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RESEARCH ARTICLE

Effects of Fe(II) on anammox community activity and physiologic response

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HIGHLIGHTS

- 0.12 mmol/L Fe(II) enhanced the total anammox activity and bacterial abundance best.
- 0.09 mmol/L Fe(II) led to the best performance on relative anammox activity.
- 0.75 mmol/L Fe(II) had an immediate but recoverable inhibition on anammox activity.
- More genes but not relative level were expressed at higher Fe(II) concentration.

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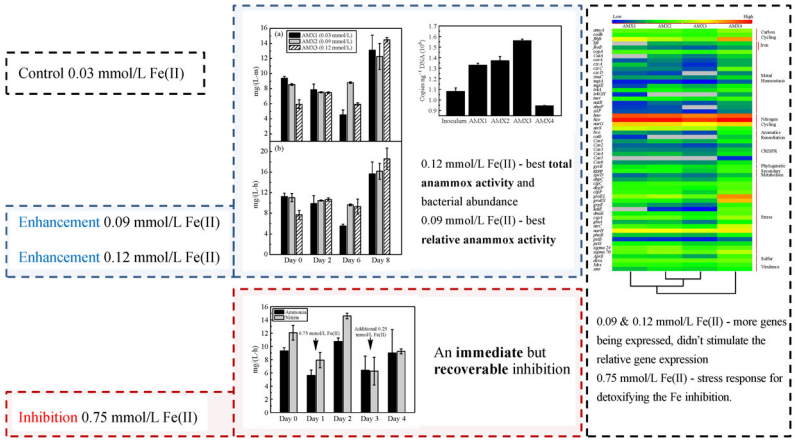
GRAPHIC ABSTRACT



ABSTRACT

Though there are many literatures studying the effects of iron on anammox process, these studies only focus on the reactor performance and/or the microbial community changes, the detailed effects and mechanisms of Fe(II) on anammox bacterial activity and physiology have not been explored. In this study, four Fe(II) concentrations (0.03, 0.09, 0.12 and 0.75 mmol/L) were employed into the enriched anammox culture. The enhancement and inhibition effects of Fe(II) on anammox process and bacterial physiology were investigated. It was discovered that the anammox process and bacterial growth were enhanced by 0.09 and 0.12 mmol/L Fe(II), in which the 0.12 mmol/L Fe(II) had advantage in stimulating the total anammox activity and bacterial abundance, while 0.09 mmol/L Fe (II) enhanced the relative anammox activity better. The anammox activity could be inhibited by 0.75 mmol/L Fe(II) immediately, while the inhibition was recoverable. Both 0.09 and 0.12 mmol/L Fe(II) induced more genes being expressed, while didn't show a stimulation on the relative expression level of functional genes. And anammox bacteria showed a stress response to detoxify the Fe inhibition once inhibited by 0.75 mmol/L Fe(II). This study provides more information about physiologic response of anammox bacteria to external influence (enhancement and inhibition), and may also instruct the future application of anammox process in treating various sources of wastewater (containing external disturbances such as heavy metals) and/or different treatment strategies (e.g. from side-stream to main-stream).

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1 Introduction

Anaerobic ammonium oxidation (anammox) process has many advantages comparing with the traditional nitrification-denitrification, such as zero consumption of organic carbon, reduced oxygen demand and CO_2/N_2O emission, low sludge yield (Op den Camp et al., 2006; Qiao et al., 2013; Zhang et al., 2019b). However, the anammox bacteria have a long doubling time of 11–20 days (Hu et al., 2010), and their activities can be greatly affected by operational conditions and interfering substances containing in the wastewater (e.g. heavy metals) (Liu et al., 2008; Zhang et al., 2019a), which restrict their application in pilot-scale plants. Thus, enhancing the growth rate, activity, and resistance to external disturbance of anammox bacteria are significant for application of anammox process in wastewater treatment.

Iron is important for anammox bacterial growth, as it may be related to energy generation or the function of some enzymes in the electron transport chain (van Niftrik et al., 2008b; Liu and Horn, 2012). The increase of Fe(II) concentration in the medium is beneficial for the synthesis of some key proteins, such as cytochrome *c* which contains heme-c groups and needs to chelate with ferrous iron for active regions formation (Qiao et al., 2013). These proteins involve in the ammonium oxidation (van Niftrik et al., 2008a), thus an increase of Fe(II) in the medium would be beneficial for the electron transfer in metabolism of anammox bacteria and can enhance anammox activity. However, heavy metals can also cause bioaccumulative toxicity because of their non-biodegradability and accumulation in organisms, thus too high concentration of Fe(II) will also cause inhibition on anammox activity. Therefore, the Fe(II) concentration in wastewater should be appropriate.

There have been some studies examining the effect of Fe(II) on anammox culture (Liu and Horn, 2012; Qiao et al., 2013; Bi et al., 2014; Huang et al., 2014; Liu and Ni, 2015; Shu et al., 2016), finding the enhancement of appropriate concentration of Fe(II) on nitrogen removal and anammox bacterial growth. Qiao et al. (2013) and Bi et al. (2014) revealed that modest increase in Fe(II) concentration facilitated more heme c synthesis and enhanced hydrazine dehydrogenase (HDH) activity. Liu and Ni (2015) constructed a model of anammox system to depict the relation between Fe(II) addition (0.03, 0.06, 0.09, 0.12, 0.18 mmol/L) and bacterial growth, and proposed that Fe(II) addition enhanced anammox activity mainly by increasing the bacterial growth rate. Besides, the effects of Fe(II) (0.02, 0.04, 0.06, 0.08, 0.10 mmol/L) on the nitrogen conversion pathways and microbial communities in an anammox system were also studied (Shu et al., 2016), and the results revealed the potential importance of the following coupling processes in the total nitrogen loss under Fe(II) stress: anammox, nitrification, dissimilartory nitrate reduction to ammonium (DNRA), anaerobic ammonium oxidation coupled with Fe(III) reduction (Feammox), and nitrate-dependent Fe(II) oxidation (NDFO). Apart from the enhancement effect, the inhibition of high Fe(II) concentration on anammox activity was also studied (Zhang et al., 2018; Mak et al., 2019). Zhang et al. (2018) gradually increased the Fe(II) concentration in a long-term operated anammox reactor, and found that the inhibition of nitrogen removal caused by 30 mg/L Fe(II) (about 0.54 mmol/L) addition could still be recovered partly, while 50 mg/L Fe(II) (0.89 mmol/L) inhibited the activity without any recovery. Though there are many reported works studying the effects of iron on anammox process, these studies only focus on the reactor performance and/or the microbial community changes, the detailed ferrous function on the activity and physiology of anammox bacteria have not been explored. However, since anammox process has attracted increasing attention in municipal wastewater treatment recently, and iron is one of the most commonly existed heavy metals in nitrogen-containing wastewater (Zhang et al., 2018; Mak et al., 2019), thus knowing the physiologic response of anammox bacteria to ferrous iron, and how the anammox bacteria perform in this kind of wastewater are important for its future application.

In this study, four Fe(II) concentrations (0.03, 0.09, 0.12 and 0.75 mmol/L) were tested to investigate the stimulation and inhibition effects of ferrous iron on anammox bacterial physiology. The performances of the batch reactors were monitored, RNAs were extracted from the cultures, and the synthesized cDNAs by reverse transcription were used to carry out GeoChip analysis, by which the functional communities and expression level differences in functional genes under different Fe(II) concentrations conditions were obtained, and the response of anammox bacteria to Fe(II) stimulation and inhibition were discussed. The results about effects of Fe(II) on anammox bacterial physiology can provide more instructions for enhancing the anammox bacterial growth and its future use in treating various sources of nitrogen-polluted wastewater.

2 Materials and methods

2.1 Batch experiments

Batch experiments were set-up and conducted in 120 mL serum bottles. Inocula were withdrawn from a laboratory-scale anammox reactor that had been operated for more than two years. The biomass in the reactor was mainly in the granular type. Anammox culture (35 mL) and culture medium (80 mL) were mixed and then added into each serum bottle in an anaerobic chamber. The medium contained (per liter) $KHCO_3$ 1.25 g, KH_2PO_4 0.025 g,

$CaCl_2 \cdot 2H_2O$ 0.3 g, $MgSO_4 \cdot 7H_2O$ 0.2 g, trace element solution I 1 mL, and trace element solution II 1 mL. The trace element solution I contained (per liter) 5 g EDTA and 5 g $FeSO_4$, and trace element solution II contained (per liter) EDTA 15 g, $ZnSO_4 \cdot 7H_2O$ 0.43 g, $CoCl_2 \cdot 6H_2O$ 0.24 g, $MnCl_2 \cdot 4H_2O$ 0.99 g, $CuSO_4 \cdot 5H_2O$ 0.25 g, $NaMoO_4 \cdot 2H_2O$ 0.22 g, $NiCl_2 \cdot 6H_2O$ 0.21 g, $NaSeO_4 \cdot 10H_2O$ 0.21 g, H_3BO_4 0.014 g. The serum bottles were then removed from the anaerobic chamber and purged with mixed gas (95% N_2, 5% CO_2) to further remove any O_2 in the bottles. Ammonium (initial concentration 50 mg NH_4^+-N/L) and nitrite (initial concentration 66 mg NO_2^--N/L) were added into the bottles during purging. The bottles were incubated in a shaker under 35°C. After several hours' stabilization, the ammonia and nitrite consumption rates were measured. To avoid substrate starvation, ammonium and nitrite were replenished two or three times a day by adding 50 mg NH_4^+-N/L and 66 mg NO_2^--N/L.

The initial Fe(II) (in the form of $FeSO_4$) concentration in all bottles was 0.03 mmol/L. On day 1, additional Fe(II) was injected into three sets of bottles to achieve Fe(II) concentrations of 0.09 (AMX2), 0.12 (AMX3), and 0.75 mmol/L (AMX4); no additional Fe(II) was added to a fourth set which served as the control (AMX1). Each experimental condition was set up in triplicate.

Ammonium and nitrite consumption rates of all of the cultures were measured on day 0, and the rates in AMX1, 2, and 3 sets were tracked on day 2, day 6, and day 8. The measured activities were called total anammox activity. The total anammox activities in these cultures on day 8 were also normalized by the quantitative PCR (qPCR) result with anammox bacterial abundance, which was considered as relative anammox activity. The activity of AMX4 set was measured on day 1 and day 2; then additional 0.25 mmol/L Fe(II) was injected into AMX 4 on day 3, and activity was tracked afterwards. The ammonium and nitrite concentrations were measured by the colorimetric method (APHA, 1998).

2.2 DNA and RNA samples preparation

On day 1 (AMX4) and 8 (AMX1, 2 and 3), 20 mL samples were collected from bottles and stored at −80°C for future RNA and DNA extraction. The day 1 sample was used to check the immediate response of anammox bacteria to Fe(II) inhibition. DNA was extracted from 5 mL sample with the MoBio PowerSoil DNA isolation kit, and RNA was extracted from 15 mL sample with MoBio PowerSoil total RNA isolation kit according to the manufacturer's instructions. Then the extracted DNA and RNA were checked about concentration and quality with a NanoDrop One spectrophotometer (Thermo Fisher, USA).

RNA was digested with DNase (New England Biolabs Inc, USA) to remove any DNA contamination prior to use in further assays. The digestion mixture containing 39 μL total RNA, 5 μL 10 μ DNase I reaction buffer, 1 μL DNase I, and 5 μL H_2O was incubated at 37°C for 1 h, 0.5 μL EDTA (0.5 mol/L) was added and then the mixture was heat inactivated at 75°C for 10 min. The RNA was then checked by Agilent 2100 Bioanalyzer (Agilent, USA) to determine the quality and concentration before use in cDNA synthesis.

2.3 cDNA synthesis and purification

To maintain consistency, 1 μg total RNA was used as the template for all samples. First strand cDNA synthesis was carried out in a reaction buffer containing 2 μL random primer, 4 μL 5 × ProtoScript II buffer, 2 μL DTT (0.1 mol/L), 1 μL ProtoScript II reverse transcriptase, 1 μL dNTP (10 mmol/L), and 0.2 μL RNase inhibitor (New England Biolabs Inc, USA). The volumes of RNA template and nuclease-free H_2O were adjusted according to the added RNA concentrations. Reaction conditions were 25°C for 5 min, 42°C for 1 h, and 65°C for 20 min.

Second strand cDNA synthesis was carried out using 20 μL of first stand cDNA template, 4 μL second strand synthesis enzyme mix, 8 μL 10 × second strand synthesis reaction buffer, and 48 μL nuclease-free H_2O (New England Biolabs Inc, USA). The reaction mixture was then incubated in a thermal cycler for 2.5 h at 16°C. The synthetic double strand cDNAs were then purified using a PCR column purification kit (Promega, USA) according to the instructions.

2.4 GeoChip analysis

GeoChip is a powerful tool for the analysis of functional microbial communities (Zhao et al., 2014a), which can directly link microbial genes with the process and function of ecosystem (He et al., 2007), and is very useful in studying the structural composition, functional diversity and metabolic potential of microbial communities (Ren et al., 2016). The GeoChip 5.0 is a newly developed version containing 167044 distinct probes which covers about 1500 functional gene families.

The synthetic double strand cDNAs were labeled and purified as described previously (Zhao et al., 2014a). Subsequently, all labeled cDNAs were re-suspended in hybridization solution and was hybridized with GeoChip 5.0. Microarray was scanned by a NimbleGen MS200 scanner (Roche, Madison, WI, USA) and signal intensities were quantified. Spots with signal-to-noise ratio (SNR) < 2 or signal intensity < 200 were removed as poor spots. The processed data was then used for further analysis. The final gene intensities were normalized based on the anammox bacterial abundance. The GeoChip data obtained from these samples has been submitted to NCBI Gene Expression Omnibus with accession number GSE120704.

2.5 Quantitative PCR

DNA samples were used to conduct qPCR with a SYBR green qPCR kit (Invitrogen, USA). The primers used for anammox bacteria were: A438F (5′-GTCRGGAGTTAD-GAAATG-3′) and A684r (5′-ACCAGAAGTTC-CACTCTC-3′) (Han et al., 2013). The qPCR protocol had an initial dissociation step at 95°C, followed by 45 amplification cycles with an annealing temperature of 55°C. Finally, a melting curve was run from 95°C to 65°C.

3 Results

3.1 Response of anammox activity to Fe(II) addition

It was noted that the anammox bacteria concentrations differed among batch reactors due to the various amounts and sizes of anammox bacterial aggregates. The original ammonium oxidation rates in the four experimental sets ranged from 5.933 to 9.386 mg/L/h, and nitrite reduction rates ranged from 7.693 to 12.072 mg/L/h (Table 1). After additional Fe(II) was added into the AMX2 (0.09 mmol/L) and AMX3 sets (0.12 mmol/L), the ammonium oxidation and nitrite reduction rates varied slightly in the first 6 days, and then increased significantly on the 8th day (Fig. 1). The final ammonium oxidation rates in AMX1, 2, and 3 ranged from 12.265 to 14.496 mg/L/h, and nitrite reduction rates ranged from 15.655 to 18.608 mg/L/h (Table 1). When compared with the initial rates (day 0), the ammonium oxidation rates at the end of the experiment (day 8) increased by 39.8%, 43.5%, and 144.3% in AMX1, 2, and 3, respectively, and the nitrite reduction rates increased by 39.2%, 46.4%, and 141.9%, respectively. These results illustrated that the increase in Fe(II) enhanced the total anammox activities in the cultures, with 0.12 mmol/L Fe(II) having the greatest enhancement effect. However, when the activities were normalized based on anammox bacterial abundance in each sample, the relative ammonium oxidation rates were 13.117, 15.041, and 13.692 mg/L/h in AMX1, 2, and 3, respectively, and the relative nitrite reduction rates were 15.655, 19.827, and 17.575 mg/L/h, respectively (Table 2), which showed that anammox bacteria under 0.09 mmol/L Fe(II) condition had the highest relative activity.

Ammonium oxidation and nitrite reduction rates were immediately inhibited by 39.5% and 34.2%, respectively, by 0.75 mmol/L Fe(II) (Table 1), but recovered on the second day (Fig. 2). When the Fe(II) concentration was further increased on the 3rd day, the anammox activity was inhibited again. Interestingly, the activity was still able to recover after 24 h. These results showed that though 0.75 mmol/L Fe(II) had an immediate inhibitory effect on the anammox activity, the inhibition could be recoverable.

3.2 Abundance of anammox bacteria

qPCR results showed that the anammox bacterial abundance increased in AMX1, 2, and 3 compared with the inoculum, but decreased significantly in AMX4 (Fig. 3), indicating that the high concentration of Fe(II) (0.75 mmol/L) inhibited the growth of anammox bacteria as well as activity. The increase of anammox bacterial abundance in AMX2 and AMX3 compared with AMX1 showed that 0.09 and 0.12 mmol/L Fe(II) concentrations were more beneficial than 0.03 mmol/L for the anammox bacterial gorwth. Additionally, anammox bacteria were most abundant in AMX3, showing that 0.12 mmol/L Fe(II) was better for anammox bacterial growth than 0.09 mmol/L Fe(II).

3.3 Changes of the detected gene phyla and categories

A total of 34241, 40938, 39749, and 44508 genes were detected by RNA-based GeoChip in AMX1, 2, 3, and 4 sets, demonstrating the induction of more genes being expressed under higher Fe(II) concentrations. These expressed genes could be grouped into 51, 52, 53, and 52 phyla, respectively, although only one or two genes were detected in several phyla. The most abundant phyla in the four cultures were *Proteobacteria*, *Firmicutes*, and

Table 1 The changes of anammox activity in the four sets, comparing individual finished rates with the start rates. Unit for the rate is mg/L/h

Set	Nitrogen in different set	Time 1	Time 2	Rate change (%)
AMX1	NH_4^+	9.386±0.255	13.117±1.994	**39.80**
	NO_2^-	11.243±0.677	15.655±2.317	**39.20**
AMX2	NH_4^+	8.549±0.109	12.265±1.734	**43.50**
	NO_2^-	11.043±0.808	16.168±1.572	**46.40**
AMX3	NH_4^+	5.933±0.571	14.496±0.272	**144.30**
	NO_2^-	7.693±0.838	18.608±2.087	**141.90**
AMX4	NH_4^+	9.311±0.475	5.634±0.804	–39.50
	NO_2^-	12.072±1.110	7.945±1.149	–34.20

Notes: time 1 and time 2 represent day 0 and day 8, respectively, for 0.03, 0.09, and 0.12 mmol/L sets, while represent day 0 and day 1 for 0.75 mmol/L set.

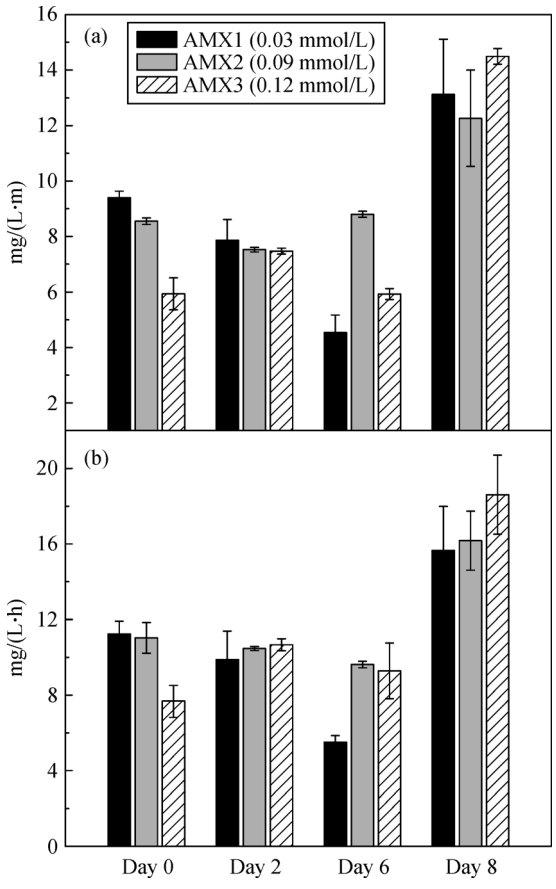

Fig. 1 Anammox activities in AMX1, 2, and 3: (a) ammonium oxidation rates; (b) nitrite reduction rates.

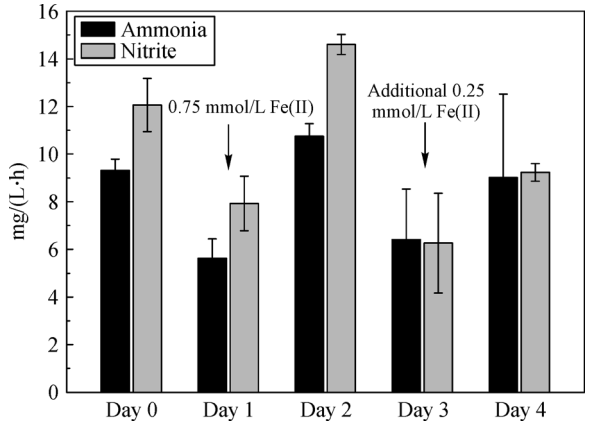

Fig. 2 The inhibition effect of high Fe(II) concentration to anammox activity.

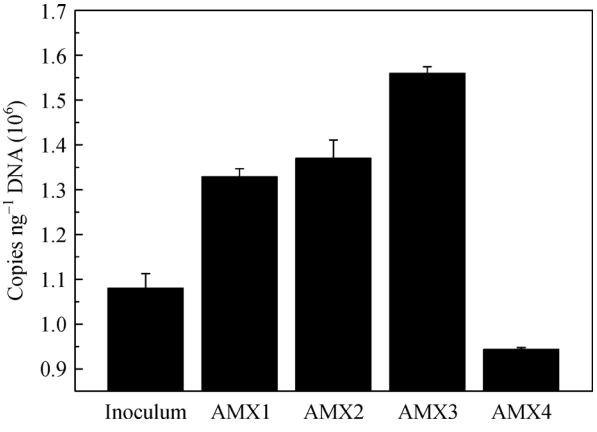

Fig. 3 qPCR results of anammox bacterial abundance in inoculum and the four experimental sets.

Actinobacteria (Fig. S1). The phyla composition was very similar in all cultures (Fig. S1), which confirms that the addition of Fe(II) didn't cause significant effects on the microbial community structure during the experimental period. The detected genes were also grouped into 12 major functional categories, the proportions of which were also very similar in the four communities (Fig. 4), indicating that the Fe(II) had also only minor effects on the metabolic pathways in the communities. However, the obviously more genes being expressed illustrated that the higher Fe(II) addition induced more active microbial processes proceeding in these systems.

Table 2 Relative anammox activity which was normalized by anammox bacterial abundance in AMX1, 2, and 3 at the finish time (day 8)

Set	NH_4^+	NO_2^-
AMX1	13.117	15.655
AMX2	15.041	19.827
AMX3	13.692	17.575

Notes: The abundance of anammox bacteria in AMX1 was set as a basis (set as 1), then the times of this abundance in AMX2 and AMX3 to AMX1 were calculated, and the ammonium oxidation and nitrite reduction rates in AMX2 and AMX3 was normalized by these obtained times. Thus, the unit for the rates is still mg/L/h, while the rates have been normalized by the anammox bacterial abundance.

3.4 Expressed genes in anammox bacteria

There were 70, 76, 75, and 88 genes from anammox bacteria detected in AMX1, 2, 3, and 4 sets, respectively. Nearly all of these detected genes belonged to *Candidatus* Kuenenia stuttgartiensis (data not shown), although there were also 50 probes provided in the GeoChip probe pool for strain KSU-1 which belongs to *Candidatus* Jettenia caeni, and more than ten probes for other anammox genus. It is thus deduced preliminarily that *Candidatus* Kuenenia stuttgartiensis might contribute significantly to the overall anammox activities in these cultures.

These detected genes in anammox bacteria could be grouped into 10 categories, among which the relatively abundant categories were stress, metal homeostasis, nitrogen, carbon cycling, and CRISPR (Table 3, Fig. 5);

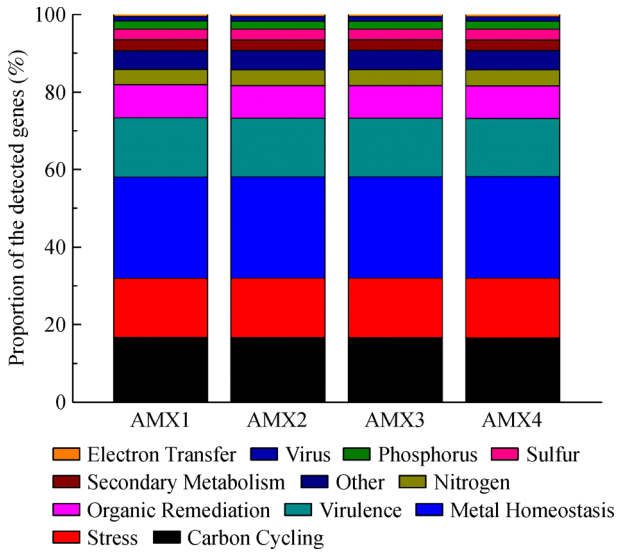

Fig. 4 Proportion changes of total genes categories in the four experimental sets.

the other 5 categories only contained 1, 2, or 3 genes each, thus were not introduced here. CRISPR (clustered regularly interspaced short palindromic repeats) is reported important for bacteria to defense the foreign genetic elements and relate with the self-immunization of bacteria (Barrangou et al., 2007; Barrangou, 2015). The detected genes related to carbon cycling were the same abundant in the four communities, while gene numbers of the stress, metal homeostasis, nitrogen, and CRISPR categories increased in the higher concentration of Fe(II) conditions (Table 3, Fig. 5). These results indicated that the higher concentration of Fe(II) had a stimulating effect on metabolic processes related to stress response, metal homeostasis, nitrogen cycling, and defense activity in anammox bacteria, but had negligible influence on the other processes, such as carbon cycling.

As shown in the Venn diagram, the four communities shared 66 genes, and the three communities with higher Fe (II) concentration (AMX2, 3, and 4) had 7 additional shared genes that were not detected in AMX1 (Fig. S2). In addition, AMX4 had 9 unique genes that were not detected in other communities. These results further suggested that higher Fe(II) concentration induced certain gene families expressing in anammox bacteria.

3.5 Functional genes expression in anammox bacteria

The intensities of some important genes in anammox bacteria were normalized by the anammox bacterial abundance and summarized in the heatmap, representing a relative gene expression level (Fig. 6). The intensities of *hao*, *narG*, and *nirS* genes didn't show the expected increase in AMX2 and AMX3 (Fig. 6). The hydroxylamine-oxidoreductase-like protein (HAO) is used by anammox bacteria to oxidize hydrazine to dinitrogen gas, *narG* gene is involved in the process of nitrite oxidation to nitrate (Strous et al., 2006), and *nirS* gene is involved in nitrite reduction to nitric oxide process. The variation trends of above genes intensities in AMX2 and AMX3 demonstrated that elevated Fe(II) didn't improve higher relative expression level of nitrogen transformation genes in anammox bacteria. The other three important genes in anammox process, namely, *hzsA*, *hdh* and *hh*, which are the functional genes of hydrazine synthase, hydrazine dehydrogenase, and hydrazine hydrolase, respectively, were not detected in this study, because there was no specific *hzsA* gene probe for *Candidatus* Kuenenia stuttgartiensis and no *hdh* and *hh* genes probes for all anammox bacteria on the GeoChip.

It has been reported that anammox bacteria are able to fix carbon via the reductive acetyl-CoA pathway while oxidizing ammonium anaerobically (Schouten et al., 2004; Strous and Jetten, 2004). However, the intensities of *codh*

Table 3 Numbers detected and proportions about the functional genes of anammox bacteria in the four experimental sets

Functional gene group	AMX1[a)]	AMX2[a)]	AMX3[a)]	AMX4[a)]	AMX1[b)]	AMX2[b)]	AMX3[b)]	AMX4[b)]
Stress	20	22	22	22	28.57	28.95	29.33	25.00
Metal homeostasis	16	18	16	25	22.86	23.68	21.33	28.41
Nitrogen	11	13	12	14	15.71	17.11	16.00	15.91
Carbon cycling	7	7	7	7	10.00	9.21	9.33	7.95
CRISPR	5	6	8	9	7.14	7.89	10.67	10.23
Phylogenetic	3	3	3	3	4.29	3.95	4.00	3.41
Secondary metabolism	2	2	2	2	2.86	2.63	2.67	2.27
Sulfur	2	2	2	2	2.86	2.63	2.67	2.27
Virulence	2	2	2	2	2.86	2.63	2.67	2.27
Organic remediation	2	1	1	2	2.86	1.32	1.33	2.27

Notes: a) detected gene numbers in the four experimental sets; b) proportions (%) of the detected genes of each group in total detected genes.

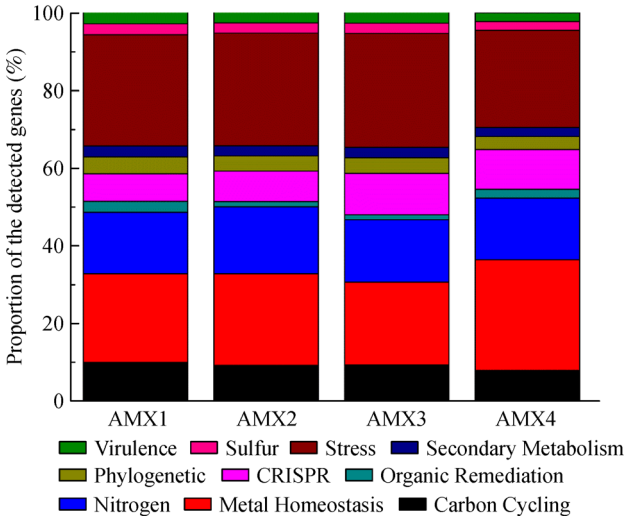

Fig. 5 Proportion changes about the functional genes in anammox bacteria in the four experimental sets.

and *fthfs* were also not improved in AMX2 and AMX3 (Fig. 6). The *codh* is functional gene of CO dehydrogenase/acetyl-CoA synthase (Strous et al., 2006), and *fthfs* is functional gene of formyltetrahydrofolate synthetase which functions in the reductive acetyl-CoA pathway (Liu et al., 2015). But the sudden rebound of the intensity of *hao*, *narG*, *fthfs* genes in AMX4 (compared with AMX3) may indicate a self-protection response of anammox bacteria to detoxify the inhibition by inducing more protein expression.

The functional gene (*bfr*) of bacterioferritin-like protein which is a heme-containing protein for iron storage (Strous et al., 2006; van Niftrik and Jetten, 2012), was not detected in AMX1, but was detected in AMX2, 3, and 4 (Fig. 6), demonstrating an induction effect of high Fe(II) concentration on its expression. Bacterioferritin is a heme-containing iron storage protein, which can dissolve and store Fe(III) in an inorganic mineral form (Andrews, 1998). The increased expression of bacterioferritin with elevated Fe(II) is consistent with previous reports which indicates a proper Fe(II) addition is beneficial for the synthesis of heme c (Qiao et al., 2013). However, the intensity of *feoB* gene that is related with iron transport was also not improved in AMX2 and AMX3 (Fig. 6). The FeoABC system is considered to be the only iron uptake system in anammox bacteria, while only gene of FeoB transporter is carried in the bacteria (Ferousi et al., 2017). However, FeoB seems to be required for normal bacterial growth and iron uptake under iron-restricted conditions, and little is known about the function of this transporter under iron sufficient conditions (Andrews et al., 2003). Thus, it is reasonable that *feoB* was more expressed under lower Fe(II) condition (0.03 mmol/L). It is likely that Fe(II) is preferred and stored in the cells at a higher concentration, and a high Fe(II) concentration hinders the Fe(II) transportation in and/or out of the cells, which may be because that the iron transporter is only needed under the low iron concentration condition to facilitate the iron utilization.

Furthermore, as shown in Fig. 6, AMX4 was clustered far from the other three samples; AMX2 and AMX3 had the nearest clustering distance in the heatmap. This showed that AMX4 had the most different gene expression in the four cultures, and gene expression in AMX2 and AMX3 were mostly similar.

4 Discussion

4.1 The relationship between anammox performance and the functional genes expression

The appropriate addition of Fe(II) accelerated anammox activity in the culture, and the enhancement effect on total anammox activity as well as the anammox bacterial abundance was most obvious with 0.12 mmol/L Fe(II) addition, while relative anammox activity was the highest under 0.09 mmol/L Fe(II) condition. Combining with the qPCR results, it indicates that the higher total activity of 0.12 mmol/L than 0.09 mmol/L Fe(II) should be caused by the enhancement of anammox bacterial abundance under 0.12 mmol/L Fe(II) condition. This means that 0.09 mmol/L was the preferred Fe(II) concentration for anammox activity in this study, while 0.12 mmol/L Fe(II) stimulated the anammox bacterial growth the best. This is slightly different with the results of Liu and Ni (2015) who tested five Fe(II) concentrations (0.03, 0.06, 0.09, 0.12, 0.18 mmol/L) and found that 0.09 mmol/L Fe(II) had the best enhancement effect on both anammox activity and growth rate (Liu and Ni, 2015). This difference could be due to species difference, as the cultures examined by Liu and Ni (Liu and Ni, 2015) were mainly *Candidatus* Jettenia caeni strain KSU-1, while in the current study, the detected strain were primarily *Candidatus* Kuenenia stuttgartiensis. This difference provides us the information that different anammox bacteria strains have different responses to the ferrous iron. Thus in the actual wastewater treatment, the bacteria strain should be taken into account for determining an appropriate Fe(II) concentration, which was not noticed and proposed in the previous studies.

More genes were indeed expressed in the higher Fe(II) conditions, while the relative gene expression level was not completely consistent with observed performance of anammox bacteria. Generally, mRNA levels are correlated with biomass activity by reflecting the abundance of related proteins. However, the inconsistency between activity and gene expression also occurred in some other cases. Wang et al. (2016) explored the effects of nitrite inhibition on functional genes expression of *Candidatus* Kuenenia stuttgartiensis, and also found the inconsistency between activity data and RT-qPCR results. They con-

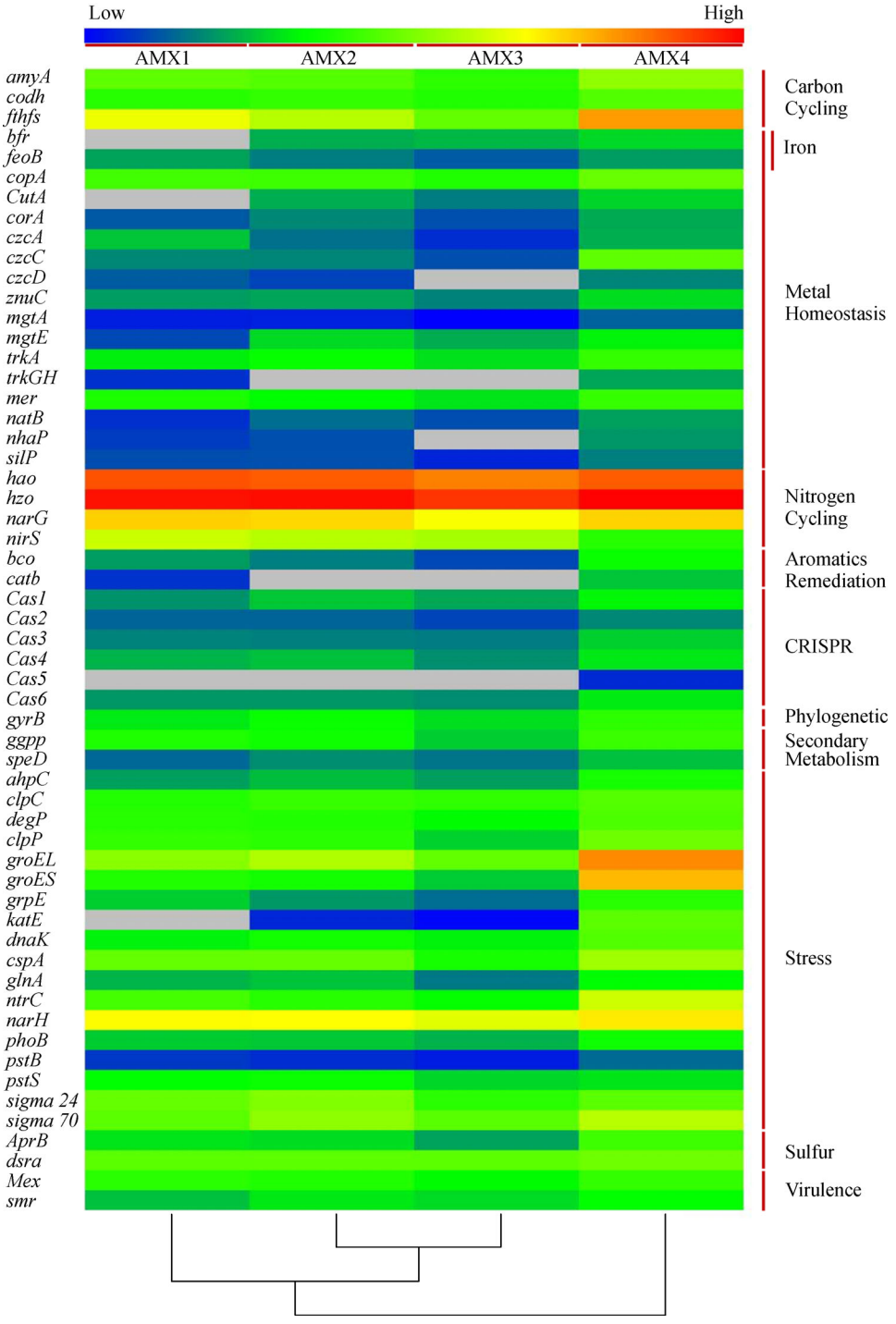

Fig. 6 Expression level of the functional genes in anammox bacteria in the four experimental sets.

cluded that the mRNA levels would not always be consistent with the biomass activity, some other factors (e.g., post-transcriptional and/or post-translational regulations) may also have influences on the biomass activity (Wang et al., 2016). Besides, Cua et al. (2011) studied the responses of several AOB strains to nitrite addition on ammonium oxidation rates and functional gene expression levels, and also encountered the inconsistency between activity results and mRNA results. Regarding the inhibition of Fe(II) on anammmox bacteria, there is no available literature providing direct discussion. Though some relevant enzymes in anammox bacteria, such as cytochrome c, need to chelate with ferrous iron for active regions formation, the activity change indicates that high

Fe(II) concentration can cause a direct and immediate inhibition to anammox activity. Meanwhile, the intensities of some functional genes in AMX4 indicated that anammox bacteria might try to increase the gene expression level under Fe(II) stress. The immediate rebound of expression intensities about nitrogen-transforming genes in AMX4 compared with AMX3 might have been stimulated as a stress response to detoxify Fe inhibition. However, as heavy metals can be accumulated in cells, even though the gene intensities increased to some extent by bacterial stress response, the protein activity was still inhibited by Fe(II), causing an obvious and immediate activity reduction. This is also similar to results from Wang et al. (2016) showing elevated gene expression in *Candidatus* Kuenenia stuttgartiensis under nitrite inhibited condition. They explained that the *Candidatus* Kuenenia stuttgartiensis might attempt to increase the mRNAs levels of energy-harvesting-related genes, to alleviate the nitrite inhibition on enzyme activities, but the activity of the synthesized proteins might be inhibited by the in situ nitrite toxicity, resulting in worse performance. Therefore, the results in this study further indicate that the future transcriptional study should combine with proteomics results, to provide certain mechanism explanation.

4.2 Fate and function of ferrous iron in anammox system with mixed culture

NDFO has been observed in many environments, and is examined and found co-occurring with anammox reaction in *Candidatus* Brocadia sinica and *Candidatus* Scalindua sp. (Oshiki et al., 2013). Besides, it is also experimentally confirmed that iron can be oxidized by *Candidatus* Kuenenia stuttgartiensis with nitrate as the electron acceptor, thus the versatility of anammox bacteria is proposed (Strous et al., 2006). So the added Fe(II) could be oxidized to Fe(III) in the mixed culture by anammox bacteria, denitrifiers, and iron-oxidizing bacteria, etc. Additionally, Fenammox is also thought contributing to nitrogen removal in anaerobic environment (Li et al., 2015), through which the Fe(III) in the condition can be reduced back to Fe(II). And anammox bacteria is also reported having the potential of using organic matters as electron donor to reduce Fe(III) to Fe(II) (Zhao et al., 2014b). As such, in the higher Fe(II) conditions, the fate of the added Fe(II) could be shown in two ways: being oxidized partly to Fe(III) through processes such as NDFO or oxidation by anammox bacteria itself, then being reduced back to Fe(II) through processes such as Feammox or reduction by anammox bacteria itself. This creates an oxidizing/reducing cycle of Fe(II)/Fe(III), making an endless existence of iron in the mixed culture.

The Fe(II)/Fe(III) cycle is also reported and considered triggering a new anammox-like process using nitrate as terminal electron acceptor to remove high concentration of ammonium (Yang et al., 2020). Thus, based on all of these studies, the appropriate addition of Fe(II) in the anammox system can actually improve nitrogen removal through the ways of enhancing the real anammox activity and anammox bacterial growth, and meanwhile providing additional nitrogen removal by various processes one of which is NDFO coupling with Feammox. Shu et al. (2016) also proposed similar thought that in an anammox system under Fe(II) stress, the coupling of anammox, nitrification, DNRA, NDFO and Feammox may contribute together to the nitrogen removal. This kind of potential complex relationships in the cultures might make the effects of ferrous iron on anammox bacteria indirect and less clear. Therefore, the more detailed effects of Fe(II) on real anammox process and bacterial physiology need to be further studied in pure cultures, to provide more precise data and conclusion.

4.3 Implication

This is the first to investigate the physiologic response of anammox bacteria (mainly *Candidatus* Kuenenia stuttgartiensis) to different Fe(II) concentrations on the transcriptional level. Combining the relative gene expression results with the published papers, we propose the thought that an enhancement/inhibition of different Fe(II) concentrations on anammox activity does not always imply corresponding increase/decrease in the mRNA abundance of certain functional genes, which improves our understanding about the physiologic characteristics and metabolic response of anammox bacteria.

The overall effects (enhancement and inhibition) of Fe(II) on the growth and activity of anammox bacteria indicate that the appropriate external addition and/or the original existence of low concentration of Fe(II) in the actual wastewater will be beneficial for an anammox system to remove nitrogen, and the bearable Fe(II) concentration in wastewater can be as high as 0.75 mmol/L. However, the most appropriate and bearable Fe(II) concentration should be considered according to both the treatment purpose and anammox bacterial strain. Anammox process has attracted increasing attentions in municipal wastewater treatment in recent years (Zhang et al., 2018), and research focus in side-stream wastewater treatment with anammox process was also moved to main-stream treatment to maximize the savings of energy and materials (operation temperature exceeds 25°C in side-stream treatment, but 10°C–25°C in main-stream treatment) (Liu and Ni, 2015). Thus how anammox bacteria response to external disturbance (ferrous iron in this study) can provide more instructions for the future use of anammox bacteria in treating various sources of nitrogen-polluted wastewater (such as containing heavy metals). Additionally, this study provides a new thinking that appropriate Fe(II) existence/addition may also improve the anammox bacterial adaptation to different external disturbance and/or different treatment strategies,

when previous studies only focused on the direct effect of single Fe(II) factor on anammox process.

5 Conclusions

The moderate increase of Fe(II) in the medium can enhance anammox activity and bacterial growth. Fe(II) at concentration of 0.09 mmol/L is more beneficial for anammox activity, but 0.12 mmol/L Fe(II) can stimulate anammox bacterial growth better. Both 0.09 and 0.12 mmol/L Fe(II) can induce more genes being expressed, while doesn't show a stimulation on the relative expression level of certain functional genes. Fe(II) at concentration of 0.75 mmol/L can cause immediate inhibition to anammox process, while the inhibition is recoverable. Besides, anammox bacteria may have a stress response under 0.75 mmol/L Fe(II) condition to detoxify the Fe inhibition.

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