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Investigation of COD and COD/N ratio for the dominance of anammox pathway for nitrogen removal via isotope labelling technique and the relevant bacteria

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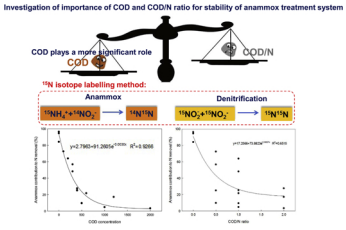
GRAPHICAL ABSTRACT



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ABSTRACT

This study aimed to investigate the importance of COD (chemical oxygen demand) and ratio of COD and nitrogen (COD/N) in influencing the dominance of anammox pathway to N-removal in anammox systems, which had been widely researched and results were not yet conclusive. Results showed that N-removal efficiency increased with increasing organic substrate, while the anammox contribution to N-removal decreased as confirmed by isotope labelling technique. Excessively high TN (total nitrogen) concentrations were detrimental to N-removal, and TN of 600 mg L^{-1} was optimized. Specific COD of 300 mg L^{-1} (a threshold value above which anammox was less active) was synergistic for N-removal. Moreover, Illumina sequencing and qPCR techniques uncovered that while the microbial community composition was relatively stable for all treatments, abundances of denitrifier were positively correlated with increase of COD, which was counter-productive for anammox abundance. Structure equation model indicated that COD was more important with respect to maintain the anammox stability than the COD/N ratio. Furthermore, experiment and model fittings revealed that anammox contributed more than 80% of N-removal when COD was below 55.7 mg L^{-1}, and approximately 50% at 220–300 mg L^{-1} COD, respectively. These data formed a reference for regulation of anammox systems in real-world applications.

1. Introduction

Anaerobic ammonium oxidation (anammox), a cost-effective and efficient technology of autotrophic biological nitrogen removal, has gained widespread attention, from laboratory-scale studies to engineering applications. The anammox, the slowly growing

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microorganisms, convert ammonium and nitrite into dinitrogen gas in the complete absence of oxygen [1]. Therefore, it possesses the advantages of no addition of organic carbon source, low oxygen consumption and sludge production. However, anammox bacteria exist low cellular yield, and anammox process and its microbial communities are easily influenced by nitrogen concentration, type of reactor, carbon sources, temperature, etc. [2–5]. Based on the anammox stoichiometric equation [6], the produced nitrate provides external substrate for denitrification. Meanwhile, COD in wastewater is ubiquitous, and provides electron donors to the denitrifying bacteria. An anammox system is able to obtain higher nitrogen removal efficiency through a synergistic effect between anammox and denitrifying bacteria by utilising the remaining nitrite/nitrate [6]. Both anammox and denitrifying bacteria were observed to be present and active in the anaerobic inner part of the biofilm [7], and *Proteobacteria* and *Planctomycete* were the dominant heterotrophic and autotrophic bacteria responsible for N-removal [8]. On the other hand, excessive COD leads to prevalence of denitrification over anammox, which is detrimental to the system. Therefore, the appropriate COD content for maintaining the stability and effectiveness of anammox/denitrification systems for long-term operation needs to be investigated.

So far, anammox has mainly been applied to treat wastewater with high ammonia concentration and low biodegradable organic substances (COD/N < 0.5) [9]. However, many studies have shown that anammox activity can be maintained at higher COD/N ratios under certain conditions. For example, anammox bacteria could coexist with heterotrophic bacteria at an elevated COD/N of 1.4 if a sufficiently high sludge retention time (SRT) was maintained [10]. Industrial wastewater with COD/N ratio of 2.2 was sufficiently low to prevent heterotrophic denitrifiers from overgrowing in an anammox system at an SRT of 46 d [11]. A high nitrogen removal was achievable at COD/N ratio of 1.5–2.7 when population ratio of denitrifying to anammox bacteria was maintained within 0.3–0.4 [12]. A 95% nitrogen removal rate was achieved at ratios less than 1.28, with anammox contribution decreasing to 51% and denitrification increasing to 49% [13]. The nitrogen removal rate could be maintained at 80%–85% by raising the COD/N ratio stepwise from 1 to 3, denitrification accounting for approximately 50% [3]. A COD/N ratio below 1.57 was critical to maintain simultaneous anammox and denitrification processes in a sequencing batch reactor [14]. Additionally, anammox activity in a partial nitrification-anammox reactor remained stable when C/N ratio increased to 2.5 [15]. According to the available literature, the appropriate COD/N ratio for synergistic effect between denitrifying and anammox bacteria on nitrogen removal seemed to be in the range of 0.5–3.

On the other hand, there exist different viewpoints regarding the effect of COD on anammox process. COD concentration over 300 mg L^{-1} was found to inactivate anammox communities [16]. Molinuevo et al. [17] pointed out that COD above 290 mg L^{-1} could result in complete cessation of an anammox process. However, in an anammox hybrid reactor, anammox bacteria could augment endurance against COD as high as 600 mg L^{-1} due to the addition of anaerobic granular sludge [18]. Moreover, the anammox process was not inactivated under COD concentration of 396.5 mg L^{-1}, probably due to the limited content of easily biodegradable substances in landfill leachate, and the average nitrogen removal rate was 85.1%, with denitrification accounting for only 14.9% [19].

To sum up, opinions on the effect of COD and COD/N ratio on nitrogen removal are both equivocal and contradictory, and results are not yet conclusive. It is obvious that organic substrate concentration significantly influences the anammox performance, but the exact levels at which synergistic and inhibitory effects balance each other remain unclear. Moreover, it should be investigate the importance of COD and COD/N ratio for the stability of anammox treatment system, to deepen understanding of the effect of organic carbon. Herein, the stability meant the dominance of anammox pathway for nitrogen removal in an anammox system, rather than the stable N-removal efficiencies only. In this study, isotope labelling (IL) and statistical techniques were firstly adopted to answer the question regarding how COD (with respect to COD/N) influences the stability of an anammox reactor. Moreover, the changes in abundance of functional gene and that of community structure corresponding to various organic substrate levels were also investigated. The results will present a reference for practical application.

Table 1
Experimental design.

Batch experiment	No.	TN (mg L^{-1})	COD (mg L^{-1})	COD/N ratio
I	1	200	0	0
	2	200	100	0.5
	3	200	200	1
	4	200	400	2
II	5	600	0	0
	6	600	300	0.5
	7	600	600	1
	8	600	1200	2
III	9	1000	0	0
	10	1000	500	0.5
	11	1000	1000	1
	12	1000	2000	2
Verification experiment	13	300	300	1
	14	400	400	1
	15	500	500	1
	16	800	400	0.5

2. Materials and methods

2.1. Experimental setup and reactor operation

To explore the effect of different levels of COD value and COD/N ratios on anammox performance, twelve rectors with different TN and COD concentrations were divided into three batch experiments to conduct, as shown in Table 1. Relatively low (200 mg L^{-1}), medium (600 mg L^{-1}), and high (1000 mg L^{-1}) concentrations of TN were investigated in the study, and the corresponding COD/N ratio was set at 0, 0.5, 1 and 2, respectively. As mentioned above, excessive COD concentration (usually COD/N > 2) facilitates heterotrophic denitrifier to outcompete the anammox bacteria in a reactor. Laboratory-scale upflow anaerobic filter (UAF) reactor was established, and filled with flexible fibre bundle filler. The effective volume was 70 mL, and hydraulic retention time (HRT) was 12 h. The seed sludge of each batch experiment was withdrawn from another anammox reactor, which has been operating stably for over 400 d under the conditions of the same TN concentration and the absence of organic carbon. The influent contained (per litre): 0.2 g of $MgSO_4{\cdot}7H_2O$, 0.25 g of $CaCl_2{\cdot}2H_2O$, 0.023 g of $FeSO_4{\cdot}7H_2O$, 1.25 g of $KHCO_3$, 1 mL of trace elements, nitrogen, and carbon source depending on the set value of each treatment. The trace element solution consisted of 20 g EDTA, 0.43 g $ZnSO_4{\cdot}7H_2O$, 0.24 g $CoCl_2{\cdot}6H_2O$, 0.22 g $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 0.99 g $MnCl_2{\cdot}4H_2O$, 0.25 g $CuSO_4{\cdot}5H_2O$, 0.20 g $NiCl_2{\cdot}2H_2O$, 20 g KH_2PO_4, and 0.014 g H_3BO_3 per litre [20]. The influent TN represented the sum of NH_4^+-N and NO_2–N (1:1), supplied by $(NH_4)_2SO_4$ and $NaNO_2$. Glucose was added, since it had no effect on the anammox bacterial metabolism [4]. Each treatment was run in three paralleled reactors, which were operated for two months at a constant temperature of 35 °C (Fig. SI1).

The ammonium (NH_4^+), nitrite (NO_2^-), and nitrate (NO_3^-) amounts were analysed using spectrophotometry, following standard methods (Chinese NEPA, 2002). COD was examined using the colorimetric method (Lianhua, China). Herein, total nitrogen (TN) removal rate represented the reduced amount of the sum of NH_4^+-N, NO_2^--N, and NO_3–N of the influent and effluent.

According to the results of batch experiment, COD seemed to be a more important factor in determining anammox contribution, which

would be present in Results and Discussion section. To further verify the detailed influence of organic substrate, additional experiments were carried out in this study. Some dubious COD levels of 300–500 mg L^{-1} were selected to test. Moreover, appropriate TN content (200 mg L^{-1} < TN < 1000 mg L^{-1}) was considered for experimental design, with the suitable COD/N ratio range of 0.5–1 for anammox survival (Table 1).

2.2. ^{15}N-labelled nitrite isotope experiment for calculating contributions of anammox and denitrification processes to nitrogen removal

An isotope experiment was conducted for an incubation time of Day 44–58 of each batch, when the reactor was operated in stable conditions. Before each isotope experiment, the reactors were flushed with the influent without N and C sources for 12 h to remove any residual N-species. 1 mg of sodium sulphite was added for oxygen depletion. The pre-treated reactors were sealed with rubber stoppers ensuring the elimination of all bubbles, considered to be airtight in other studies [21]. Subsequently, N_2-purged stock solutions of $^{14}NH_4^+$ + $^{15}NO_2^-$ (^{15}N at 99.24%, Shanghai Research Institute of Chemicalindustry Co. Ltd) and glucose were injected to maintain the final concentrations at the set values for each treatment. The isotope labelled reactors were incubated in a shaker at 250 rpm at 35 °C and sampled at the end of incubation period by transferring 200 μL of supernatant to He-flushed, 12.6-mL glass vials. The product $^{29}N_2$ was derived from anammox pathway by combining one molecule of $^{15}NO_2$- and one molecule of NH_4^+, whereas $^{30}N_2$ was generated from two molecules of $^{15}NO_2^-$ via denitrification. $^{29}N_2$ and $^{30}N_2$ were measured by isotope-ratio mass spectrometry (Thermo Delta V Advantage, Germany). One example of mass spectra was present in Fig. SI 2. The potential contribution of anammox and denitrification to N_2 formation was calculated [21]. The stability meant the dominance (more than 50%) of anammox pathway for nitrogen removal in an anammox system according to the results of isotope labelling technology.

2.3. DNA extraction, quantitative PCR, and amplicon sequencing

Aiming to illustrate the effect of organic substrate on the abundance of functional bacteria and community structure, sludge samples collected at Day 58 of each operation were subjected to DNA extraction using a FastDNA SPIN kit (MP Biomedicals, Santa Ana, CA) following the manufacturer's protocol. The DNA quality was measured with a NanoDrop spectrophotometer (ND-1000, NanoDrop Technologies, Wilmington, DE), and stored at −20 °C for further analysis. The functional genes of denitrifying and anammox bacteria were amplified with specific primer pairs (nosZ 1F:1R for denitrification, hzsA 1597F:1857R for anammox) (Table SI 1), representing the absolute abundance of denitrifier and anammox bacteria, respectively. Each Sample was run in triplicate in a 96-well plate using a LightCycler 480II PCR system (Roche). Amplification procedure was as follows: 94 °C for 5 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s, and a final melting curve analysis. Standard curve for quantitative PCR was linear (Supplementary Information, $r^2 > 0.99$) with a PCR efficiency between 85.0% and 102.7%. The absolute abundances were calculated according to the standard curve.

A set of primer (515F:806R) was used for 16S rRNA gene amplification (Table SI 1). The 25 μL reaction mixture consisted of 12.5 μL of Phusion High-Fidelity PCR Master Mix with GC buffer (New England Biolabs, Beverly, MA, USA), 0.4 μM of each primer and 10 ng of the extracted DNA template. The PCR reaction conditions were 94 °C for 5 min; 30 cycles of 94 °C for 60 s, 57 °C for 45 s and 72 °C for 60 s, and a final extension at 72 °C for 5 min. Each sample was amplified in triplicate. The PCR products were quantified with a Qubit 2.0 fluorometer (Invitrogen, USA). The amplicons from different samples were pooled in equal amount. High-throughput sequencing of V4 hyper variable region of the bacterial 16S rRNA gene was performed using an Illumina Hiseq 2500 platform. This part work was conducted by Novogene Bioinformatics Technology Co., Ltd (Beijing, China).

Raw sequence data were merged, quality controlled, and aligned using MOTHUR v.1.33.3 [22]. The sequences were assigned to operational taxonomic units (OTUs) at 97% similarity level. For our data, we used a randomly selected subset of 64,601 sequences from each sample to normalize sequencing effort across samples. The final total data set retained 2324 OTUs at 97% identity. The raw sequence data have been deposited into the GenBank sequence read archive (SRA) under the accession numbers SRP171955.

2.4. Data analysis

Structure equation model (SEM) was adopted to assess the significance of various parameters using path analysis. The Spearman's coefficient was used to establish a correlation matrix between groups of parameters. An initial model was included with all plausible pathways between operation effectiveness and other parameters (e.g. TN, COD, COD/N, denitrifier and anammox abundances, etc.). The significance of each path coefficient was tested by critical ratio ($p < 0.05$). Non-significant paths were trimmed from the model until all remaining paths were significant. The final model was evaluated with the goodness-of-fit index (GFI) and the adjusted goodness-of-fit index (AGFI). Both indices close to 1 implied a better fit. The SEM analysis was performed using the software package AMOS version 17.

The differences between the experimental treatment and the control in one batch experiment were conducted by one-way analysis of variance (ANOVA), and correlation was conducted by spearman analysis.

3. Results and discussion

3.1. Reactor performance under different TN and COD concentrations

TN removal efficiency is a critical parameter to assess the reactor performance, contributed by both heterotrophic denitrifier and autotrophic anammox bacteria (Fig. 1). For the treatment of TN = 200, 600, and 1000 mg L^{-1}, the TN removal rates were 52.3 ± 6.7%, 56.0 ± 10.0, and 27.8 ± 12.2%; 59.1 ± 8.5, 71.5 ± 7.7, and 40.7 ± 13.0%; 61.8 ± 8.8, 70.4 ± 7.6, and 54.0 ± 7.6%; and 81.8 ± 6.2, 76.3 ± 7.9, and 61.3 ± 8.9%, respectively, for COD/N values 0, 0.5, 1, and 2. TN removal rates generally increased with increasing COD/N ($p < 0.05$). Additionally, the TN removal rates fluctuated relatively remarkably, and were lower at COD = 0 compared to those (77% on average) in seed-sludge reactor operated under the same conditions. Both of these could be attributed to the small UAF reactor, which could not maintain the strictly anaerobic and flow conditions as in the larger seed-sludge reactor, and was easily influenced by the external environmental conditions. Nevertheless, TN removal rate showed an increasing trend with the increase of organic substrate concentration in the influent. The denitrifying bacteria conducting denitrification utilised the glucose as a carbon source, due to which, the increasing organic substrate promoted the denitrifier growth and nitrogen removal. Treatment of TN = 600 mg L^{-1} at different COD/N levels achieved better performances than those in the treatment of TN = 200 mg L^{-1} and TN = 1000 mg L^{-1} as a whole. Neither excessively high nor too low TN concentration in the influent was beneficial to nitrogen removal in the anammox reactor. According to an application survey, anammox bacteria favoured high nitrogen loading (500–1500 NH_4^+-N L^{-1}) with respect to the majority of the full-scale installations [23]. Therefore, anammox-based system applied for treating mainstream municipal wastewater was considered a great challenge. Simultaneously, increased nitrogen loading seemed to have an adverse effect on the nitrogen removal performance. Ma et al. [2] indicated that TN concentration higher than 550 mg L^{-1} induced a significant drop in TN removal efficiency and effluent pH, and TN removal efficiency decreased by 50% when TN concentration reached 725.3 mg L^{-1}, both of

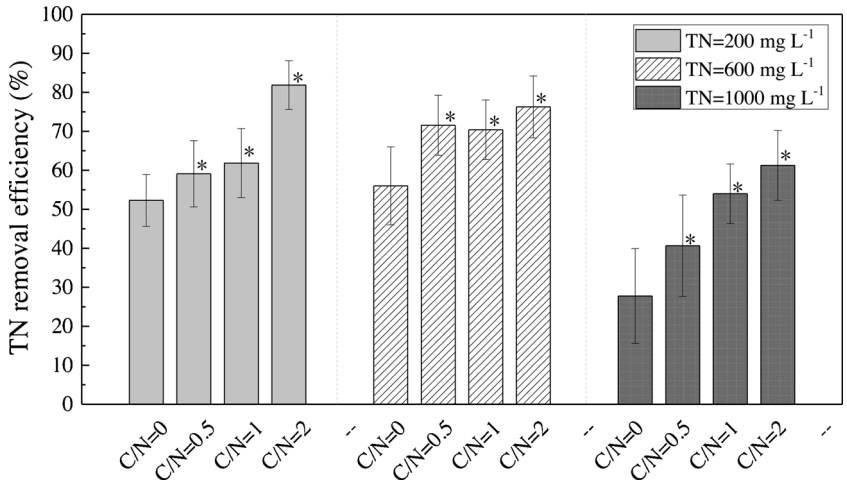

Fig. 1. TN removal efficiencies under different N and C loading during long-term incubation. Values represented the mean ± SEM. Asterisk represented $p < 0.05$ compared with the control of each batch experiment.

which were ascribed to the combined effect of high free ammonia (FA) and free nitrous acid (FNA). However, the half-inhibitory concentration (IC_{50}) of ammonia was generally higher than that of nitrite [24,25]. Research has proved that IC_{50} values of nitrite and FNA were in the range of 350–400 mg L^{-1} and 0.011–0.11 mg L^{-1}, respectively [24,26]. Therefore, high nitrite concentration was speculated to be responsible for the reduced TN removal rate in this study, with consistent with the other research [27]. Till date, the effective substrate form (ammonia vs. FA; nitrite vs. FNA), inhibiting anammox activity, remains controversial [25,26].

It was noteworthy that TN removal rate was relatively higher at TN = 600 mg L^{-1}, COD = 300 mg L^{-1} than others, indicating a synergistic effect between the denitrifier and anammox bacteria for nitrogen removal. Both the functional microorganisms coexisted well in a system, rather than competing for substrate. Ni et al. [28] also found an effective strategy utilising this coexistence to treat wastewater containing high levels of nitrogen and COD value of 308 mg L^{-1}. However, COD of 300 mg L^{-1} was thought to be the inhibitory level in previous studies [16,17]. In this study, the seed sludge partly constituted of granular sludge, and the carriers were installed inside the UAF reactor. On the one hand, anammox granules was considered to have higher tolerance to organic substrate than flocculent sludge [28]. On the other hand, the biofilm carriers may offer a refuge site for inactive cells, and anammox bacteria were observed in high numbers deeper in the biofilm [29]. These observations reported by others may give an explanation of good performance obtained at the COD of 300 mg L^{-1} in our study.

3.2. Contributions of anammox and denitrification to nitrogen removal

The proportion of anammox and denitrification pathways during the biological nitrogen removal process could be calculated by measuring the produced mass of $^{29}N_2$ and $^{30}N_2$ using isotope-ratio mass spectrometry. Organic substrate level was a key factor determining the N removal processes. The dominance of anammox pathway gradually diminished with increasing concentration of COD, and denitrification prevailed over anammox (Fig. 2). The anammox pathway dominated in nitrogen removal when no COD was added to the influent. As for the treatment of TN = 1000 mg L^{-1}, anammox process contributed 84.1 ± 4.3% to nitrogen loss, which was less than those seen in the other two treatments (95.9%–98.6%). Therefore, excessively high TN concentration was proved again to be detrimental to the anammox bacteria. The dead bacteria may offer a carbon source, which could explain the relatively large denitrification contribution in the treatment of TN = 1000 mg L^{-1}. Subsequently, the proportion of anammox contribution negatively correlated with glucose dosage. When COD/N was 2, anammox contribution reached the bottom of each treatment, only accounting for 3.4%–27.6%. The contribution of anammox and denitrification varied to some extent in spite of the same COD/N ratio. For example, the anammox contributions were 72.4%, 56.7%, and 9.8% under the same COD/N ratio of 0.5, with the corresponding COD concentrations of 100, 300, and 500 mg L^{-1}, respectively. It seemed that COD played a more important role in determining the interspecies relationship between the denitrifying and anammox bacteria. The latter could maintain the dominance at COD levels < 200 mg L^{-1} (e.g. TN = 200 mg L^{-1}, C/N = 1), whereas the denitrifier outcompeted the anammox bacteria at COD levels > 400 mg L^{-1}, with the denitrification accounting for 72.4% (TN = 200 mg L^{-1}, C/N = 2) of N removal, even reaching up to 90.2% at a COD level of 500 mg L^{-1} (TN = 1000 mg L^{-1}, C/N = 0.5). For the treatment of TN = 600 mg L^{-1} and COD = 300 mg L^{-1}, a better performance could be achieved since the anammox and denitrification contributed almost equivalently, indicating coexistence of both bacteria and stability of the anammox system in a long-term operation.

To explore the actor responsible for nitrogen removal in an anammox system, mass balance based on influent/effluent quality [11,13,14], molecular biological technique, such as quantitative PCR, FISH [10,16], ex-situ enzyme activity test, and specific anammox activity test [16,30]were usually adopted to depict stability of the anammox system. However, mass balance is not deterministic in the present work, since the calculation does not take nitrification effect on ammonium and nitrite reduction and COD removal by other heterotrophic bacteria into account, thereby leading to a misestimate of the different process contributions. Molecular biological technique only reflected the bacterial profile without quantitative calculation. Compared to these, the ^{15}N isotope labelling method distinguished the amount of $^{29}N_2$ from that of $^{30}N_2$ according to different reaction substrates, in order to essentially quantitatively estimate the pathway contribution to nitrogen loss when reactors were exposed to COD existence. The results in this study were more direct and reliable when other interferences were reduced. Nowadays, the ^{15}N isotope labelling method had been already applied for assessment of anammox effect in natural system, e.g., coastal marine sediments [31] and estuarine sediments [32], and traditional municipal wastewater treatment plants [33].

3.3. Changes in functional bacteria and community structure

Physiological change of the biomass was observed during the study. The sludge colour changed from reddish brown to black when COD increased. The abundance of functional microorganism in the reactors

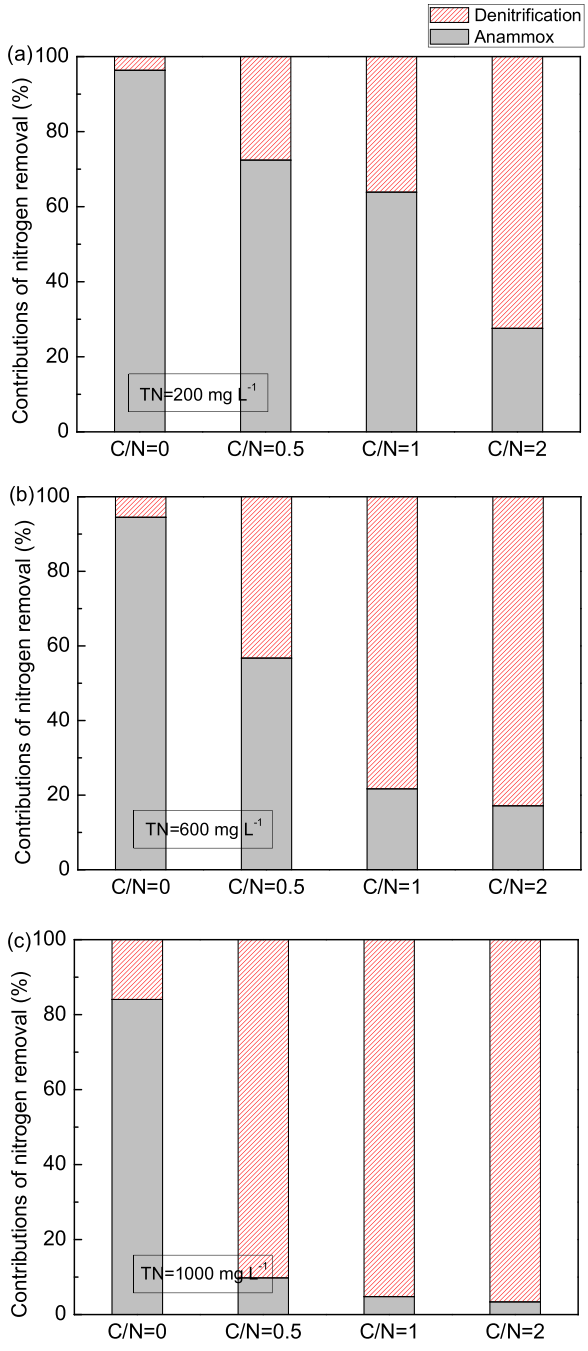

Fig. 2. Contributions of anammox and denitrification processes to nitrogen removal in different treatments.

at the later stage of operation was shown in Fig. 3. Overall, the autotrophic anammox bacteria were negatively correlated with the increasing glucose dosage ($p < 0.05$), which was positively correlated with the heterotrophic denitrifier ($p < 0.05$). Ni et al. [28] found a reduction in the number of anammox bacteria and an increase in denitrifier quantity when 400 mg L^{-1} of COD-containing influent was applied. For the treatments of TN = 600 mg L^{-1}, the copy numbers of anammox bacteria were higher than those of denitrifier in the corresponding treatments. On the contrary, the denitrifier abundance was higher than that of anammox in the treatment of TN = 1000 mg L^{-1} in presence of COD. The anammox bacteria are known to have a cell-density dependent phenomenon, the anammox activity appears when the cell concentration is higher than 10^{10}–10^{11} cells mL^{-1} [34]. Taking the treatment of TN = 600 mg L^{-1} and COD = 300 mg L^{-1} as an example, where good performance and anammox dominance in nitrogen removal was recorded, anammox abundance was two orders of magnitude higher compared to that of the denitrifier. The higher anammox abundance seemed to better maintain the anammox activity and reactor operational stability. Moreover, since the highest abundance of anammox occurred in the treatment of TN = 600 mg L^{-1} and COD = 0, in comparison with the other two treatments without COD addition, the appropriate TN concentration was assumed to be beneficial to anammox growth, consistent with the TN removal performance discussed in section 3.1.

The OTU coverage was sufficient for community diversity, with a coverage estimate of above 0.99 for each library. The OTU number was in the range of 687-1327. The treatment of TN = 1000 mg L^{-1} had the richest OTU number, consecutive of TN = 200 mg L^{-1}, and TN = 600 mg L^{-1} had the lowest. Changes of Shannon and Chao 1 indices was consistent with those of OTU (Table SI 2). It was speculated that anammox worked well and prevailed at the treatment of 600 mg L^{-1}, so as to achieve a relatively low diversity. However, the bloom of heterotrophic bacteria at the treatment of TN = 1000 mg L^{-1} may cause an increase in diversity.

The phylum-level composition of bacterial communities at the end of the incubation showed that majority of the sequences belonged to *Proteobacteria*, *Chlorobi*, *Chloroflexi*, and *Firmicutes*, with *Bacteroidetes* and *Planctomycetes* also making contributions to the community (Fig. 4). In addition, *Deferribacteres*, *Armatimonadetes*, *Parcubacteria*, and *Deinococcus-Thermus* were listed in the top 10 in relative abundances. The phyla *Proteobacteria*, *Chlorobi*, *Chloroflexi* and *Bacteroidetes* were most commons in anammox bioreactors and comprised a large proportion of microbial community [35]. It was reported that the predominant denitrifying microorganisms belonged to the phyla *Proteobacteria* [36]. *Chloroflexi* refers to the filamentous species, known to provide biofilm structural integrity [37], and reported to scavenge metabolites derived from anammox under anoxic conditions [38], thereby explaining their occurrence throughout the sludge. *Chlorobi* dominated the abundance of the anammox granule community, consistent with other study [35. The relative abundance of *Chlorobi*, which was heterotrophic bacteria [35], increased with increasing COD, especially in the treatments of TN = 200 and 600 mg L^{-1}. And it was reported that nitrite in the influent may also lead to an increase of OTUs associated with *Chlorobi* [39]. Chlorobi-affiliated bacteria had the ability to degrade extracellular proteins produced by anammox bacteria to affect consortium aggregation [40], therefore, the increasing abundance of Chlorobi was probably detrimental to anammox growth, according with changing pattern of anammox of qPCR results in this study. A large number of *Firmicutes* were noted in the treatment of TN = 1000 mg L^{-1}, which probably metabolised the dead cells caused by nitrite toxicity. The target functional bacteria belonged to the phylum *Planctomycetes* in the anammox systems. Sequencing results showed that *Candidatus Kuenenia* was the dominant species possessing anammox ability, accounting for 1.69% of the total bacteria on an average. Desloover et al. [11] also found that the genus *Kuenenia* constituted 3.1% of the biomass treating practical digested industrial wastewater. Other species playing a role in anammox systems included *Denitratisoma*, *Ignavibacterium*, *Comamonas*, and *Xanthomonas*, similar with the founding reported by Agrawal et al. [35] and Wen et al. [36]. The microbial community towards increasing substrate availability showed little change in composition in this study. Persson et al. [29] also observed small change in community composition under the conditions of downshifts in substrate concentration. The impact of the influent substrate concentrations on the shift of the community was mainly about the changes of abundance of heterotrophic bacteria, since the presence of COD enabled the survival and predominance of denitrifiers [41].

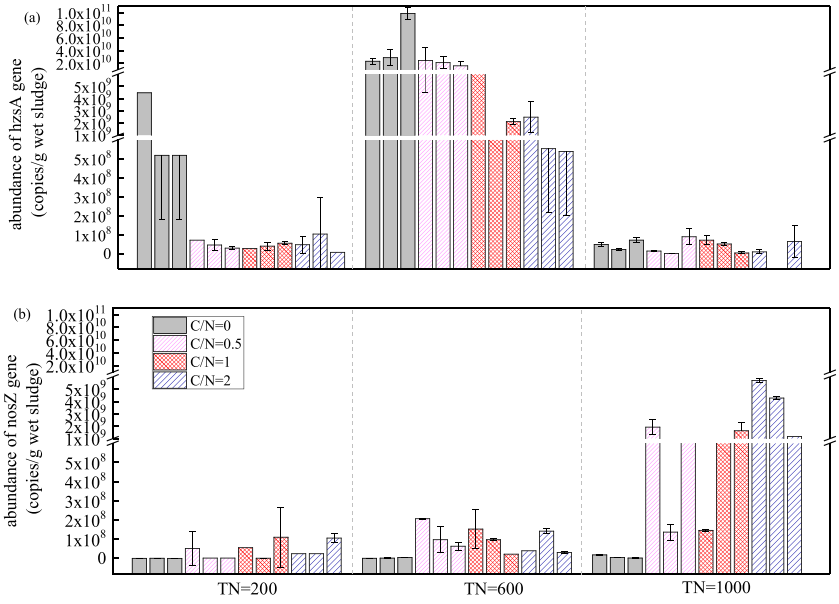

Fig. 3. Changes in abundance of (a) anammox and (b) denitrifying bacteria.

3.4. Drivers of nitrogen removal pathway according to SEM

To further prove the relationship between anammox contribution to N removal and operation parameters, SEM was developed (Fig. 5). The indices of the modifier model (GFI = 1.00, AGFI = 1.00) suggested a good fit of the model to the original data. COD concentration, COD/N ratio, and anammox abundance had direct effect on anammox bacteria involved in N removal, and COD/N ratio had an indirect effect on anammox contribution due to their effect on anammox abundance. Numbers on each arrow indicated partial correlation coefficient of each causal relationship [42]. Therefore, it was suggested that COD concentration was more remarkably associated with the anammox contribution to N removal. SEM analysis validated that the COD had more influence on the competition between anammox bacteria and denitrifier.

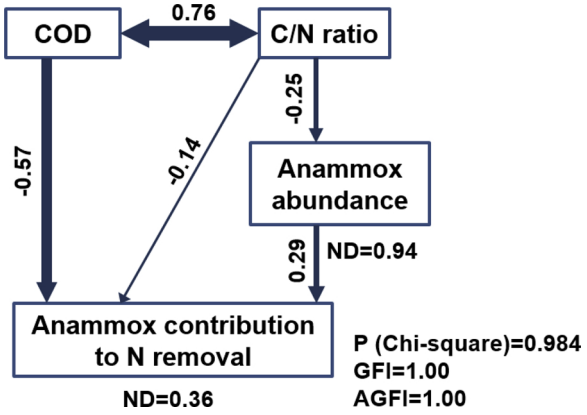

Fig. 5. Final path by SEM analysis. Significance of the chi-square test was given. Both indices of GFI and AGFI were 1, indicating the best fit. Numbers on each arrow showed partial correlation coefficients related to each causal relationship, and arrow thickness was proportional to the partial correlation value. The coefficient of non-determination (ND = 1-R^2) suggested that the fraction could not be explained by the model.

3.5. Verification experiments and fitting equations

According to the results from systematic experiments, COD was concluded to be a more significant factor in determining anammox contribution. However, TN of 1000 mg L^{-1} was considered as

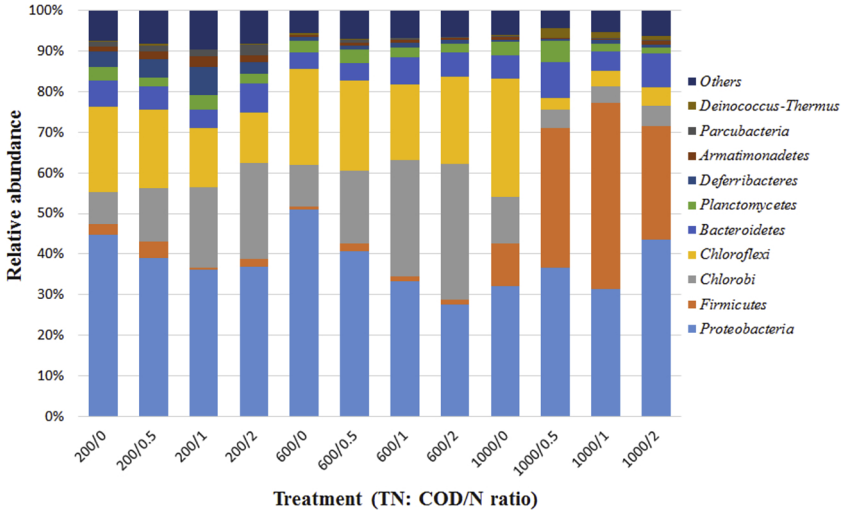

Fig. 4. Bacterial community composition in different treatments.

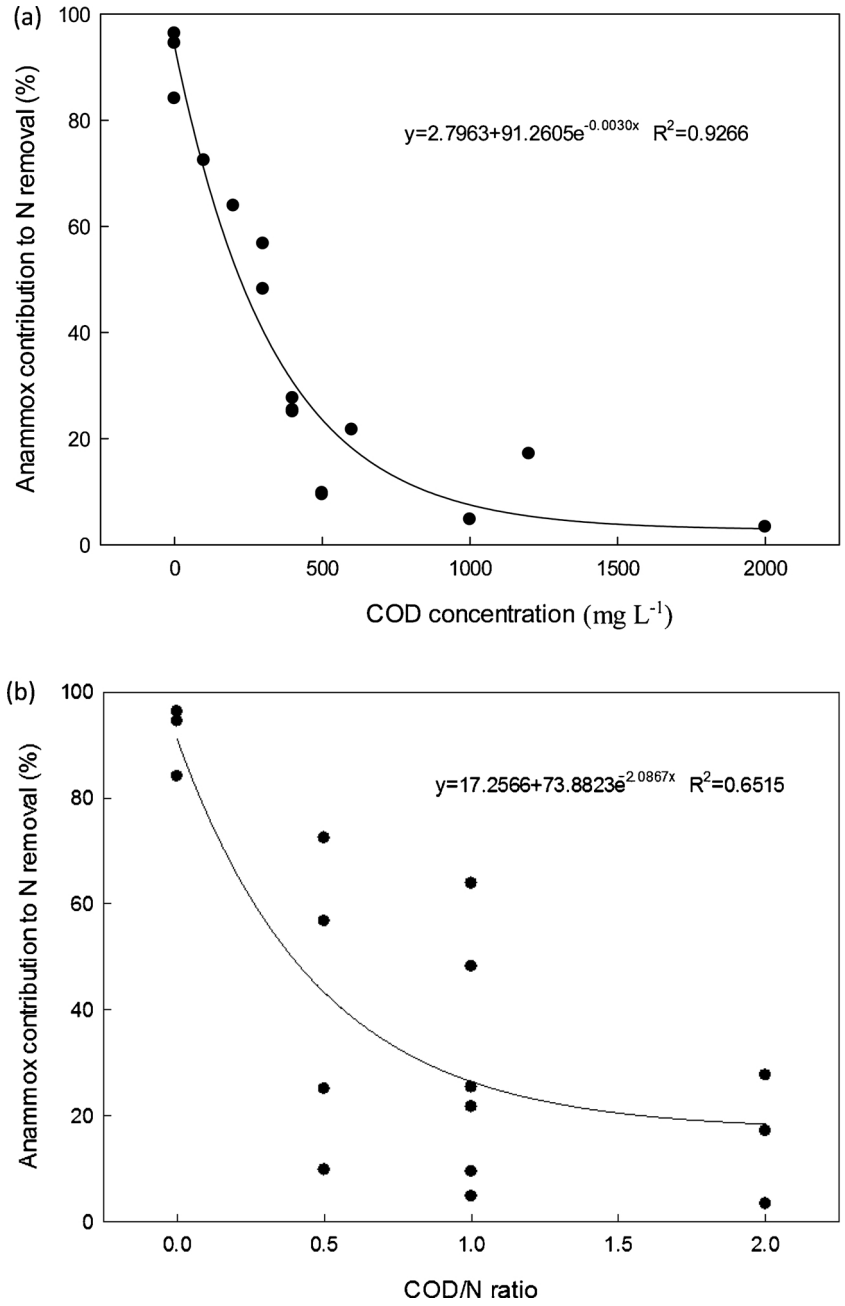

Fig. 6. Fitting curves between anammox contribution and (a) COD or (b) COD/N ratio.

excessively high nitrogen concentration, detrimental to anammox system. COD of 300 mg L^{-1} seemed to be a threshold value to retain the dominance of anammox. Additionally, there were relatively wide gaps of nitrogen load among the set treatments. Therefore, considering all factors mentioned above, to further investigate the influence of organic substrate on the nitrogen removal pathways, some additional experiments with more appropriate of nitrogen laod of 300–800 mg L^{-1} and COD of 300–500 mg L^{-1} were designed and conducted (see Table 1). During the two month-operation, TN removal rates in the treatment of TN = 300 mg L^{-1}, 400 mg L^{-1}, and 500 mg L^{-1} with COD/N = 1 were 73.5 ± 10.1%, 51.5 ± 9.8%, and 57.9 ± 12.7%, respectively. Meanwhile, there was lower nitrogen removal efficiency (23.2–45.0%) when treated with lower COD/N ratio (TN = 800 mg L^{-1} and COD = 400 mg L^{-1}), which could also be ascribed to deterioration of bacteria under high nitrogen loading (Fig. SI 3).

The results of ^{15}N-labelled isotope experiments with respect to verification experiment were summarised in Fig. SI 5; it clearly showed that the contribution of anammox pathway decreased stage-wise with increasing COD concentrations. Anammox contribution accounted for more than 70% of N removal when COD concentration in the influent was 100 mg L^{-1}; this value reduced to 63.9% at COD of 200 mg L^{-1}. Subsequently, anammox pathway contributed 48.2%–56.7% at COD of 300 mg L^{-1}, 25.0%–27.6% at COD of 400 mg L^{-1}. This proportion reduced only to approximately 10% under the condition of 500 mg L^{-1} COD, at which N removal was accomplished mainly through denitrification. Anammox dominated in nitrogen removal when COD was below 200 mg L^{-1}, and anammox reactor could be operated stably at COD of 300 mg L^{-1}, with anammox and denitrification contributing equivalently.

Anammox contribution showed a good correspondence with concentrations of organic substrate. As shown in Fig. 6(a) and (b), the R^2 value of fitting curve, based on the COD values, was larger than that of

COD/N ratio, indicating that COD was more important with respect to determine relative contributions of different nitrogen removal pathways, which was less correlated with COD/N ratio. Synergistic effect between anammox and denitrifying bacteria is known to achieve a better performance of nitrogen removal under an appropriate range of organic substrate, beyond which denitrification may outcompete anammox. A COD threshold value for operational stability of anammox defined the anammox pathway contributing more than 80% to nitrogen removal. According to the fitting equation, the corresponding COD threshold value was 55.7 mg L^{-1}. It was reported anammox system achieved a stable and efficient performance at COD concentrations of 140 mg L^{-1} [43] and 95–121 mg L^{-1} [17], respectively. When the definition was 50%, the COD threshold value was 219.7 mg L^{-1}, consistent with the reports that anammox contributed 51.2%–55.3% to nitrogen removal by mass balance calculations, when the influent COD concentration was 284.1 mg L^{-1} [44,45]. Anammox system deteriorated rapidly when COD was over 300 mg L^{-1}. However, in actual wastewater treatment system, anammox activity can be obtained at high COD if the surrounding environment offers a refuge site for anammox bacteria that are less sensitive to COD loading [6]. For example, simultaneous partial nitrification, anammox, and denitrification (SNAD) could endure higher COD loading, since anammox bacteria were present in the anaerobic inner part of the SNAD biofilm [7].

4. Conclusions

Results of ^{15}N isotope labelling experiments, combined with SEM analysis, showed that COD was more important in maintaining the stable operation of anammox reactor than the COD/N ratio. In the batch experiments, specific COD concentration of 300 mg L^{-1}, which balanced synergistic and inhibitory effects of anammox and denitrification to N-removal, exhibited satisfactory performance. Moreover, considering the results of experiment and fitting together, quantitative anammox contribution to N-removal was achieved, that was, anammox contributed more than 80% when COD threshold value was below 55.7 mg L^{-1}; anammox contributed more than 50% when COD was 220–300 mg L^{-1} and the system deteriorated rapidly when COD was over 300 mg L^{-1}. Even so, anammox activity may be maintained at high organic substrate concentrations in actual wastewater treatment system, if measures are taken to reduce the exposure to organic carbon, such as anaerobic pre-treatment and biofilm. Additionally, abundance of denitrifiers were positively correlated with increase of COD, which was counter-productive for the anammox bacterial abundance. These findings supplied a reference to regulate the anammox system in a real application.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jhazmat.2018.12.036.

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