Responses of anammox process to Ni(II): Reactor performance, Mechanisms and Inhibition Recovery

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Article

Keywords: Anammox, Ni(II) concentration, Exposure time, Inhibitory mechanisms, Cumulative effect

Posted Date: May 19th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1611852/v1

Abstract

The Responses of anammox process to Ni(II) were studied here. Anammox was the dominant reaction with Ni(II) concentrations no more than 25 mg/L. 1 mg/L of Ni(II) addition promoted nitrogen removal by Anammox. The higher the Ni(II) concentrations and longer exposure time, the more inhibition for anammox bacteria was gotten. The IC_{50} of Ni(II) to Anammox was determined as 83.86 mg/L by an exponential regression equation. The relative abundance of *Planctomycetia* in an anammox system with 100 mg/L Ni(II) addition decreased by 18.45% compared with that without Ni(II) addition. The inhibition of Ni(II) on anammox activity was mainly attributed to intracellular accumulation Ni(II) inhibition to HDH activity and microbial diversity. Two times increase of IC_{50} after 4 times circles of domestication suggests multiple intermittent domestication can increase the tolerance of anammox bacteria to Ni(II). EDTA washing can eliminate the inhibition of anammox activity by Ni(II) with Ni(II) addition no more than 25 mg/L. Our study provides an insight for practical engineering applications of anammox to treat ammonia-rich sewage containing Ni(II).

1 Introduction

Anammox bacteria (*Planctomycetales*)[1] can use nitrite nitrogen (NO_2^--N) as an electron acceptor to directly convert ammonia nitrogen (NH_4^+-N) into nitrogen under anaerobic conditions. Compared with traditional processes of nitrogen removal, Anammox process is more environmentally friendly and economical, making it suitable for wastewater treatment with high ammonia. However, anammox bacteria require a long generation time (9–11 days)[2] and are sensitive to environmental factors, e.g., metals[3,4], antibiotics[5,6], dissolved oxygen[7,8] and sulfides[9]. Thus, the application of anammox bacteria in wastewater treatment is limited. Some high ammonia nitrogen wastewaters[10], such as, landfill leachate, metal smelting wastewater, and new energy production wastewater, often contain high concentrations of heavy metals (Cu(II), Zn(II), Pb(II), Ni(II), Co(II), or Mo(II)[11–13]. These metals may seriously inhibit the nitrogen removal performance of anammox systems by interference with anammox bacteria activity[3,14,15]. Therefore, it is necessary to clarify the effect of metals on anammox process.

Many studies have explored the effects of heavy metals during various nitrogen removal processes. As an important cofactor of metalloproteinases and some enzymes, Ni(II) plays an important role in the growth and metabolism of microorganisms[16]. However, excessive heavy metals combined with enzymes are inhibitory or even toxic to biochemical reactions and microorganisms, and cause the disruption of enzymatic structure and activities[17,18]. The concentration of Ni(II) in industrial wastewater and municipal sewage treatment plants typically ranges from 0.1 to 1000 mg/L[19,20]. The Ni(II) concentrations in some wastewater are much higher than the concentration required for microbial life activities. Therefore, it is important to study the effect of Ni(II) on the inhibition threshold and microbial activity in anammox systems for anammox application in practical sewage treatment process.

Gutwinski et al.[21] investigated the effects of different mixed heavy metals on anammox process performance during long-term experiments, and pointed out the mixture of Zn^{2+}, Cu^{2+}, and Ni^{2+} at concentrations of 0.8, 0.075, and 0.04 mg/L caused a rapid inhibition in anammox process. Wu et al.[22] found an anammox system could maintain a superior performance at 10 mg/L Ni(II) during a long operation, and Ni(II) had a greater impact on the microbial community composition. An IC_{50} of Ni(II) on anammox bacteria was determined as 48.6 mg/L in batch experiments[23]. Different IC_{50} values of Ni(II) on anammox bacteria were found in different biomass reactors or different operation process. Existing reports about the effects of Ni(II) on Anammox are most focus on single Ni(II) or mixed heavy metal on the inhibition phenomenon and inhibition degree about anammox activity. The inhibition mechanism is limited known. And also, less information can be found about adaptability and domestication of Anammox under Ni(II).

Hence, the main objectives of this study: (1) determine the dominant reaction in the anammox system under Ni(II) shock; (2) investigate the effects of Ni(II) concentration and exposure time on nitrogen removal by Anammox; (3) analyze Ni(II) inhibition mechanism according to anammox activity and the changes of microbial community under Ni(II) shock; and (4) explore the cumulative inhibitory effect of Ni(II) and recovery of anammox activity of anammox systems with different Ni(II) concentration.

2 Materials And Methods

2.1 Anammox sludge and synthetic wastewater

The sludge used in batch experiments was obtained from a laboratory-scale up-flow anaerobic sludge blanket (UASB) reactor with a nitrogen load of approximately 0.5 kg-N/g-VSS/h. Before batch experiments, the sludge was washed three times using a buffer solution (pH 7.0 and 0.1 M NaCl) to remove residual matrix. Results of high-throughput pyrosequencing showed the dominating bacterial groups in the anammox sludge was *Planctomycetia* (48.44%). The compositions of the synthetic wastewater used in our batch experiments were listed in Table 1. NH_4^+-N and NO^{2-}-N in the synthetic wastewater were provided by NH_4Cl and $NaNO_2$, respectively. According to the anammox reaction equation, the ratio of NH_4^+-N to NO_2^--N is 1:1.32. Ni(II) was provided in the form of $NiCl_2{\cdot}6H_2O$.

Table 1
The compositions of the synthetic wastewater

Compositions	Concentration(mg/L)
$NaHCO_3$	1000
KH_2PO_4	27.2
$MgSO_4{\cdot}7H_2O$	200
$CaCl_2$	300
NH_4^+-N	Add as need
NO_2^--N	Add as need

2.2 Batch experiments

Batch experiments were performed in serum bottles with a total volume of 250 mL and a liquid phase volume of 180 mL. The initial pH of reaction systems in serum bottles was adjusted to 7.0 ± 0.2 using 0.1 M HCl or 0.1 M NaOH. These systems were needed to aerate with high purity nitrogen for 5 min to remove oxygen. After then, the serum bottles were quickly sealed with a rubber stopper and aluminum crimp. Finally, the serum bottles were placed in a constant temperature shaking incubator with 35 ± 1°C and 120 rpm. Samples in the serum bottles were extracted using a syringe.

2.2.1 Effects of Ni(II) concentration on Anammox

The concentration of NH_4^+-N and NO_2^--N in batch experiments were 50 and 66 mg/L, respectively. Ni(II) concentration was set as 0, 1, 2.5, 5, 10, 25, 50, 75, and 100 mg/L, respectively. At the end of each batch experiment, sludge samples were retained to measure mixed liquor volatile suspended solids (MLVSS), hydrazine dehydrogenase (HDH) activity, distribution of Ni(II) and high-throughput pyrosequencing.

2.2.2 Effects of exposure time on Anammox

The effect of exposure time of anammox bacteria to Ni(II) on the nitrogen removal and the anammox recovery was studied here. The initial concentrations of NH_4^+-N and NO_2^--N in batch experiments were the same as that in 2.2.1 chapter. Ni(II) concentration was 50 mg/L. Exposure time were set as 0 h, 12 h, 24 h and 48 h, respectively. The sludges exposed to 50 mg/L of Ni(II) concentration with different exposure time were washed with a buffer solution (pH 7.0 and 0.1 M NaCl) according to a modified EDTA wash procedure[24] and fresh substrate was added to assess the anammox recovery performance.

2.2.3 Cumulative effect experiments

Two different Ni(II) concentrations, 25 and 75 mg/L, were applied in batch experiments to investigate Ni(II) cumulative effect on Anammox. Four different cycles were carried out here. Each cycle was about 24 h, including 12 h experimental phase and 12 h intermittent phase. The initial concentration of NH_4^+-N and NO_2^--N were 50 and 66 mg/L, respectively. Liquid samples were taken every 3 hours to analyze nitrogen components concentration.

2.2.4 Recovery experiments about anammox activity

The sludges exposed to different Ni(II) concentration in section 2.2.1 were washed using a modified EDTA wash procedure as the same as that in section 2.2.2. Fresh substrate without Ni(II) addition was added to assess the anammox recovery performance after the EDTA washing.

2.3 Analytical methods

NH_4^+-N, NO_2^--N, NO_3^--N and MLVSS were determined by standard methods[25]. Other indicators were determined as described in separate sections below.

2.3.1 Ni(II) determination

Two same anammox sludges (1 g wet weight) were taken from each bottle at the end of batch experiments. The samples used to measure intracellular Ni(II) concentration were washed with a modified EDTA washing procedure[26] to remove Ni(II) adsorbed on sludge surface. The other was not treated by EDTA washing. With or without EDTA-treated sludge sample was all dissolved in a 4M nitric acid solution with incubation at 120 rpm for 25 min. Then, the mixture was centrifuged at 12,000 rpm for 15 min to obtain a supernatant. The supernatant was filtrated through 0.2 mm membrane. Then, filtrated supernatant was used to determined Ni(II) concentration using an ICP Instrument Perkin Elmer OES Optima 2000 DV. Ni(II) from EDTA-treated sludge was about intracellular Ni(II). Extracellular Ni(II) was calculated according the subtraction result of untreated sludge by EDTA washing and EDTA-treated sludge.

Liquid samples in anammox reactors were also needed to centrifuged at 12,000 rpm for 15 min and filtrated through 0.2 mm membrane to measure Ni(II) concentration in liquid.

2.3.2 Measurement of HDH activity

In order to measure HDH activity of anammox cells, 5 g (wet weight) anammox sludge were taken from each bottle at the end of each experiment, and then centrifuged at the condition of 12,000 rpm, 4°C for 20 min. The centrifuged sludge samples were washed twice times using sodium phosphate buffer (20 mM, pH 7.0). The washed sludge samples were resuspended in 20 mL buffer to frozen (-20°C) for 24 h, and then sonicated (225 W, 4°C; Ultrasonic processor CPX 750, USA) for 30 min. The supernatants (-20°C) were centrifuged at 12,000 rpm for 30 min and used to analyze HDH activity. HDH activity was measured by spectrophotometry at the wavelength of 550 nm and expressed as the rate of production of reduced cytochrome c (μmol cytochrome reduced/min/mg protein).

2.3.3 High-throughput pyrosequencing

High throughput pyrophosphate sequencing was performed using the MiSeq sequencing platform (Illumina, Inc., San Diego, CA, USA). More than 20,000 sequences of 410 base pairs were obtained from each sample and compared with the reference microorganisms from the Silva database (http://www.arb-silva.de).

2.4 Mathematical calculation

2.4.1 Determination of Specific Anammox Activity

The specific anammox activity (SAA) was calculated by the following equation (Eq. 1).

$$SAA = \frac{(C_{inf} - C_{eff}) \times V}{24h \times MLVSS} \quad (1)$$

where C_{inf} or C_{eff} is the total nitrogen concentrations (mg/L) at the beginning or the end of our experiments, respectively; V is the effective volume of the serum bottle (mL); MLVSS is the sludge concentration in anammox systems (g/L).

2.4.2 Determination of IC_{50}

An exponential regression equation (Eq. 2) was used to describe Ni(II) inhibition on nitrogen removal by Anammox.

$$IP(\%) = 100 \times \left(1 - \frac{1}{1 + \left(\frac{C_{Ni}}{IC_{50}}\right)^{b}}\right)$$

2

where C_{Ni} is Ni(II) concentration in anammox systems (mg/L); IC_{50} is the inhibition coefficient with an 50% inhibition of anammox activity; and b is a fitting parameter.

2.4.3 Biological toxicity model

In order to study the inhibitory mechanisms of Ni(II) to Anammox, the inhibition form of the Monod equation was used as below,

$$IP(\%) = \frac{IP_{max} C_{Ni}}{K_{Ni} + C_{Ni}}$$

3

where IP_{max} is the maximum percentage of inhibition (100%); C_{Ni} is the intracellular Ni(II) concentration (mg/gVSS); and K_{Ni} is the inhibition coefficient (mg/gVSS) associated with an 50% inhibition of anammox activity.

3. Results And Discussion

3.1 Dominant reaction of Anammox system under Ni(II) shock

According to the stoichiometric equation of the anammox bioreaction process proposed by Strous et al.[27], the ratio of NO_2^--N consumed and NH_4^+-N consumed (Rs) is 1.32, and the ratio of NO_3^--N produced and NH_4^+-N consumed (Rp) is 0.26 in anammox process. The two stoichiometric ratios are usually applied to indirectly judge whether anammox reaction was the main reaction in biological nitrogen removal systems[28]. Figure 1 describes the change of Rs and Rp values in our experiments with Ni(II) addition. As shown in Fig. 1, the values of R_S and R_P were relatively stable and close to these stoichiometric values (1.32 and 0.26) when Ni(II) concentration was no more than 25 mg/L. However, the values of R_S and R_P fluctuated sharply with a further increasing of Ni(II) concentration. It suggests anammox reaction was the main bioreaction for nitrogen removal in the systems with Ni(II) addition no more than 25 mg/L. The average values of Rs and Rp were about 1.68 and 0.181 with the Ni(II) concentration no more than 25 mg/L, respectively.

The Rs value of 1.68 in our experiment is a little higher than the stoichiometric Rs value of 1.32. The Rp value of 0.181 is a little lower than the stoichiometric Rp value of 0.26. As we all know, microorganisms in wastewater biological treatment system are mixed flora. Special deaeration measures were not used in our experiment and the presence of nitrifying bacteria can convert NO_2^--N oxide into NO_3^--N. This results in the excessive removal of NO_2^--N and the higher value of Rs. The lowed Rp value was because part of nitrate was used by denitrifying bacteria in our experiment systems. The values of Rs and Rp with Ni(II) addition equal or higher than 50 mg/L were seriously larger than those stoichiometric values. This indicates Ni(II) addition with high concentration has a much greater impact on anammox bacteria than on nitrifying bacteria. The anammox bacteria were sensitive to external environment Ni(II) addition with high concentration.

Other studies have also reported that Rs and Rp ratios were not strictly consistent with these stoichiometric ratios in anammox systems[29–31]. Zhou et.al[32] pointed out the stoichiometric ratios Rs and Rp in anammox system seeded with a single type of denitrified granular sludge were 1.20 and 0.34, respectively. And the stoichiometric ratio Rs and Rp in anammox system seeded with a mixed sludge of denitrified granular sludge and aerobic nitrification sludge was 1.26 and 0.21. As shown in Fig. 2, the anammox sludge kept blood-red at the end of experiments in the systems with Ni(II) addition no more

than 25 mg/L, which is a significant feature of the anammox reaction. The close to stoichiometric value of R_S and R_P and the blood-red sludges suggest anammox reaction was the dominant reaction in experiment with Ni(II) addition no more than 25 mg/L.

3.2 Effect of Ni(II) on Anammox system

3.2.1 Effect of Ni(II) concentration on Anammox system

The changes of NH_4^+-N and NO_2^--N concentrations with time under different Ni(II) concentrations were shown in Fig. 3. When the Ni(II) concentration was lower than 50 mg/L, the NH_4^+-N and NO_2^--N concentration stabilized at about 14 h with an 83%-99% removal efficiency of NH_4^+-N and a 94%-95% removal efficiency of NO_2^--N. When Ni(II) concentration was equal or higher than 50 mg/L, the NH_4^+-N and NO_2^--N concentration reach steady state at about 9 h. This stabilized time was shorten by 5 h than that with Ni(II) addition lower than 50 mg/L. Similar phenomena was also observed by Yang et al.[12], who found out the stabilized time with Cu(II) concentration higher than 25 mg/L was shortened by one hour compared with that with 10 mg/L Cu(II) addition in an anammox reactor. When Ni(II) concentration equal or higher than 50 mg/L, the removal efficiency of NH_4^+-N and NO_2^--N were 36%-50% and 60%-86%, respectively. Comparing with low Ni(II) concentrations, high Ni(II) concentrations had a more critical inhibition for nitrogen removal performance by anammox bacterial. The difference of inhibition degree between NH_4^+-N and NO_2^--N removal efficiency is obvious under high Ni(II) addition. The inhibition degree of high Ni(II) concentration on anammox bacteria was more serious than that on nitrifying bacteria.

An interesting phenomena was found in the system with 1 mg/L Ni(II) addition. When 1 mg/L of Ni(II) was added to the system, the concentrations of NH_4^+-N and NO_2^--N at the ending of reaction were 0.11 mg/L and 3.04 mg/L, respectively. The concentrations of NH_4^+-N and NO_2^--N without Ni(II) addition were 0.98 mg/L and 4.34 mg/L, respectively. The removal efficiencies of NH_4^+-N and NO_2^--N with 1 mg/L Ni(II) addition increased by 8.75% and 5.84% than that without Ni(II) addition, respectively. It exposes that Ni(II) addition with lower concentration can certainly promote the activity of anammox bacteria and was beneficial for the removal of NH_4^+-N and NO_2^--N.

To further quantify the inhibitory effect of Ni(II) concentration on the nitrogen removal performance by anammox bacteria, specific anammox activity (SAA) and Ni(II) concentration were fitted using an exponential regression model, as described in Table 2. SAA was calculated according to the Eq. 1. The high value of the fitting correlation coefficient (R^2 = 0.9974) indicates that the exponential regression model was suitable to describe the relationship between SAA and Ni(II) concentration. An 83.86 mg/L value of IC_{50} was gotten by the exponential regression model.

Table 2
Regression equation fitting

Model	Regression equation	R^2
Exponential regression	$IP = 100 \times \left(1 - \frac{1}{1 + \left(\frac{C_{Ni}}{83.86}\right)^{2.0235}}\right)$	0.9974
Intracellular Ni(II) and inhibition fitting	$\mathrm{IP} = \frac{0.8604 M_{Ni}}{0.07233 + M_{Ni}}$	0.9850

3.2.2 Effect of exposure time on anammox activity

The effect of exposure time of anammox bacteria to Ni(II) on anammox activity was shown in Fig. 4. The SAA decreased from 11.19 mg-TN/g-VSS/h to 7.86 mg-TN/g-VSS/h with the increase of exposure time from 0 h to 12 h. Extending of the exposure time to 48 h, the SAA further decreased to 3.09 mg-TN/g-VSS/h. The stronger inhibition of Ni(II) on anammox activity was existed with the longer exposure time.

The recovery performance of anammox bacteria after exposing to Ni(II) were also studied here. The SAA of exposed sludge under different exposure time were all improved after EDTA washing (Fig. 4). After EDTA washing, the SAA with 12 h exposure time and with 24 h exposure time recovered from 7.60 mg-TN/g-VSS/h to 12.1 mg-TN/g-VSS/h and from 4.53 mg-TN/g-VSS/h to 10.7 mg-TN/g-VSS/h. The SAA with 0 h exposure time was about 11.2 mg-TN/g-VSS/h. The anammox bacteria with exposure time no more than 24 h basically restored the anammox ability that without contacted with Ni(II). When the exposure time extended to 48 h, there was a tiny digital difference between the SAA values with or without washing. Therefore, prolonging the exposure time of sludge to Ni(II) caused particularly serious inhibition of Ni(II) to anammox bacteria. Zhen et.al.[33]pointed out the heavy metal entering the sludge by active transport grow with the extending of exposure time. The accumulated heavy metal in anammox microorganisms cannot been removed by simple washing. Most of the anammox cells may be substantially damaged when the anammox bacteria exposed to Ni(II) for 48 h. These lead to a bad anammox activity recovery with 48 h the exposure time. The inhibition of anammox activity by Ni(II) with exposure time no more than 24 h can be eliminated after EDTA washing.

3.3 Mechanism analysis of the effects of Ni(II) on anammox

3.3.1 Microbial community of anammox with different Ni(II) addition

Sample OUT number and diversity index of all samples under different Ni(II) concentration are listed in Table 3. The coverage index above 0.98 proved the sequencing covered most of the eukaryotic microorganisms in the samples and the data results were reliable and accurate. 115201, 105278, 73992, 68681, 64243 and 50165 sequences were obtained from 6 samples with different Ni(II) addition, respectively. The corresponding OTU number of the 6 samples were 1973, 1950, 1764, 1571, 1570 and 1416, respectively. The sequence and OTU number both decreased with the increase of Ni(II) concentration. 5 mg/L of Ni(II) had a negligible impact on the values of the sequence and OTU number. Shannon index reflects the biodiversity in biological systems. The Shannon index of our samples also reduced with the raise of Ni(II) concentration. The higher Ni(II) concentration resulted in the less biodiversity. The addition of high Ni(II) concentration led to cell death and vanish of some inadaptable microorganisms.

Table 3
Sample OUT number and diversity index table

Sample	Sequence numbers	OTU number	Shannon index	Coverage index
C_0	115201	1973	4.150666	0.991279
C_1	105278	1950	4.054089	0.988259
C_2	73992	1764	3.919178	0.99388
C_3	68681	1571	3.893829	0.99323
C_4	64243	1570	3.890371	0.990987
C_5	50165	1416	3.800503	0.989931
(C_0: Ni(II) = 0 mg/L, C_1: Ni(II) = 5 mg/L, C_2: Ni(II) = 10 mg/L, C_3: Ni(II) = 25 mg/L, C_4: Ni(II) = 50 mg/L, C_5: Ni(II) = 100 mg/L)				

All effective sequences of each sample were further assigned to the corresponding taxonomy levels using Silva database. The taxonomic results in class level are shown in Fig. 5. Nitrogen removal related bacteria mainly includes *Planctomycetia*, *Betaproteobacteria* and *Alphaproteobacteria*. The relative abundance of *Planctomycetia* of samples with different Ni(II) addition were 34.07%, 34.77%, 32.74%, 29.53%, 29.19% and 15.62%, respectively. Most of the microorganisms involved in anammox process belong to *Planctomycetia*. The abundance of *Planctomycetia* gradually decreased with the increase of Ni(II) concentration. It suggests the increasing of Ni(II) concentration resulted in the decreasing of anammox activity. The abundance of *Proteobacteria* (*Betaproteobacteria* and *Alphaproteobacteria*) in sample C_5 was 42.20%. While *Proteobacteria* only accounted for 22.32% in C_0 without Ni(II) addition. Most of the microorganisms involved in nitrification and denitrification process belong to *Proteobacteria*. The high Ni(II) led to the death of some organisms, and then the dead was may utilized by denitrification process as the organic substrate. This leaded to an increased abundance of *Proteobacteria*. The reducing

of *Planctomycetia* and the increasing of *Proteobacteria* expose the reducing of nitrogen removal by anammox process and the increasing of nitrogen removal by nitrification and denitrification process in high Ni(II) concentration. This is consistent with the changes of Rs and Rp in anammox systems with high Ni(II) addition.

3.3.2 Relationship between SAA and HDH activity

As shown in Fig. 6(a), the SAA increased from 8.46 mg-TN/g-VSS/h to 9.11 mg-TN/g-VSS/h with Ni(II) concentration increasing from 0 mg/L to 1 mg/L. The SAA kept falling with a further increasing of Ni(II) concentration. Similar change trend was appear between HDH activity and Ni(II) concentration (Fig. 6(b)). The SAA and HDH activity with 1 mg/L Ni(II) addition were enhanced by 11.14% and 8.56% than that without Ni(II) addition, respectively. Ni(II) is an indispensable trace element for microbial metabolism, and is involved in protein synthesis and electron transfer processes[34] HDH is the most important enzyme in the anammox process, and can convert the intermediate hydrazine (N_2H_2) into nitrogen[35,36] The enhancement of HDH activity accelerated the reaction of nitrogen conversion. Ni(II) addition with low concentration may stimulate electron transfer processes of anammox and promoted anammox activity, resulting in a more nitrogen removal by anammox bacteria. 1 mg/L Ni(II) addition improved the nitrogen removal performance of anammox.

3.3.3 Relationship between the distribution of Ni(II) and SAA

Ni(II) existed mainly in the forms of soluble Ni(II), extracellular adsorption Ni(II) and intracellular accumulation Ni(II) in anammox systems. The content of Ni(II) distributed to different three regions in sludges all increased with a raise of Ni(II) addition (Fig. 6(c)). When 25 mg/L Ni(II) was added, the content of intracellular accumulation Ni(II) was about 1.73 mg/L. After then, the intracellular accumulation Ni(II) content rapidly grows with a further increasing of Ni(II) addition. Intracellular accumulation Ni(II) content was very little in anammox systems with Ni(II) addition no more than 25 mg/L. When Ni(II) concentration was no more than 25 mg/L, nitrogen removal was mainly attributed to anammox reaction and higher NH_4^+-N removal efficiency (83%-99%) and NO_2^--N removal efficiency (94%-95%) were gotten in our experiments (Figs. 1 and 3). The different experimental phenomenon appeared with a further increasing of Ni(II) concentration. This indicates that the increasing of intracellular accumulation Ni(II) may cause the change of nitrogen removal in the anammox systems with more than 25 mg/L Ni(II) addition. Extracellular adsorption Ni(II) content had not change much with Ni(II) addition between 25 mg/L and 50 mg/L. Hence, the anammox activity inhibited by Ni(II) with more than 25 mg/L addition may be mainly brought by intracellular accumulation Ni(II).

To further clarify the mechanism of Ni(II) inhibition on anammox bacteria, the relation of intracellular accumulation Ni(II) and SAA inhibition percent was investigated and summarized in Table 2. A high R^2 of 0.9850 was gotten by using a biotoxicity model (Eq. 3) to simulate the relation of intracellular accumulation Ni(II) and SAA inhibition percent. The biotoxicity model was suitable to describe the

relation of intracellular accumulation Ni(II) and SAA inhibition percent. This further exposes SAA inhibition by Ni(II) was more closely related to the intracellular accumulation.

Ni(II)ingested by microorganisms can disrupt protein with functional groups [18]. In general, the toxicity of heavy metals to biological systems is due to the effect of heavy metals on enzyme and protein functions. Although Ni(II) is a trace element required for the growth of anammox bacterial, excessive Ni(II) in anammox bacterial damaged protein structure and reduced activity of HDH, resulting in the deterioration of nitrogen removal performance. When excessive Ni(II) was present in anammox system, the Ni(II) content of intracellular accumulation was the dominant factor for SAA inhibition.

3.4 Cumulative effect

Experiments with four different cycles were carried out to explored the bioaccumulation effect of Ni(II) on anammox systems. Each cycle was about 24 h, including 12 h experimental phase and 12 h intermittent phase. As shown in Fig. 7(a), TN cumulative removal without Ni(II) addition was 449 mg-TN/g-VSS after four times cycles. Corresponding, the TN cumulative removal with 25 mg/L Ni(II) and 75 mg/L Ni(II) addition were about 303 mg-TN/g-VSS and 103 mg-TN/g-VSS, respectively. When Ni(II) addition was 25 mg/L, the TN cumulative removal after four times cycles obviously increased than that after three times cycles. When Ni(II) addition was 75 mg/L, there was no significant difference about TN cumulative removal between three times cycles and four times cycles. And, the TN cumulative removal with 75 mg/L Ni(II) addition was only 31.57% of that without Ni(II) addition. The inhibition of anammox microorganisms by Ni(II) addition was reduced after multiple cycles domestication with 25 mg/L Ni(II) addition. With a higher Ni(II) addition (75 mg/L), the inhibition did not eliminate well with multiple cycles domestication.

The inhibition response to Ni(II) accumulated in the sludges with 25 mg/L Ni(II) addition was shown in Fig. 7(b). The relation of the inhibition response and Ni(II) concentration in anammox systems with different times cycles were exponentially fitted by Eq. 2. The fitting equations, fitting coefficient R^2 and IC_{50} calculated by Eq. 2 were summarized and listed in Table 4. The IC_{50} values for one-, two-, three- and four-times cycles were 10.83, 30.89, 82.08, and 165.43 mg/L, respectively. The IC_{50} values increased with a raise in the number of cycles. Therefore, with low Ni(II) concentration (≤ 25 mg/L) addition, anammox microorganisms can extend their tolerance to Ni(II) by multiple intermittent exposures to Ni(II).

Table 4
IC_{50} under different domestication cycles

domestication cycles	Inhibition regression equation	R^2	IC_{50}
1	$IR = 100 \times \left(\frac{1}{1 + \left(\frac{c}{10.83871} \right)^{0.89781}} \right)$	0.99968	10.83
2	$IR = 100 \times \left(\frac{1}{1 + \left(\frac{c}{30.89777} \right)^{0.95712}} \right)$	0.9985	30.89
3	$IR = 100 \times \left(\frac{1}{1 + \left(\frac{c}{82.08358} \right)^{1.68673}} \right)$	0.9993	82.08
4	$IR = 100 \times \left(\frac{1}{1 + \left(\frac{c}{165.43606} \right)^{1.72543}} \right)$	0.98375	165.43

3.5 Recovery of anammox activity after Ni(II) shock

Recovery of anammox activity in anammox systems with different Ni(II) addition were presented in Fig. 8. The sludges exposed to different Ni(II) concentration were washed by EDTA washing. When Ni(II) concentrations were 2.5, 5, 10, and 25 mg/L, the SAA recovered to 98.49%, 95.61%, 97.42%, and 93.25% of the SAA without Ni(II) addition. When Ni(II) concentrations were 75 and 100 mg/L, only 53.47% and 46.49% of SAA were gotten after EDTA washing. When Ni(II) concentration was no more than 25 mg/L, Ni(II) not in liquid most stayed outside of the sludges through extracellular adsorption (Fig. 6(c)). Little Ni(II) entered into the inside of the sludges with Ni(II) addition no more than 25 mg/L. The inhibition of anammox activity by Ni(II) with lower Ni(II) addition can be eliminated after EDTA washing. However, Ni(II) content of intracellular accumulation increased with a further raise of Ni(II) concentration. High Ni(II) concentration may lead to an irreversible deactivation of the anammox bacteria. Intracellular accumulation Ni(II) is not easy to clean by EDTA washing. A poor recovery of anammox activity was gotten by EDTA washing in anammox systems with Ni(II) addition higher than 25 mg/L.

4. Conclusion

Anammox was the dominant reaction in systems with Ni(II) addition no more than 25 mg/L. Ni(II) addition with low concentration (1 mg/L) may stimulate electron transfer processes of anammox and promoted anammox activity, resulting in a more nitrogen removal by anammox bacteria. High Ni(II) concentration and long exposure time resulted to poor nitrogen removal performance in anammox systems. The IC50 of Ni(II) was determined as 83.86 mg/L. The abundance of *Planctomycetia* gradually decreased with the increase of Ni(II) concentration. The higher Ni(II) concentration resulted in the less microbial diversity biodiversity. When Ni(II) addition was higher than 25 mg/L, anammox activity was inhibited and HDH activity decreased. Intracellular accumulation Ni(II) was the dominant factor for anammox activity inhibition. Multiple cycles domestication and EDTA washing can eliminate the inhibition of anammox activity by Ni(II) with Ni(II) addition no more than 25 mg/L.

Declarations

Acknowledgements

The authors gratefully acknowledge the funding of the National Natural Science Foundation of China (51978009), the Open Fund of State Key Laboratory of Water Resource Protection and Utilization in Coal Mining (Grant No. KFJJ2018072), and the Beijing Postdoctoral Research Foundation (2021ZZ118).

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Figures

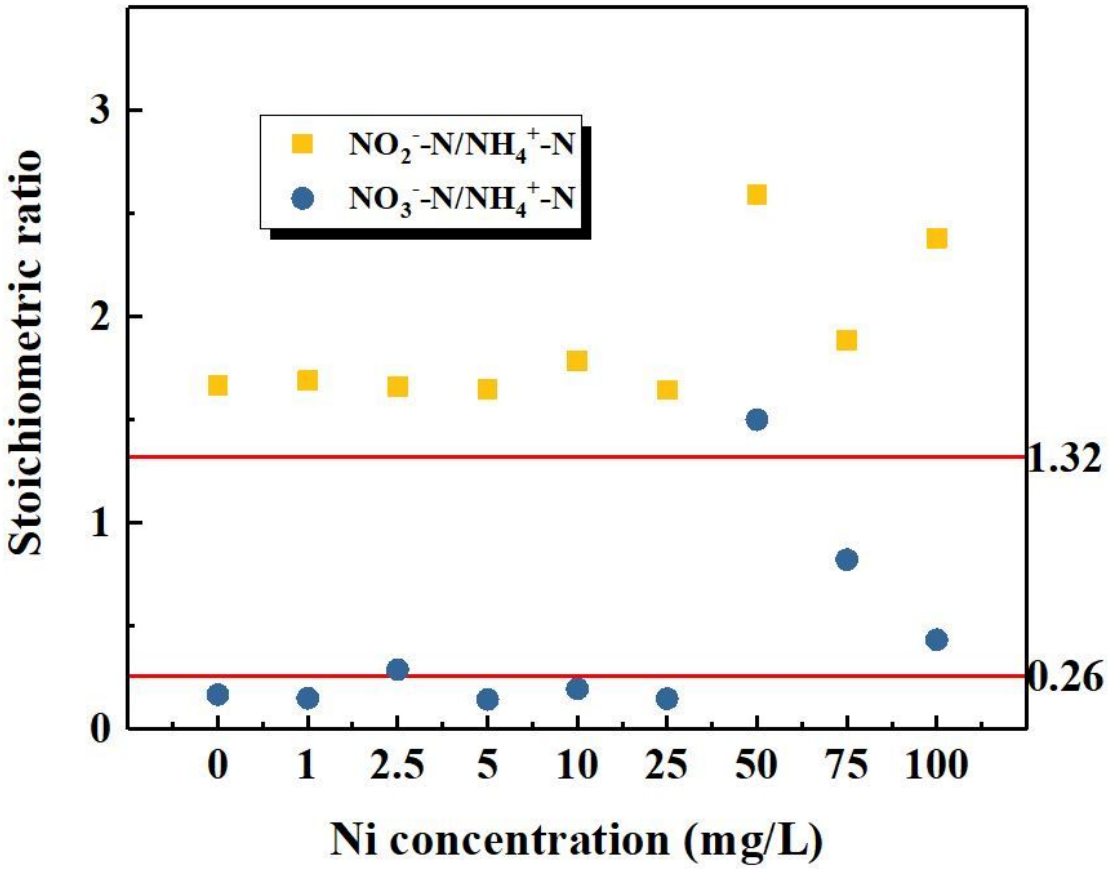

Figure 1

Stoichiometric ratio of anammox system under different Ni(II) concentrations

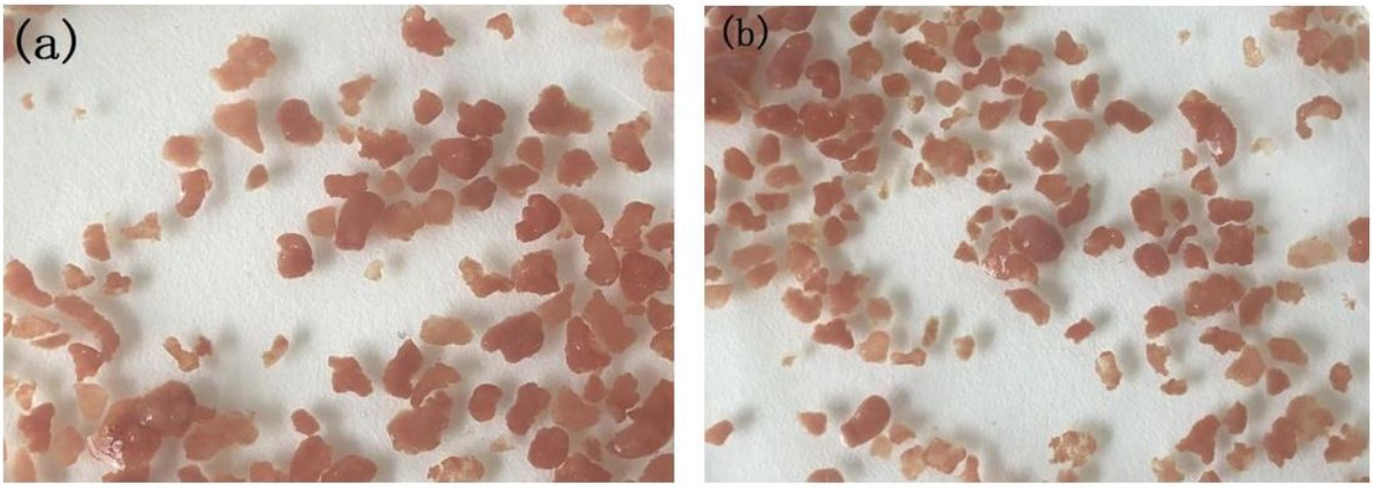

Figure 2

The anammox sludge at the end of experiments with different Ni(II) addition: 0 mg/L (a) and 25 mg/L (b)

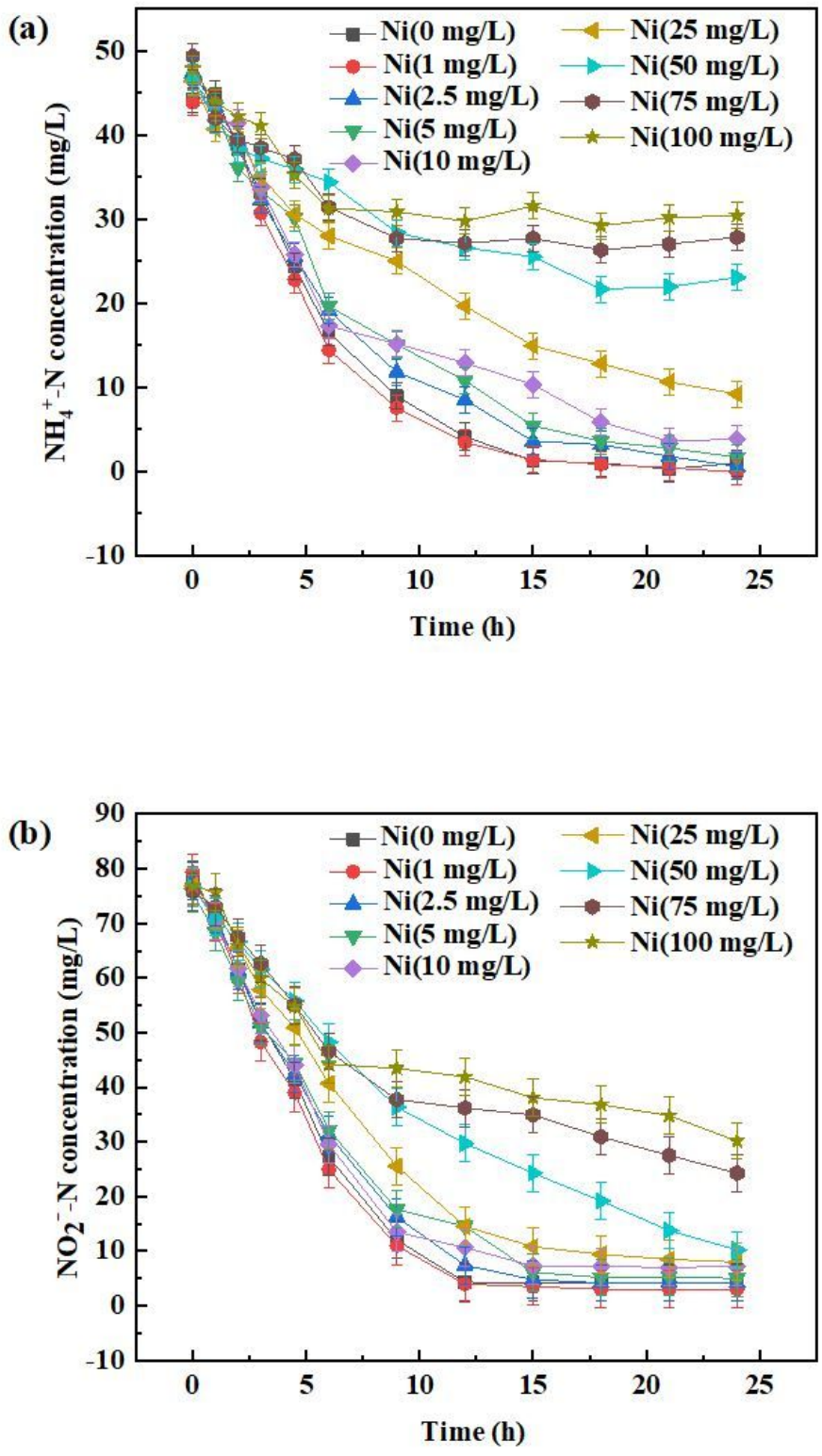

Figure 3

Effects of Ni(II) concentration on anammox systems performance with time: (a) NH_4^+-N concentration and (b) NO_2^--N concentration.

Figure 4

Changes of SAA at different exposure time

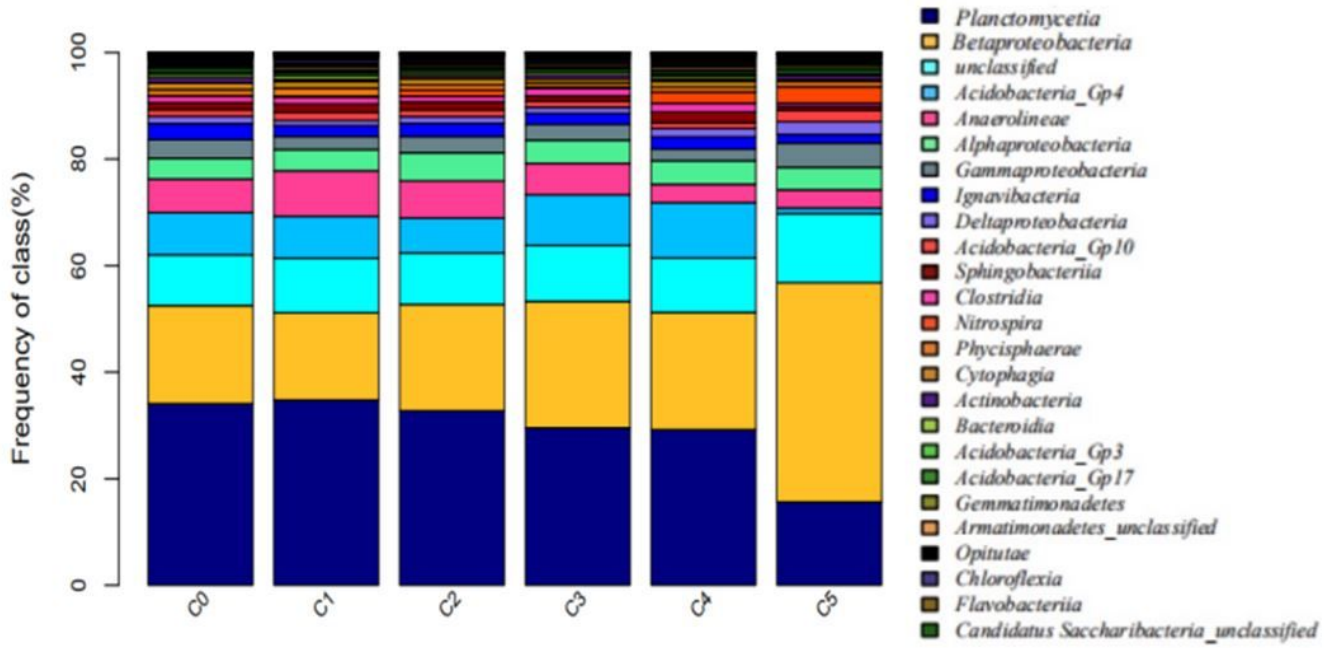

Figure 5

Microbial class horizontal structure of anammox with different Ni(II) addition. (C_0: Ni(II)=0 mg/L, C_1: Ni(II)=5 mg/L, C_2: Ni(II)=10 mg/L, C_3: Ni(II)=25 mg/L, C_4: Ni(II)=50 mg/L, C_5: Ni(II)=100 mg/L)

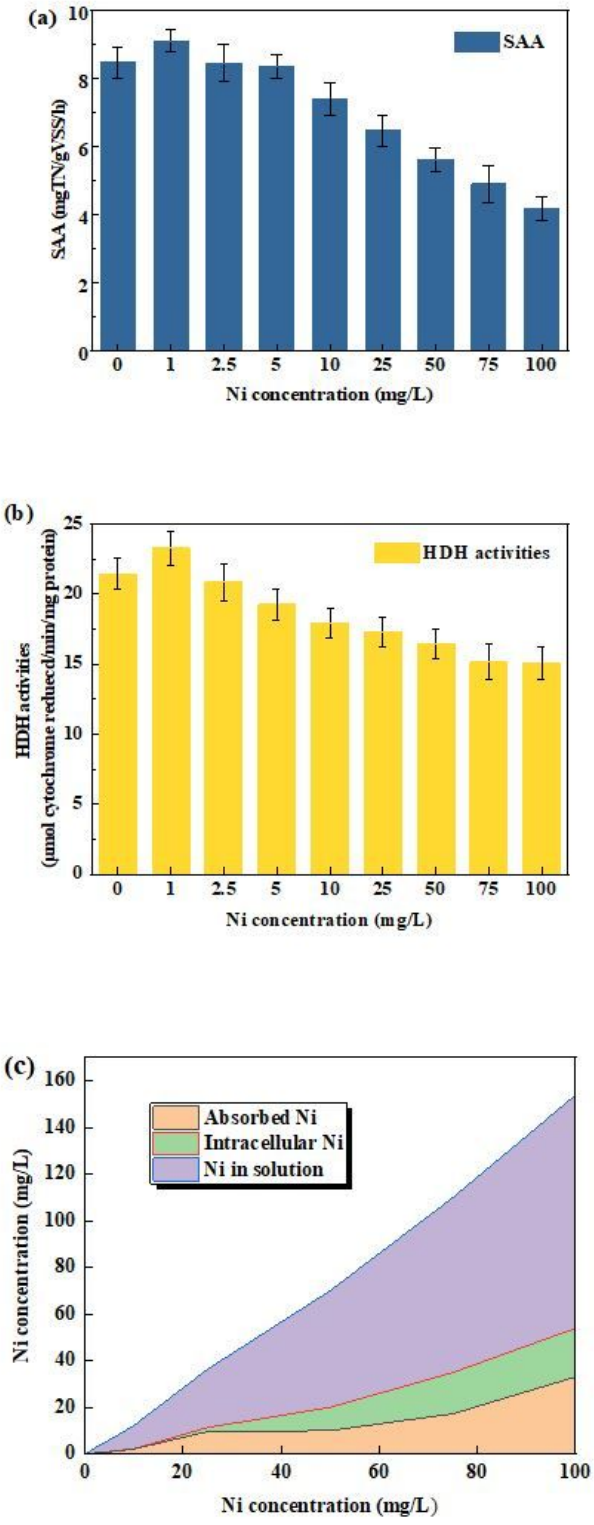

Figure 6

(a) Changes of SAA, (b) Changes of HDH activity, and (c)The distribution of Ni(II) with different Ni(II) addition

Figure 7

(a) Changes of TN cumulative removal at different Ni(II) concentrations, and (b) Inhibition response of multiple cycles domestication with 25 mg/L Ni(II) addition

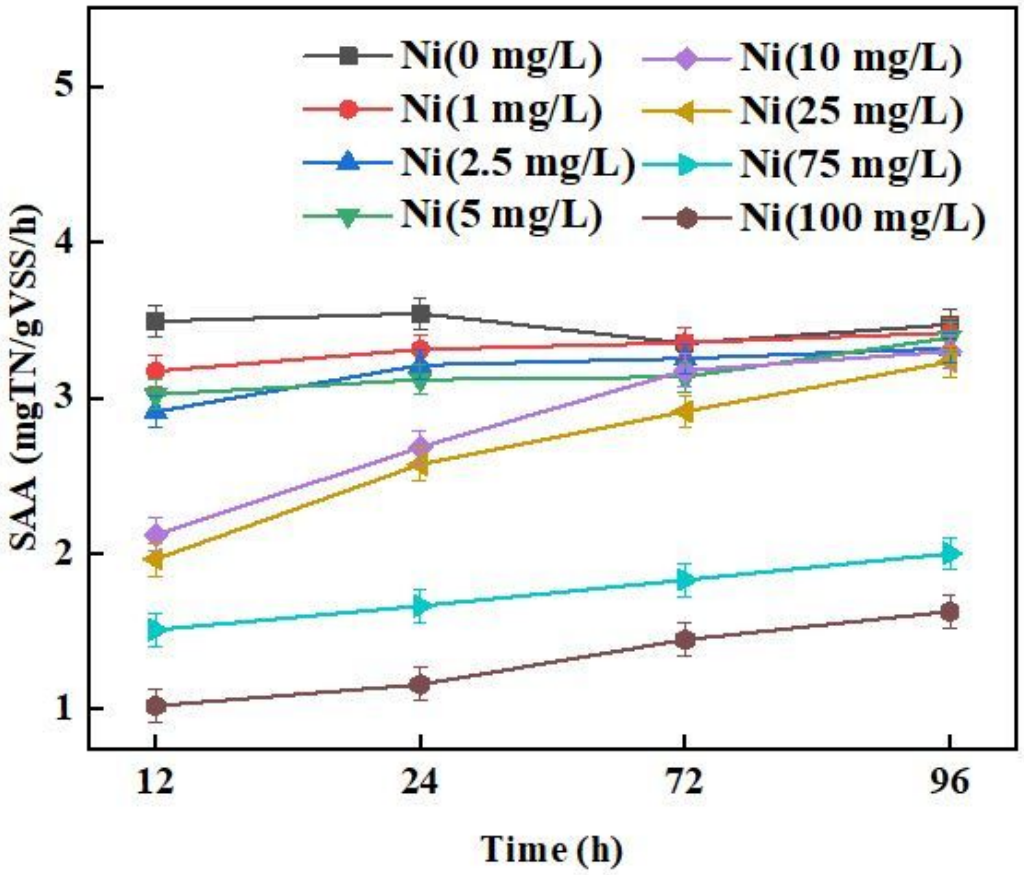

Figure 8

The recovery of anammox activity under different Ni(II) addition