

The short- and long-term inhibitory effects of Fe (II) on anaerobic ammonium oxidizing (anammox) process

Cherh Yih Mak, Jih Gaw Lin, Wen Hsing Chen, Choon Aun Ng and Mohammed J. K. Bashir

ABSTRACT

The application of the anammox process has great potential in treating nitrogen-rich wastewater. The presence of Fe (II) is expected to affect the growth and activity of anammox bacteria. Short-term (acute) and long-term effects (chronic) of Fe (II) on anammox activity were investigated. In the short-term study, results demonstrated that the optimum concentration of Fe (II) that could be added to anammox is 0.08 mM, at which specific anammox activity (SAA) improved by 60% compared to the control assay, 0.00 mM. The inhibition concentration, IC_{50} , of Fe (II) was found to be 0.192 mM. Kinetics of anammox specific growth rate were estimated based on results of the batch test and evaluated with Han-Levenspiel's substrate inhibition kinetics model. The optimum concentration and IC_{50} of Fe (II) predicted by the Han-Levenspiel model was similar to the batch test, with values of 0.07 mM and 0.20 mM, respectively. The long-term effect of Fe (II) on the performance of a sequencing batch reactor (SBR) was evaluated. Results showed that an appropriate Fe (II) addition enhanced anammox activity, achieving 85% NH_4^+ -N and 96% NO_2^- -N removal efficiency when 0.08 mM of Fe (II) was added. Quantitative polymerase chain reaction (qPCR) was adopted to detect and identify the anammox bacteria.

Key words | anammox, Fe (II), Han-Levenspiel model, IC_{50} , stimulation, wastewater treatment

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INTRODUCTION

The process of anaerobic ammonium oxidizing (anammox) has become essential at the global level as it contributes to the oceanic nitrogen loss level and has multiple advantages compared to the conventional nitrification/denitrification process in the removal of nutrients from wastewater. The anammox process, as a cost-effective and environment-friendly nitrogen removal method, has revealed potential for treating nitrogen-rich wastewater (Li *et al.* 2018). The anammox process is considered as one of the most cost-effective biological nitrogen removal processes, and the advantages of the anammox process over conventional nitrification/denitrification process are: (i) a saving of 90% on the operation cost (aeration is reduced by 64%, sludge production by 80–90% and exogenous electron donor (e.g. organic carbon) by 100%) (Jetten *et al.* 2010; Jin *et al.* 2012) and (ii) reduced CO_2 or N_2O emissions. The anammox process is a promising method for treating nitrogen rich and low chemical oxygen demand (COD) content wastewater such

as landfill leachate and metal refinery wastewater. However, these type of wastewaters contain a high concentration of heavy metals (Wang *et al.* 2010; Kieu *et al.* 2011). Accordingly, anammox-related nitrogen removal processes provide a new perspective for the future wastewater treatment. Nevertheless, several challenges including strong sensitivity to outer environmental effects, weak tolerance to substrate concentrations, long doubling time and low cell yield still hinder further application of anammox practice (Li *et al.* 2018). With regard to wastewater treatment using the anammox process, heavy metals such as iron, zinc, copper, nickel, and cobalt can either affect stimulation, inhibition or even be toxic in biochemical reactions depending on the concentrations and species (Wang *et al.* 2010; Aktan *et al.* 2018; Mak *et al.* 2018). In general, heavy metals are not biodegradable and can accumulate in organisms, causing bioaccumulative toxicity. Some nitrogen-rich wastewater, such as landfill leachate, typically has high

concentrations of heavy metal ions. Understanding the role heavy metals in inhibition of anammox activity will help remove nitrogen from these wastewaters (Xie *et al.* 2017). Fe (II) is one of the essential nutrients for growth of anammox. Since the discovery of the anammox process by Van de Graff *et al.* (1996), the Fe (II) concentration was set as 0.03 mM or 0.04 mM in most of the feeding media of enriched anammox sludge systems. Energy dispersive X-ray (EDX) analysis revealed that several electron-dense anammoxosome particles contained iron. The possible functions of these anammoxosome particles are for energy generation, and acting as an iron storage facility for the heme-c enzyme involved in the electron transport chain (van Niftrik *et al.* 2008). Therefore, it is crucial to obtain more knowledge about the effect of Fe (II) on the microbial activity and the performance of the reactor. Fe (II) can be the potential factor influencing the growth and activity of anammox bacteria. This study investigates: (i) the concentration of Fe (II) affecting the increase of anammox activity; (ii) the concentration of Fe (II) causing disruption to the anammox process (including IC50). Furthermore, the recovery of the anammox process was observed in a long-term test with a sequencing batch reactor (SBR).

In summary, the short-term study was conducted on the effect of Fe (II) on the microbial activity and the performance of the reactor, while the long-term study was conducted to investigate the recovery efficiency of the anammox process.

MATERIAL AND METHODS

Short-term effect of Fe (II) on anammox activity

Specific anammox activity (SAA) test

The batch tests were performed to study the effects of Fe (II) on anammox activity. Eleven sets (each set in triplicates) of batch tests with different concentrations of Fe (II), which are 0.02 mM, 0.04 mM, 0.06 mM, 0.08 mM, 0.09 mM, 0.10 mM, 0.12 mM, 0.14 mM, 0.16 mM, 0.18 mM and 0.20 mM, were carried out. The anammox seed sludge used to study the effects of Fe (II) on anammox activity were harvested from a full-scale landfill leachate treatment plant in Xinfeng, Taiwan. According to Daverey *et al.* (2014), the effect of heavy metal is more prominent when the biomass concentration is low (<2,000 mg-MLVSS L⁻¹) (MLVSS = mixed liquor volatile suspended solids). Thus the concentration of the biomass was kept below 2,000 mg-MLVSS L⁻¹ to evaluate the short-term effect of Fe (II) on anammox activity. The specific anammox

activity (SAA) tests were performed according to Dapena-Mora *et al.* (2007) methods, where each batch test was carried out in a 67 ml serum bottle that was a completely closed vial, with 57 ml of liquid volume and 10 ml headspace. The initial concentrations of substrate NH₄⁺-N and NO₂⁻-N were both 70 mg L⁻¹. Fe (II) was added together with the substrate in the form of FeSO₄·7H₂O. Anammox sludge was inoculated in the serum bottle, then washed and re-suspended in buffer solution (0.14 g KH₂PO₄, 0.75 g K₂HPO₄, and 0.5 g KHCO₃). Serum bottles containing washed and re-suspended anammox sludge along with substrate were then placed in an incubator with a fixed temperature of 30 °C, and stirred at 125 rpm. The initial pH was fixed at 7.8, and argon gas was purged to maintain an anaerobic condition. The production of N₂ gas was tracked by measuring the overpressure in the headspace with a time-frequency depending on the biomass activity in each batch test.

Calculation of SAA

The total amount of N₂ gas produced was calculated from the overpressure measured using a pressure meter in the headspace of each serum bottle at the end of the assay by using the ideal gas law equation. The N₂ gas production rate, dN₂/dt, was calculated from the maximum slope of the curve describing the pressure increase in the vial through time (α) (Equation (1)) (Dapena-Mora *et al.* 2007):

$$\frac{dN_2}{dt} = \frac{\alpha \times V_G}{R \times T}, \text{ mol } N_2 \text{ hr}^{-1} \quad (1)$$

where α = slope of pressure increase in the bottle through time (atm hr⁻¹)

V_G = volume of gas phase (0.01 L)

R = ideal gas constant 0.0820575 (atm L mol⁻¹ K⁻¹)

T = temperature (K)

Then, SAA was calculated by dividing the N₂ gas production rate, dN₂/dt, by the concentration of biomass in the serum bottle, X (g VSS L⁻¹) (Equation (2)) (Dapena-Mora *et al.* 2007):

$$SAA = \frac{dN_2}{dt} \times 28}{X \times V_L} \times 24, \text{ g } N_2 \text{ g VSS}^{-1} \text{ d}^{-1} \quad (2)$$

where 28 = molecular weight of N₂ (g N/mol)

24 = unit conversion factors from hour to days.

X = biomass concentration in the bottle (g VSS L⁻¹)

V_L = volume of liquid phase in the bottle

Percentage of activity increase and IC₅₀

The short-term effect of Fe (II) on the anammox activity was measured in terms of the percentage of activity increase (Equation (3)):

$$\text{SAA}(\%) = \frac{\text{SAA} - \text{SAA}_0}{\text{SAA}_0} \times 100\% \quad (3)$$

where SAA (gN₂ gVSS⁻¹d⁻¹) = specific anammox activity of the test sample with Fe (II)

SAA₀ (gN₂ gVSS⁻¹d⁻¹) = specific anammox activity of the control assay (in the absence of Fe (II))

The IC₅₀ is the concentration of the tested compound that corresponds to 50% activity compared with the control assay.

Kinetic analysis of anammox specific growth rates

Based on the results of the batch tests, the specific growth rate of anammox bacteria at different concentrations of Fe (II) was estimated to study the effect of Fe (II) on anammox activity. The ammonium consumption rate for every gram of biomass was calculated based on the SAA values from batch tests following the ratio from the stoichiometric equation of the anammox process (Equation (1)). The specific growth rate of anammox was then estimated by multiplying by the yield of anammox, 0.14 g VSSa/g NH₄⁺-N (Strous *et al.* 1998). Cell loss by decay is negligible in the estimation since the chance of biomass loss attributed to endogenous decay over the 5-hour batch test is minuscule.

Substrate inhibition kinetics of Han-Levenspiel model

The inhibition factor in the real world could be caused by high concentrations of cell, product, substrate or other inhibitory substances. Fe (II) is one of the essential substrates for anammox growth. However, inhibition can happen if it is overdosed. Hence, the inappropriate addition of Fe (II) can be considered as substrate inhibition. In this study, the substrate inhibition kinetics equation of the Han-Levenspiel model (Equation (4)) is used to examine the specific growth rate kinetics of anammox bacteria. For substrate inhibition:

$$\frac{r_c}{C_c} = k \left(1 - \frac{C_A}{C_{A^*}} \right)^n \frac{C_A}{C_A + C_M(1 - C_A/C_{A^*})^m}, d^{-1} \quad (4)$$

where r_c = growth rate of cells, g VSS d⁻¹

C_c = cell concentration, g VSS

k = reaction rate constant, d⁻¹

C_A = substrate concentration, mM

C_{A^*} = critical inhibitor concentration above which reaction stops, mM

n, m = empirical constants

C_M = Monod constant, mM

R^2 , or the coefficient of determination, is used to calculate the quality of fitting of a model in linear regression models. However, this method could not provide comparable analysis for nonlinear regression because it is common to have a variable number of parameters from one model to another in nonlinear regression. Therefore, the quality of nonlinear regression models is assessed by adopting an adjusted R^2 .

Long-term effect of Fe (II) on anammox process

Reactor start-up

An SBR having a volume of 1 L, as shown in Figure 1, was started up with 3,000 mg/L of seed sludge obtained from the Xinfeng landfill leachate plant, Taiwan. The SBR was placed in a dark incubator at a constant temperature of 35 °C with HRT of 5 days. Complete mixing of the SBR was achieved with a magnetic stirrer at 70 rpm. The pH of the SBR was continuously monitored, and the pH of the feeding medium was controlled at between 7.8 and 8.0 by manually adding 1 M H₂SO₄. The anaerobic condition was maintained by flushing with argon gas and the dissolved oxygen (DO) concentration was maintained in the range of 0 and 0.5 mg O₂ L⁻¹. Influent to the SBR was synthetic wastewater with composition as presented in Table 1. KHCO₃ was added to act as an inorganic carbon source and a buffering agent. All of the compounds in the synthetic wastewater remained the same except for Fe (II) concentrations. The Fe (II) levels during a different experimental phase are shown in Table 2.

The SBR was operated in a 24 hour cycle consisting of 22 h 30 min of reaction (including 30 min of feeding), 1 h for settling, followed by 30 min of decanting and sampling.

RESULTS AND DISCUSSION

Short-term effect of Fe (II) on anammox activity

It is crucial to understand the effect of different Fe (II) concentrations on anammox activity before applying anammox

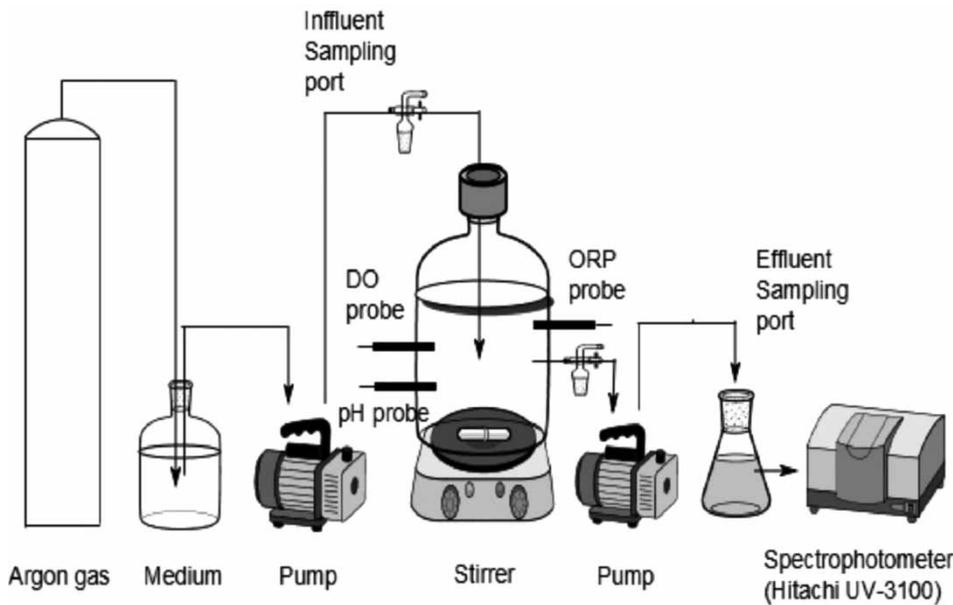


Figure 1 | Schematic diagram of reactor.

Table 1 | Synthetic wastewater composition (Dapena-Mora et al. 2004; Daverey et al. 2014)

Compound	Concentration (g L ⁻¹)
(NH ₄) ₂ SO ₄	0.236
NaNO ₂	0.246
KHCO ₃	1.25
NaH ₂ PO ₄	0.025
CaCl ₂ ·2H ₂ O	0.3
MgSO ₄ ·7H ₂ O	0.2
FeSO ₄ ·7H ₂ O	0.0115 (0.04 mM of Fe (II))
EDTA	0.00625
Trace elements solution ^a	1.25 ml L ⁻¹

^aDescribed by Van de Graaf et al. (1996).

Table 2 | Fe (II) concentrations during different experimental phase

Phase	Time (d)	Concentration of Fe (II) in Influent (mM)
I	190–213 (24)	0.04 (control)
II	214–251 (38)	0.08
III	252–311 (60)	0.20
IV	312–379 (68)	0.04 (recover)

process in treating nitrogen-rich wastewater containing Fe (II). Therefore, the SAA test was carried out on anammox bacteria under a stressed condition with different concentrations of Fe (II). Figure 2 shows SAA and

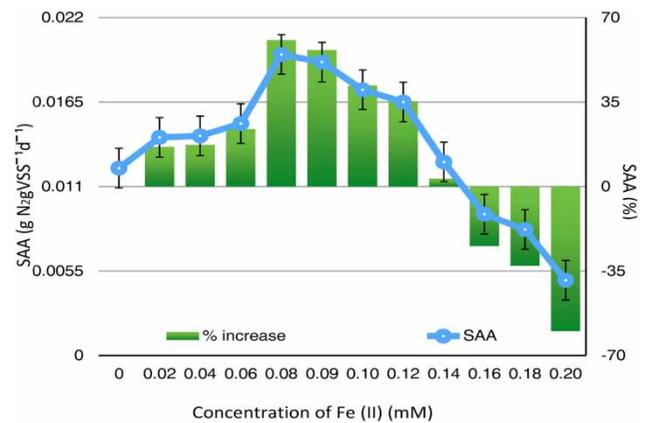


Figure 2 | SAA and SAA (% of increase) under various concentrations of Fe (II).

percentage of SAA increase under the various concentrations of Fe (II). It is evident from the data shown in Figure 2 that Fe (II) had both stimulating and inhibitory effects on anammox activity. Average SAA is relatively higher with 0.08 mM of Fe (II), which increased the SAA percentage by 60% (compared with the control assay, 0.00 mM). Further increase of Fe (II) concentration to 0.16 mM starts to cause an inhibitory effect. The inhibitory effect of Fe (II) on anammox activity increased as the concentration of Fe (II) increased. Linear regression analysis was carried out to calculate the IC₅₀ of Fe (II), and the result is shown in Figure 3. The IC₅₀ of Fe (II) was found to be 0.192 mM. The high value of R², which is 0.9833, indicated that the result fits well with the regression line.

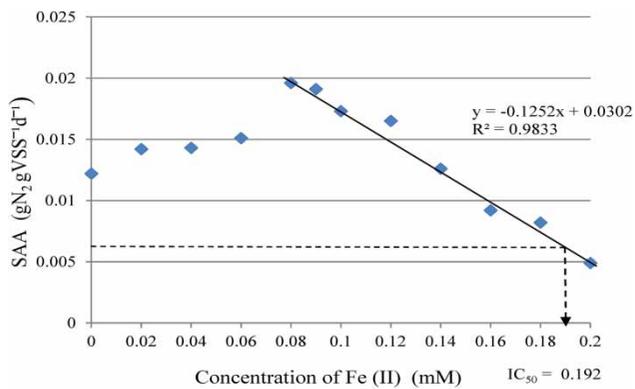


Figure 3 | IC_{50} of Fe (II) on anammox.

Numerous researchers, Liu & Ni (2015), Qiao *et al.* (2013) and Bi *et al.* (2014), have observed a similar stimulation effect of Fe (II) on anammox activity. However, the inhibitory effect on anammox activity was lower, as 0.18 mM of Fe (II) did not show an inhibitory effect in Liu & Ni (2015) and Qiao *et al.* (2013), while in this study, 0.16 mM of Fe (II) started to cause an inhibitory effect. The inhibitory effect of heavy metals depends on the biomass concentration and state of microbial growth (Wang *et al.* 2010). Therefore, the inhibitory effect of Fe (II) at 0.16 mM (compared to the control assay) in this study was evident as this study used lower biomass concentration (1,125 mg MLVSS/L) to study the inhibitory effect of Fe (II) on anammox activity compared to Qiao *et al.* 2013 and Liu & Ni 2015 with biomass of (1,850 mg MLVSS/L).

Hans-Levenspiel's substrate inhibition kinetics model was evaluated to examine the specific growth rate kinetics of the anammox bacteria under various Fe (II) concentrations. The calculated constant value of the Han-Levenspiel model, C_M (Monod constant, mM), C_A^* (the critical concentration above which a reaction cannot proceed, mM), k (reaction rate constant, d^{-1}) and two dimensionless empirical constants, n and m , were 0.067, 0.417143, 0.002959, 2.982315 and 5.9704 respectively. The relationship of anammox specific growth rate and Fe (II) concentration in this study is well described using Han-Levenspiel's substrate inhibition kinetics model as shown in Figure 4 with SSE and an R^2 value of 1.93E-07 and 0.82 (Table 3). The IC_{50} of Fe (II) on anammox is predicted to be 0.20 mM by the Han-Levenspiel model, which is similar to the measured value, 0.192 mM.

Based on the results obtained from the current study and a literature survey, a comparison was carried out to exhibit the IC_{50} inhibition concentration via various heavy metals on anammox bacteria based on SAA, as shown in Table 4.

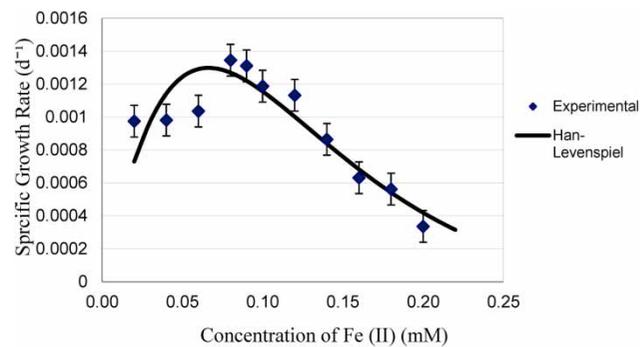


Figure 4 | Fitting experimental data using the substrate inhibition kinetics of the Han-Levenspiel model.

Table 3 | Parameter values of the Han-Levenspiel model

Equation	$\frac{r_c}{C_c} = k \left(1 - \frac{C_A}{C_A^*}\right)^n \frac{C_A}{C_A + C_M(1 - C_A/C_A^*)^m}, d^{-1}$
R^2	0.82
k	0.002959
C_A^*	0.417143
n	2.982315
m	5.9704
C_M	0.067
IC_{50} (mM)	0.20

Table 4 | Half inhibition concentration of heavy metals on anammox bacteria

Heavy metal ions	Half inhibition concentration, IC_{50} (mg/L)	References
Cadmium, Cd^{2+}	11.2	Bi <i>et al.</i> (2014), Li <i>et al.</i> (2014)
Copper, Cu^{2+}	1.9–30	Lotti <i>et al.</i> (2012), Yang & Jin (2012), Li <i>et al.</i> (2014), Zheng <i>et al.</i> (2016)
Lead, Pb^{2+}	NT	Li <i>et al.</i> (2014)
Mercury, Hg^{2+}	60.35	Bi <i>et al.</i> (2014)
Molybdate, MoO_4^{2+}	NT	Li <i>et al.</i> (2014)
Nickel, Ni^{2+}	48.6	Li <i>et al.</i> (2014)
Silver, Ag^+	11.52	Bi <i>et al.</i> (2014)
Zinc, Zn^{2+}	3.9–25	Lotti <i>et al.</i> (2012), Zheng <i>et al.</i> (2016), Daverey <i>et al.</i> (2014), Li <i>et al.</i> (2014)
Fe (II), Fe^{2+}	55.6 (0.20 mM)	This study

NT = not toxic at the highest concentration tested (75 mg Pb/L and 23.8 mg Mo/L).

Accordingly, the inhibitory effects of various heavy metals investigated by the literature including cadmium (Cd^{2+}), copper (Cu^{2+}), lead (Pb^{2+}), mercury (Hg^{2+}), molybdate (MoO_4^{2+}), nickel (Ni^{2+}), silver (Ag^+) and zinc (Zn^{2+}) were compared with the inhibitory effect of ferrous ions (Fe^{2+}) achieved by the current study. Among the nine heavy metals, the lead ion, Pb^{2+} , had the lowest inhibitory effect on the anammox process, in which even 75 mg/L of lead ions did not cause any inhibitory effects on anammox bacteria, while the copper ion, Cu^{2+} might have the greatest inhibitory effect on anammox with the IC_{50} inhibition concentration range starting from as low as 1.9 mg/L. In this study, the IC_{50} inhibition concentration of Fe (II) was found to be approximately 0.20 mM, which, after conversion, is equal to 55.6 mg/L. Thus, by comparison, the inhibitory effects of Fe (II) on the anammox process are not as intense as most of the heavy metals tested and the inhibitory effects of Fe (II) are lower than cadmium (Cd^{2+}), copper (Cu^{2+}), nickel (Ni^{2+}), silver (Ag^+) and zinc (Zn^{2+}).

Long-term effect of Fe (II) on the reactor performance

The SBR system

The long-term effect of Fe (II) on anammox was investigated in a 1 L SBR. Before investigating the long-term effect of Fe (II), 189 days of anammox cultivation were carried out in the SBR. During 189 days of cultivation, the Fe (II) concentration added in the influent was 0.04 mM (regular Fe (II) concentration) (Dapena-Mora *et al.* 2004; Daverey *et al.* 2014). The long-term effect of Fe (II) on the nitrogen removal efficiency of the SBR was investigated from day 190 until day 379 in four phases (I-IV) with various Fe (II) concentrations in the feeding medium.

Nitrogen removal performance

The nitrogen removal performance of the SBR is as shown in Figure 5 and the nitrogen removal efficiency is as illustrated in Figure 6. During phase I, 0.04 mM of Fe (II) (regular Fe (II) concentration) was added in the influent. The average $\text{NH}_4^+\text{-N}$ and $\text{NO}_2^-\text{-N}$ concentrations in the effluent were kept below 10 mg/L and 7 mg/L respectively. Also, the average $\text{NH}_4^+\text{-N}$ and $\text{NO}_2^-\text{-N}$ removal efficiencies are approximately $82\% \pm 3$ and $87\% \pm 4$ respectively. The average $\text{NO}_3^-\text{-N}$ produced in this stage was about 3.6 mg/L.

During phase II, the concentration of Fe (II) in the influent was set at 0.08 mM, which is the optimum concentration of Fe (II) on anammox activity observed from the short-term

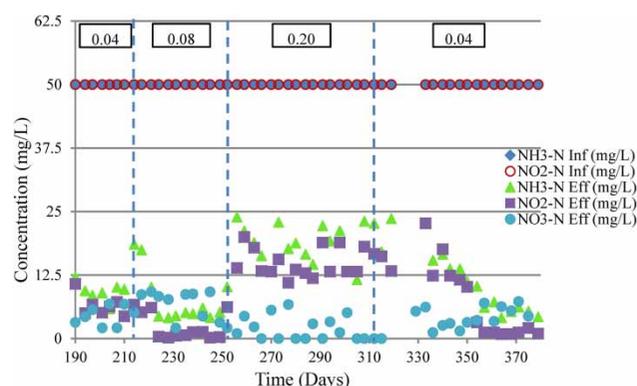


Figure 5 | Variance inorganic nitrogen compounds in the reactor.

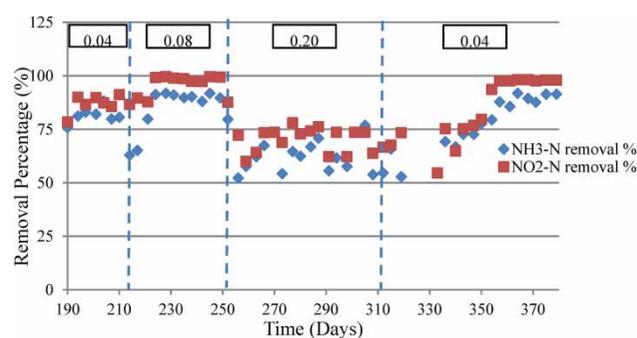


Figure 6 | Nitrogen removal percentage in different phases.

test. The average $\text{NH}_4^+\text{-N}$ and $\text{NO}_2^-\text{-N}$ removal efficiencies had improved compared to phase I, to approximately $85\% \pm 3$ and $96\% \pm 2$ respectively, and remained superior during the whole of phase II. The average $\text{NH}_4^+\text{-N}$ and $\text{NO}_2^-\text{-N}$ concentrations in the effluent were almost as low as 8 mg/L and 3 mg/L, respectively. The results suggested that appropriate Fe (II) addition is beneficial to anammox activity by increasing nitrogen removal efficiency. This could be explained by Fe (II) helping in the formation of an active region of the heme-c enzyme, which catalyzes the anammox process. Furthermore, the average $\text{NO}_3^-\text{-N}$ produced in this phase was also higher than in phase I, with a concentration of approximately 7.2 mg/L.

During phase III, the reactor performance under IC_{50} of Fe (II) on anammox activity, which is about 0.20 mM as observed in the batch test, was investigated. The average $\text{NH}_4^+\text{-N}$ and $\text{NO}_2^-\text{-N}$ removal efficiencies in phase III reduced to approximately $64\% \pm 6$ and $71\% \pm 5$, respectively, with effluent $\text{NH}_4^+\text{-N}$ and $\text{NO}_2^-\text{-N}$ concentrations increasing to approximately 18 mg/L and 15 mg/L, respectively. Despite the fact that 0.20 mM of Fe (II) caused 50% inhibition of anammox activity during the short-term test, the inhibitory effect under 0.20 mM of Fe (II) was

suppressed during the continuous test, suggested that the SBR could resist IC_{50} of Fe (II) after the acclimatization of the sludge. Also, the average NO_3^- -N produced in this phase decreased to approximately 2.2 mg/L.

During Phase IV (the recovery phase), the concentration of Fe (II) added in the influent was reduced to 0.04 mM. The SBR performance remained suppressed for 39 days before recovery (from day 312–349) with $64\% \pm 4$ and $69\% \pm 3$ average NH_4^+ -N and NO_2^- -N removal efficiencies. The average NH_4^+ -N, NO_2^- -N and NO_3^- -N concentrations in the effluent were around 19 mg/L, 16 mg/L and 2.3 mg/L, respectively. From day 350–379, the reactor performance started to recover by achieving average NH_4^+ -N and NO_2^- -N removal efficiencies of approximately $87\% \pm 4$ and $95\% \pm 3$, respectively, while the average NH_4^+ -N and NO_2^- -N concentrations in the effluent were kept below 7 mg/L and 3 mg/L, respectively. These results indicated that the SBR performance had recovered to the previous level when 0.08 mM of Fe (II) was added (average NH_4^+ -N and NO_2^- -N removal efficiencies of approximately $85\% \pm 3$ and $96\% \pm 2$, respectively), implying that the bacterial communities were well acclimated to the Fe (II) in the SBR. After the reactor performance had recovered, the anammox activity was still observed for more than three cycles of HRT (5 days) to assure the stability of the reactor. Besides that, the average NO_3^- -N produced before recovery was almost same with phase III, with a concentration of approximately 2.3 mg/L. Fortunately, after the reactor performance started to recover (from day 350–379), the average NO_3^- -N produced increased to around 4.7 mg/L. The temperature of the reactor was controlled at $35 \pm 1^\circ C$ during the whole of the long-term test to ensure the best temperature environment for anammox bacteria. DO was kept as low as 0–0.02 mg/L throughout the experiment, and most of the time the DO in the reactor approached 0 mg/L.

Anammox identification

On day 304 of the continuous experiment, a sludge sample was taken out for anammox identification. The reactor was mixed well before the sample was collected. After collection, the sample was stored with an equal amount of 98% ethanol and stored at $4^\circ C$ before transportation. During transport, the sample was carefully kept in an insulator bag to ensure the temperature was as undisturbed as possible. Of the various way of detecting and identifying anammox bacteria, quantitative polymerase chain reaction (qPCR) was adopted in this study to determine the anammox bacteria. PCR is a type of molecular technique that identifies anammox bacteria

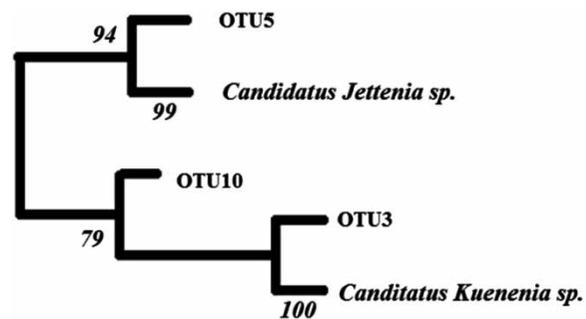


Figure 7 | Phylogeny of anammox bacteria based on *hzsB* gene amplification and sequence analysis.

based on nucleic acid analysis. Figure 7 shows the phylogeny of anammox bacteria based on hydrazine synthase subunit B (*hzsB*) protein sequences, gene amplification, and sequence analysis, which involved 10 clones sequenced for each sample. To date, five genera ('*Candidatus Brocadia*', '*Candidatus Kuenenia*', '*Candidatus Scalindua*', '*Candidatus Anammoxoglobus*' and '*Candidatus Jettenia*') have been described based on 16S rRNA analysis. Two types of genera, *Kuenenia* and *Jettenia*, were detected in the reactor of this study. Through qPCR analysis, the abundance of anammox bacteria in the reactor was found to be 10^6 copies/ μL DNA.

CONCLUSIONS

This experiment systematically investigated the effects of Fe (II) addition on anammox activity at different dosing levels based on the kinetics analysis of the specific growth rate using data from batch tests. The results evidently showed that appropriate addition of 0.08 mM of Fe (II) could enhance anammox activity.

Both the short-term and long-term experiments proved that 0.08 mM is the optimum concentration of Fe (II) by improving the anammox activity under the tested conditions. The anammox activity increased up to 60% compared to control assay (without Fe (II)) in the short-term analysis and reactor performance was enhanced by achieving $85\% \pm 3$ NH_4^+ -N and $96\% \pm 2$ NO_2^- -N removal efficiency compared to $82\% \pm 3$ NH_4^+ -N and $87\% \pm 4$ NO_2^- -N removal efficiency at the regular Fe (II) level (0.04 mM) in the long-term test.

The relationship between anammox-specific growth rate and Fe (II) concentration in this study is well described using Han-Levenspiel's substrate inhibition kinetics model.

The SBR system had shown resistance to IC_{50} of Fe (II) (i.e. 0.20 mM) with a relatively higher nitrogen removal

percentage than the batch test and had recovered from inhibition condition after acclimatization of the biomass. Quantitative polymerase chain reaction (qPCR) was adopted to detect and identify the anammox bacteria. The genera *Kuenenia* and *Jettenia* were detected in the reactor, and the abundance of anammox bacteria in the reactor was found to be 10^6 copies/ μL DNA through qPCR analysis.

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