$ (300 mg/L) and $CaCl_2{\cdot}2H_2O$ (180 mg/L).

2.2. Continuous experiments

Two identical upflow fixed-bed column reactors, R1 (the control reactor, without RGO addition) and R2 (with RGO addition of 100 mg/L) were applied for continuous experiments. The working volumes were about 0.3 L with the inner diameter of 5 cm and the height of 15 cm. The two reactors contained 30 g (wet weight) seed activated sludge resulting in an initial MLVSS concentration of 4960 mg/L for each reactor. Unlike R1, 30 mg GO was uniformity mixed with seed sludge and then added into reactor R2. The optimal dose of 100 mg/L was determined by previous research [12]. At the beginning of the reactors starting up, the influent NH_4^+-N and NO_2^--N dose were 50 mg/L and 65 mg/L. The initiative hydraulic retention time (HRT) was 6 h corresponding to the nitrogen loading rate (NLR) was 460 g-N/(m^3 d). Both the two reactors were continuously fed with the same media, and the influent was purged with 99.5% N_2 to maintain dissolved oxygen (DO) below 0.5 mg/L. Influent pH was adjusted to 7.0 ± 0.2 by dosing 2 M HCl and the temperature was maintained at 35 ± 1 °C using a water bath. Fig. 1 shows the schematic diagram of the continuous experiments.

2.3. Analytical methods

Concentrations of nitrite and nitrate were determined using an ion-exchange chromatography (ICS-1100, DIONEX, AR, USA) with an IonPac AS18 anion column. Prior to the detection, all samples must be filtered by 0.22 μm pore size membranes. Measurement of pH was done using a digital pH meter (PHS-25, Leici Company, China), while DO was measured by a digital DO meter (YSI, Model 55, USA). NH_4^+-N and MLVSS concentrations were measured according to the Standard Methods [15].

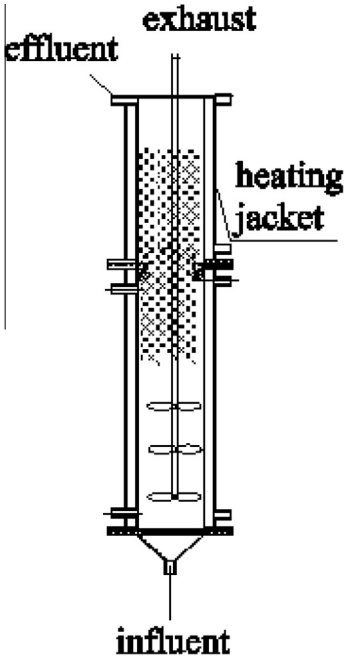

Fig. 1. Schematic diagram of the identical anammox reactor.

2.4. FISH assay

The anammox biomass samples for fluorescence in situ hybridization (FISH) analysis were taken from reactors R1, R2 on day 0 and 200. FISH analysis was carried out according to the methods described by Duan et al. [16]. FISH assay was carried out in the reactor using a fluorescein isothiocyanate (FITC)-labled Amx820 probe (5_-AAAACCCCTCTACTTAGTGCCC-3_) and 4_,6-diamidino-2-phenylindole (DAPI) to visualize the variation of anammox bacterial quantities. The probes were purchased from TaKaRa Biotechnology Co., Ltd. (Japan). The sludge samples were fixed in 4% paraformaldehyde phosphate-buffered saline at 4 °C for 24 h. Fixed cells (about 5 μL) were spotted on gelatin-coated slides and dried in a sterile room temperature space. Hybridization was performed at 46 °C for 2 h. After hybridization, unbound oligonucleotides were removed by rinsing with a washing buffer containing the same components as the hybridization buffer except for the probe. Then, samples were additionally stained with DAPI to detect the total bacteria. The slides were examined with an Olympus Inverted Microscope (Olympus ZX71, Japan), and then using a digital camera (Nikon D7000, Nikon Corporation, Japan) captured the digital images of the slides. Image Pro-Plus software was utilized for FISH analysis.

2.5. Preparation of biomass extracts and determination of enzymes activities

Firstly, the anammox biomass samples taken from each reactor were centrifuged at 8,000 rpm at 4 °C for 20 min. Secondly, 2 g cell (wet weight) was weighted after removing the supernatant followed by washing twice with sodium phosphate buffer solution (20 mM, pH 7.0). Thirdly, the washed pellets were resuspended in 20 ml of the same buffer and lysed by freezing and thawing followed by sonication (225 W, at 4 °C for 30 min, Ultrasonic processor CPX 750, USA). Lastly, Cell debris was separated by centrifugation (22,000 rpm), at 4 °C for 30 min. The supernatant

was stored at 4 °C and used as cell extract in the determination of protein and enzymes activities. Protein concentration was measured according to the Bradford procedure [17], using bovine serum albumin (BSA) as a standard. Hydrazine dehydrogenase activity (HDH) was determined according to the methods described by Shimamura et al. [18], and the reactions were depicted as an increase in the absorbance of cytochrome c at 550 nm in the standard mixture using a spectrophotometer (V-560 UV/VIS Spectrophotometer, Jasco, Japan). The HDH activity was expressed as μmol of cytochrome *c* reduced/mg protein/min.

2.6. Quantitative PCR (qPCR) assay

Real-time PCR quantification of the anammox bacteria used Amx 809-F and Amx 1066-R as primer pairs. The reaction volume of 25 μL contained 12.5 μL SYBR® Premix Ex Taq™ (TaKaRa, Dalian, China), 0.4 mg/mL BSA, 0.5 μL Rox reference dye, 200 nM final concentration of each primer and 2 μL extracted DNA as a template. Three replicates were analyzed for each sample. The PCR program was as follows: denaturation for 2 min at 95 °C, followed by 40 cycles of 5 s at 95 °C, annealing for 30 s at 62 °C and elongation for 30 s at 72 °C. Only one peak at Tm = 87.0 °C were shown by melting curve analysis. No detectable peaks that were associated with primer–dimer artifacts and no other nonspecific PCR amplification products were observed. The plasmid DNA concentration was determined on a Nanodrop® ND-1000 UV–Vis Spectrophotometer (NanoDrop Technologies, USA). And the anammox bacterial 16S rRNA gene copy number was calculated directly from the concentration of extracted plasmid DNA. Sixfold serial dilutions of a known copy number of the plasmid DNA were subjected to q-PCR assay in triplicate to generate an external standard curve.

3. Results and discussion

3.1. Start-up period

Firstly, at the beginning of the anammox reactors' startup, the conversion of organic nitrogen to ammonia could appear due to the cells which were maladjusted could divide. Thus, ammonium concentration was even much higher than that of influent. As shown in Fig. 2, the peak value of effluent ammonium in both

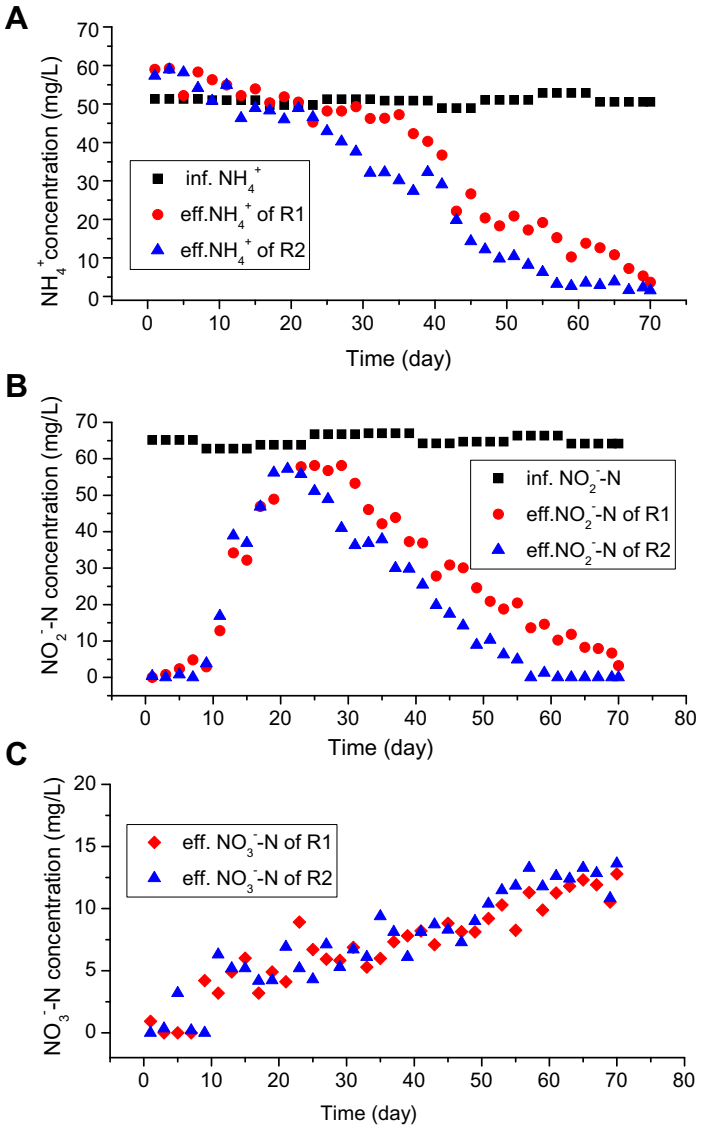

Fig. 2. Time course of influent and effluent N concentration of each reactor during start-up period. R1, control reactor; R2, reactor with 100 mg/L RGO addition. A, NH_4^+-N; B, NO_2^--N; C, NO_3^--N.

Table 1
The duration of every phase for each reactor during start-up period.

	Incubation time (days)	
Reactor	R1	R2
Cell lysis phase	19 (day1–19)	15 (day1–15)
Lag phase	17 (day20–36)	10 (day16–25)
Activity elevation phase	31 (day37–67)	24 (day26–49)
Total start up period	67	49

reactors reached 58 mg/L. Bi et al. named this period the cell lysis phase (with effluent ammonium concentration > influent one) [19]. Meanwhile, since heterotrophic denitrifying bacteria grew much faster than autotrophic anammox bacteria, denitrifying bacteria might predominate in the first stage, which resulted in the removal of much nitrite in this phase. As depicted in Table 1, the cell lysis phase of R1 lasted 20 days. However, only on day 16, ammonium concentration of R2 with RGO addition in effluent decreased lower than influent and nearly finished the microorganism lysis gradually. Besides, with the continued consumption of organic matter, the nitrite removal of both reactors offered a downward trend because denitrifying bacteria activity decreased.

Secondly, some fluctuations of effluent ammonium concentration were detected, but the average value was lower than the influent ammonium concentration. The phase was similar as lag phase by Bi et al. reported [19]. The anammox activity occurred as both ammonia and nitrite being removed simultaneously, as shown in Fig. 2. This phase lasted for 17 days (day 20–36) and 10 days (day 16–25), respectively, for R1 (control) and R2 (RGO addition), On day 35, the average ammonium removal rates for two reactors in this phase were calculated as 14.7 and 82.8 g-N/(m^3 d). The effluent nitrate of R1 and R2 reached 5.99 and 9.38 mg/L, showing a juvenile anammox activity in both reactors (Fig. 2C).

Lastly, both the ammonium and nitrite removal rate increased rapidly and nitrate accumulating proportionally. Take R2 for example, from day 26 to 49, the ammonium removal rate shot up from 33.5 to 165 g-N/(m^3 d), while the nitrite removal rate reached from 62.4 to 223 g-N/(m^3 d). After 49 days cultivation, both the ammonium and nitrite concentration in effluent of R2 were stably lower than 10 mg/L. And from day 49 to 70, the production of nitrate reached 8.99–13.6 mg/L, the stoichiometric molar ratio of nitrate production versus ammonium consumption was calculated as 0.22–0.28. These marks meant the activity elevation phase was finished by Bi et al. [19]. This ratio and the nitrogen removal performance indicated that anammox process in R2 with RGO addition was started up successfully. However, the same phenomenon appeared in R1 until 18 days later. On day 67, the startup processes of anammox reactor without RGO addition could be completed. The activity elevation phases of R1 and R2 lasted for 24 (day 37–67) and 31 days (day 26–49), respectively. Obviously, the RGO addition accelerated the startup of anammox reactor from conventional activated sludge. The start-up period of anammox process, could be shortened from 67 to 49 days by RGO addition, which was reduced 26.9% compared with that of control.

3.2. Operational stability

Then the two reactors were running smoothly to investigate the operational stability under the same circumstances of nitrogen loading rate (NLR) after the anammox reactors were started up successfully. This investigation was divided into two phases including influent nitrogen concentration increasing and HRT shortening, as shown in Fig. 3. From day 70 to 165, influent ammonium and nitrite concentration, increased stepwise from 50 to 100 mg/L and HRT was maintained at 6 h, corresponding to the total NLR ranging from 460 to 920 g-N/(m^3 d) for both reactors. On day 165, the nitrogen removal rate (NRR) of R2 with RGO addition was calculated as 715 g-N/(m^3 d), while the NRR of R1 only was 576 g-N/(m^3 d). Afterward, the HRT was shortened from 6 h to 3.7 h to increase the NLR of the two reactors from day 165–

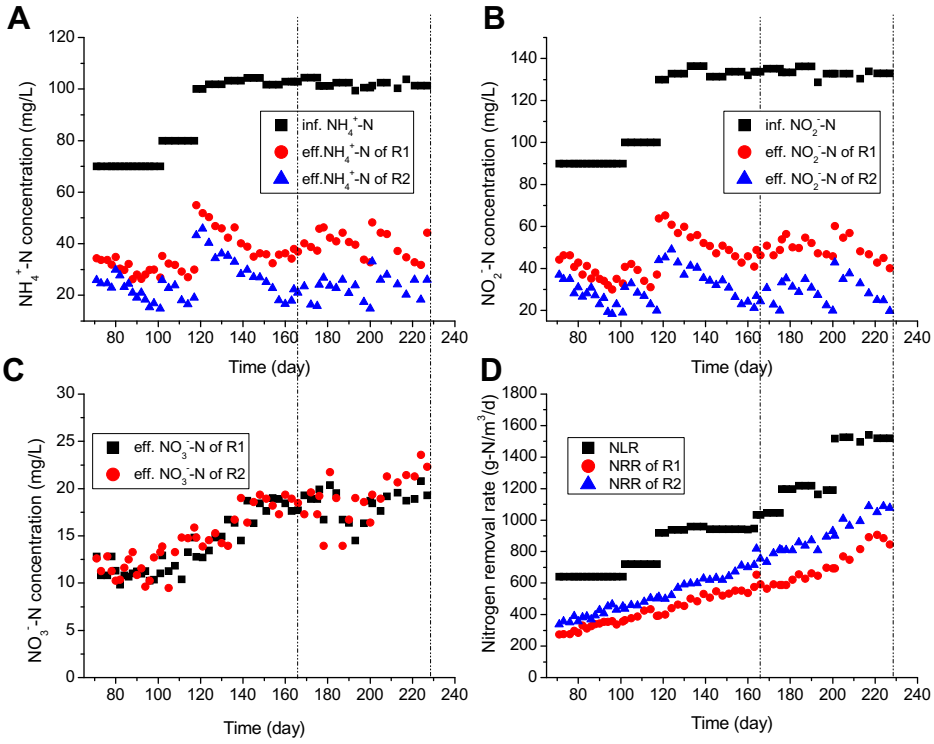

Fig. 3. Influent and effluent N concentration of each reactor during stable operation period . R1, control reactor; R2, reactor with 100 mg/L RGO addition. A, NH_4^+-N; B, NO_2^--N; C, NO_3^--N; D, NLR and NRR. Nitrogen concentration increasing: day 70–165; HRT shortening: day 166–223.

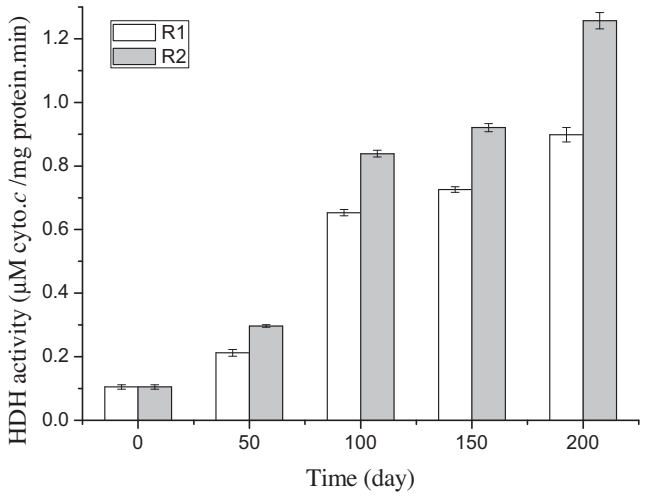

Fig. 4. Comparison of the variation of HDH activities in the whole incubation period. R1, control reactor; R2, reactor with 100 mg/L RGO addition.

229. After other 65 days cultivation, the NLRs of both two reactors were increased to 1.52×10^3 g-N/(m^3 d). And the NRRs of two reactors reached 846 and 1.08×10^3 g-N/(m^3 d), respectively. Comparison of the NRRs of two reactors, R2 with RGO addition was 27.4% higher than that of control. Moreover, both in the two phases of NLR increasing, the effluent ammonium and nitrite concentration of R2 were steadily lower than 20 mg/L, while those in R1 were about 40 and 50 mg/L under the same condition. This phenomenon could be attributed to the relatively higher anammox bacteria activity and stronger stability against the high NLR impacting in R2, which was closely related to the RGO addition. Actually, our preliminary experimental results [12] demonstrated that anammox activity could be increased by RGO addition. The operational stability investigation of new initiated anammox reactors was coincident with our previous research.

3.3. Variation of key enzymes activities

Fig. 4 depicted the variation of HDH activities of two anammox reactors during the operation period. Before the activated sludge added into the anammox reactor, the HDH activity of seed sludge was only determined as 0.11 μM cytochrome *c*/(mg protein.min). In the long term incubation period, the enzymes activities of two reactors showed a stable increasing tendency. On day 200, the HDH activities of R1 and R2 reached 0.89 and 1.16 μM cytochrome *c*/(mg protein.min), respectively, which were 8.1 and 10.5 folds higher than that of seed sludge. Distinctly, samples from R2 with RGO addition exhibited relatively higher HDH activity all the time, which was closely consistent with the nitrogen removal performance. Compared with R1, the HDH activities of R2 enhanced 42.1% on day 200. Based on the above results, the RGO addition enhanced the HDH enzyme activity of anammox biomass greatly. The increment of HDH activity might be tightly tied to excellent electron transfer ability of RGO (discussion later).

3.4. Variation of cell quantities

3.4.1. qPCR results

qPCR results can reflect the amount of anammox cell versus the total bacteria and indirectly reflect the levels of anammox bacteria in both reactors. Samples from both reactors on day 0, 50, 100, 150 and 200 were also investigated the effects of RGO on the variation of anammox cell quantities during the whole incubation period. Fig. 5 illustrated the variation of the 16S rRNA copy numbers of anammox bacteria from both reactors. At the beginning of startup, the 16S rRNA anammox bacterial copy numbers in seed sludge was measured as 9.91×10^7 copies/g biomass. With experiments going, the 16S rRNA anammox bacterial copy numbers in two reactors both exhibited an increase trend. The copy numbers of R1 reached 1.34×10^9 copies/g biomass on day 200, which was 14.9 folds higher than that of seed sludge. It was indicated that the anammox biomass got a great growth rate in the perfect cultivation environment during the start-up period. Also, the copy numbers of R2 with RGO addition relative to R1 can exhibit a higher growth rate. On day 200, the value of R2 was measured as 1.84×10^9 copies/g biomass, which was 37.3% higher than that of R1 (on day 200) and increased 18.6 folds compared to the seed sludge. These results inferred that the flora density of two reactors made a great growth during the continuous running period and RGO addition could accelerate the growth of anammox cell.

3.4.2. FISH analysis

FISH analysis was applied to visualize the variation of anammox bacterial quantities in the two reactors during the incubation period. Sludge samples were taken from two reactors on day 0 and day 200. Microbial groups, including anammox bacteria and all

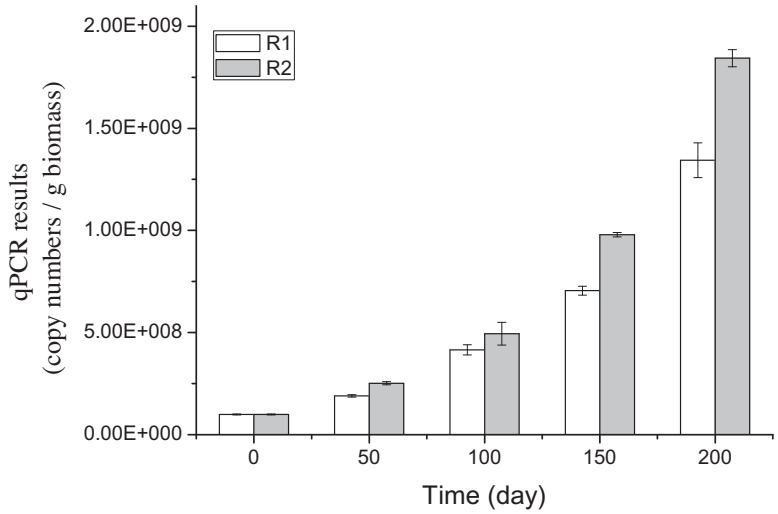

Fig. 5. Comparison of the qPCR results in the whole incubation period. R1, control reactor; R2, reactor with 100 mg/L RGO addition.

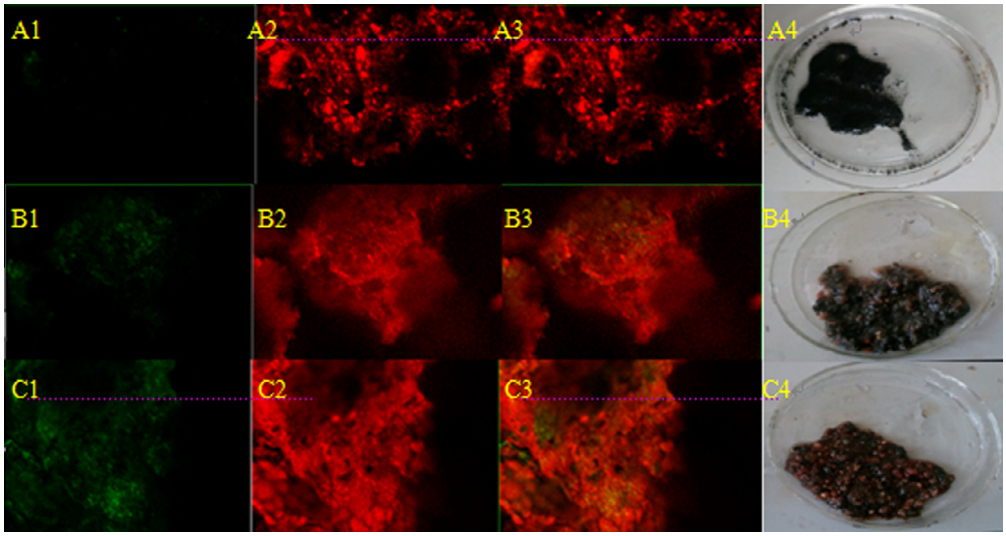

Fig. 6. FISH analysis of sludge samples from the anammox reactors. Red and green zones were total biomass and anammox biomass region. A1–A4 was taken from seed sludge; B1–B4 was taken from R1 on day 200; C1–C4 was taken from R2 on day 200. A4, B4, C4 were the physical photos of seed sludge, R1 and R2 on day 200. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

bacteria, were investigated in this study. The Amx368 probe and EUB338 (contains EUB338I-III) were used to target anammox bacteria and all bacteria, separately. As Fig. 6A1–A3 showed that there was only a few bacteria hybridized with Amx368 (green zone), indicating that the portion of anammox bacteria in the seed sludge was quite small. Image Pro-Plus software determined that the percentage of anammox bacteria was 1.47 ± 0.47% of total bacteria (red zone). After 200 days cultivation, anammox bacteria became the dominant population. Fig. 6B and C showed the green zone increased distinctly. With Image Pro-Plus software, the percentage of anammox bacteria were calculated to be 51.3 ± 7.47% and 62.9 ± 5.92% of total bacteria in R1 and R2, respectively. Synthesized the qPCR results and FISH analysis, both the anammox quantities and the percentage versus the total bacteria had a great improvement. Also, the physical photos showed that the red anammox cells in R2 (Fig. C4) were much more R1 (Fig. B4) and seed sludge (Fig. A4). Evidently, it was concluded that the anammox process was successfully started up from conventional activated sludge. Furthermore, the contrast of R1 and R2 in cell quantities proved that RGO addition accelerated the growth of anammox biomass.

Based on these experimental results, there were some reasonable speculations for affecting the startup of anammox process by RGO addition. RGO possesses similar properties of redox mediates because of epoxide, carboxyl, and hydroxyl groups on their basal planes and edges, which can lead to a good electron transfer ability and microbial affinity as redox mediates [10,20]. Compared with coenzyme Q (CoQ), RGO as an essential component participated in the electron transfer from HDH to cytochrome *bc*1 complex due to the faster electron transfer ability [12,21]. Thus, the enhancement of enzyme activity enhanced with RGO addition, which directly accelerated the ATP synthesis and the catabolism of anammox biomass [22]. Moreover, the acceleration of substance metabolism would generated because of the improvement of the catalysis of key enzymes and energy metabolism, which further brought about the consumption of more substrates, such as ammonium and nitrite. In addition, Ruiz et al. [23] predicted that RGO could be acted as a scaffold for bacteria attachment. Coincidentally, it is necessary to form macroflocs for enhancing the cell density due to the anammox bacteria were not active until the cell concentration was higher than 10^{10}–10^{11}cells/mL [24]. Thus, both the increment of enzymes activities and the formation of macroflocs could present more prominent TN removal performance. To the best of our knowledge, the anammox biomass yield was proportional to their nitrogen consumption (0.066 ± 0.01 mol/mol ammonium) [25]. With optimal RGO dose addition, the NRR of R2 always had relatively higher efficiency during the incubation period corresponding to the long-term acceleration of growth rate of anammox biomass. Therefore, appropriate RGO addition shortened the start-up time of anammox process from conventional activity sludge.

To date, there are so many studies to enhance the activity of anammox biomass with additives, such as MnO_2 [26], ferrous ion [27], and acyl homoserine lactones [28]. However, many kinds of these additives could result in secondary pollution. RGO was more like a catalyst which could long-term exist stably in reaction system and hardly could be degraded in our study. Regarding hydrophobic of the graphene materials, RGO could not discharge along with effluent avoiding secondary pollution effectively. It could be infer that RGO would be a potential additive to apply in anammox process for mass cultivation of anammox sludge in the continuous experiments.

4. Conclusion

The start-up time of anammox reactor from conventional activity sludge could be reduced significantly by RGO addition. It was confirmed that RGO enhanced the HDH activity, which was regarded as the intrinsic factor for accelerating the start-up period of anammox process. The immediate cause of fast start-up of the anammox process was that the growth rate of anammox bacteria in R2 with RGO addition was much faster than the control group.

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