

Short-term effects of monoethanolamine and copper on the activities of Anammox bacteria

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Key Words: Anammox, copper, heavy metal, inhibition, organic solvent

ABSTRACT

Organic solvents and heavy metals are commonly found in industrial wastewater. These compounds may affect or inhibit the activities of microbes involved in biological wastewater treatment process. Monoethanolamine (MEA) is an organic nitrogen containing solvent used in thin-film transistor liquid crystal display industry as stripper. MEA is usually recognized as slowly biodegradable organic solvent. Copper is the most common heavy metals found in swine wastewater. The presence of copper in wastewater can stimulate or inhibit the microbial processes involved in the biological treatment process.

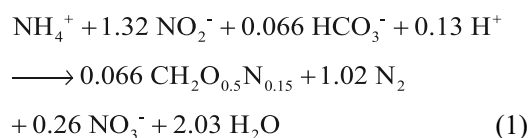
In this study, the effects of MEA and copper on the activities of Anammox (anaerobic ammonium oxidation) bacteria were investigated in batch tests. Synthetic wastewaters containing $\text{NH}_4^+\text{-N}$ (70 mg L^{-1}) and $\text{NO}_2^-\text{-N}$ (70 mg L^{-1}) were spiked with either MEA (0 to 500 mg L^{-1}) or copper (0 to 10 mg L^{-1}) at different concentrations and used as media for batch tests.

Results of batch tests revealed that MEA, at all concentrations inhibited the activity of Anammox bacteria. The IC_{50} value of MEA was calculated to be 175 mg L^{-1} . The noncompetitive inhibition model ($R^2 = 0.81$) and linear inhibition model ($R^2 = 0.87$) were fitted well with the experimental data of Anammox inhibition at different MEA concentrations. On the other hand, activity of Anammox bacteria was strongly inhibited by the presence of copper at mixed liquor volatile suspended solids (MLVSS) of $< 2000 \text{ mg L}^{-1}$. The IC_{50} value of copper was calculated to be 4.2 mg L^{-1} . Under the experimental conditions, inhibition of Anammox bacterial activity increased with concentration of copper (1 to 10 mg L^{-1}) and exposure time (3 to 24 h) at lower biomass concentration ($< 2000 \text{ mg L}^{-1}$ VSS). However, the specific Anammox activity is positively affected by copper at higher biomass concentration ($> 2500 \text{ mg L}^{-1}$ of VSS) and longer exposure time. The inhibition of Anammox bacterial activity by copper was found to be higher at 25°C compared to at 35°C . Overall, the results suggested that effect of copper on specific Anammox activity was influenced by concentrations of copper and biomass, exposure time and temperature.

INTRODUCTION

Anaerobic ammonium oxidation (Anammox) is a shortcut method to convert ammonium to nitrogen gas by Anammox bacteria, which use nitrite as electron acceptor to oxidize ammonium. Anammox bacteria are specific group of microorganisms belong to the bacterial phylum Planctomycetes. The stoichiometry equation of Anammox process is given in Eq. 1 [1]. Since Anammox bacterium uses inorganic carbon (CO_2) for biomass synthesis and it does not require oxygen, this process has been regarded as the most

economical and efficient biological nitrogen removal method [2].



However, Anammox process requires long start-up time, which limits its wide spread applications in treating nitrogen rich wastewaters. The main reasons for long start-up times are very slow growth rate and low biomass yield of Anammox bacteria. The

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maximum specific growth rate and biomass yield of Anammox bacteria have been reported to be 0.0027 h^{-1} (doubling time 11 d) and $0.066 \text{ C-mol mol}^{-1}$ ammonium (Eq. 1) [3]. Moreover, Anammox process is inhibited by various factors. Nitrite ($> 100 \text{ mg-N L}^{-1}$), free ammonia ($> 25 \text{ mg-N L}^{-1}$), free nitrous acid ($> 4.4 \text{ mg-N L}^{-1}$), and dissolved oxygen have been considered as potential inhibitors of Anammox process [4-6]. The presence of inhibitors in influent wastewater would further make it difficult to culture Anammox bacteria. Therefore, it is important to identify the potential inhibitors and toxic substances affecting Anammox process before its application on real wastewater treatment.

The high-technology industrial wastewaters such as semiconductor industries and optoelectronic industries are rich in nitrogen and contains many toxic and refractory chemicals such as organic solvents, acids and bases [7,8]. Monoethanolamine (MEA, $\text{C}_2\text{H}_5\text{ONH}_2$), an organic nitrogen containing solvent is abundantly used as a stripper in thin-film transistor liquid crystal display industry. In natural gas industry, MEA is commonly used in "sweetening" process to remove acid gases from natural gas [9]. MEA is usually recognized as slowly biodegradable organic solvent [10]. However, no information is available in literature regarding the effects (inhibition or stimulation) of MEA on Anammox process.

Apart from organic solvents, heavy metals are also common in high-technology industrial wastewaters. Heavy metals are also common in various other nitrogen rich wastewater streams such as landfill leachate and livestock wastewater. Heavy metals are known to inhibit various microbes. Inhibition of nitrifying bacteria has been reported by chromium, nickel, copper, zinc, lead and cadmium [11]. The inhibition effects of heavy metals depend on various parameters such as type of metal, concentration of metal, concentration of biomass, and state of microbial growth [12]. The low or trace concentrations of heavy metals such as Cu, Zn, Ni, and Co, which are cofactors of enzymes, stimulate the growth and activities of microorganisms, while excessive concentrations of these metals cause inhibition or toxicity to microorganisms [12-14]. However, the effects (inhibition or stimulation) of heavy metals on Anammox process have not been discussed thoroughly [15]. Therefore, the objective of this study was to evaluate the effects of MEA and copper on the activities of Anammox bacteria to check the feasibility of Anammox process in treating nitrogen rich wastewater containing MEA or copper.

MATERIALS AND METHODS

1. Anammox Biomass Sludge

The biomass sludge was collected from a pilot

scale reactor treating semi-conductor wastewater in Hsinchu Science Park, Hsinchu, Taiwan to study the effect of MEA on the activities of Anammox bacteria. While, the biomass sludge collected from a full-scale landfill leachate treatment plant located in Bali, Taiwan was used to study the effect of copper on the activities of Anammox bacteria. The pilot scale reactor has been operated as completely autotrophic nitrogen removal over nitrite process and therefore the sludge contains aerobic ammonia oxidizing bacteria (AOB) and Anammox bacteria. The full-scale landfill leachate treatment plant has been operated as simultaneous partial nitrification, Anammox and denitrification process and therefore, the sludge collected had AOB, Anammox and denitrifying bacteria. The sludge biomass was washed three times with phosphate buffer ($0.14 \text{ g L}^{-1} \text{ KH}_2\text{PO}_4$ and $0.75 \text{ g L}^{-1} \text{ K}_2\text{HPO}_4$ and 0.5 g L^{-1} of KHCO_3) in order to remove the organic and nitrogenous substances. The sludge was kept in phosphate buffer solution before using it for the experiments.

2. Substrates, MEA and Copper

The two substrates for Anammox bacteria are ammonia and nitrite. In this study, NH_4Cl and NaNO_2 were used as the source of NH_4^+ and NO_2^- , respectively. Stock solutions of NH_4Cl (2300 mg N L^{-1}) and NaNO_2 (2300 mg N L^{-1}) in deionized water were prepared. Stock solutions of test solvent (MEA, 2000 mg L^{-1}) and heavy metal (CuSO_4 , 1000 mg L^{-1}) were prepared in deionized water.

3. Short-term Effect of MEA and Copper on the Activities of Anammox Bacteria

Specific Anammox activity (SAA) tests were conducted to study the short-term effect of MEA and copper on the activity of Anammox bacteria. The SAA tests were performed in serum bottles (67 mL) closed with gas-tight septum as described earlier [16]. In brief, each serum bottle was filled with 53.6 mL of sludge sample. The NH_4Cl (1.7 mL) and NaNO_2 (1.7 mL) from the stock solutions were added to each serum bottle. The final concentrations of $\text{NH}_4^+\text{-N}$ and $\text{NO}_2^-\text{-N}$ inside the bottles were 70 mg N L^{-1} , respectively. The MEA or copper was added to each serum bottle according to the experimental plan shown in Tables 1 and 2. The initial pH value of each serum bottle contents was 7.7. The liquid-phase and headspace of each serum bottle were flushed with argon gas to maintain the anaerobic condition during the test. The serum bottles were incubated in an incubator shaker at 125-150 rpm and $35 \text{ }^\circ\text{C}$ until stabilization (about 30 min). After initial equalization to the atmospheric pressure, each serum bottle was incubated in an incubator shaker at 125-150 rpm and $25 \text{ }^\circ\text{C}$ (otherwise stated). The headspace pressure inside the bottle was

measured periodically by using a manometer (Copal Electronics model PG-100N). The SAA ($\text{g N}_2 \text{g}^{-1} \text{VSS d}^{-1}$) was calculated from the N_2 gas production rate divided by the volatile suspended solids (VSS) concentration inside the bottle. All SAA tests were performed in duplicate. Additional sets of abiotic and negative controls experiments were also carried out for each test.

4. Activity Percentage and IC_{50}

The short-term effect of MEA or copper on Anammox was measured as percentage of activity and calculated using Eq. 2.

$$\text{SAA} (\%) = \frac{\text{SAA}_i}{\text{SAA}_0} 100 \quad (2)$$

where SAA_0 is the SAA in the control assay (in absence of MEA or copper) and SAA_i is the SAA of the tests with MEA or copper. The IC_{50} is the concentration of the tested compound, which corresponds to 50% activity compared with control assay. The IC_{50} values of MEA and copper were obtained by using Probit regression analysis.

5. Analytical Methods

Concentrations of MLSS and MLVSS were measured to calculate the SAA according to the Standard Methods (Method 2540 D and 2540 E, respectively) [17].

RESULTS AND DISCUSSION

1. Short-term Effect of MEA on the Activities of Anammox Bacteria

Figure 1 shows the inhibition of SAA at different concentrations of MEA. The initial SAA of the control sample (in absence of MEA) was $0.169 \text{ g N}_2 \text{g}^{-1} \text{VSS d}^{-1}$ and this was regarded as 100% Anammox activity at tested conditions. The SAAs were slightly decreased to 0.16 (5% inhibition) and $0.156 \text{ g N}_2 \text{g}^{-1} \text{VSS d}^{-1}$ (8% inhibition) in the presence of 30 and 70 mg L^{-1} of MEA, respectively. However, a sharp inhibition was observed at 150 mg L^{-1} of MEA and the Anammox activity decreased to 59% (41% inhibition). The Anammox activity was dropped down less than 10% (93% inhibition) when the concentration of MEA increased to 500 mg L^{-1} (115 mg N L^{-1}). The IC_{50} value

of MEA was calculated to be 175 mg L^{-1} using the Probit regression analysis.

An empirical noncompetitive inhibition model (described by Eq. 3) and a simple linear model were fitted to the experimental data (Fig. 2). The results suggest that both the noncompetitive inhibition model ($R^2 = 0.81$) and linear inhibition model ($R^2 = 0.87$) were fitted well with the experimental data. These models can be used to predict the inhibition of Anammox activity at any concentration of MEA. The value of K_i obtained from the noncompetitive inhibition model was 166 mg L^{-1} , which is very near to the IC_{50} value of MEA calculated by using the Probit regression analysis (175 mg L^{-1}).

$$\text{Inhibition} = \frac{100}{1 + \frac{K_i}{[I]}} \quad (3)$$

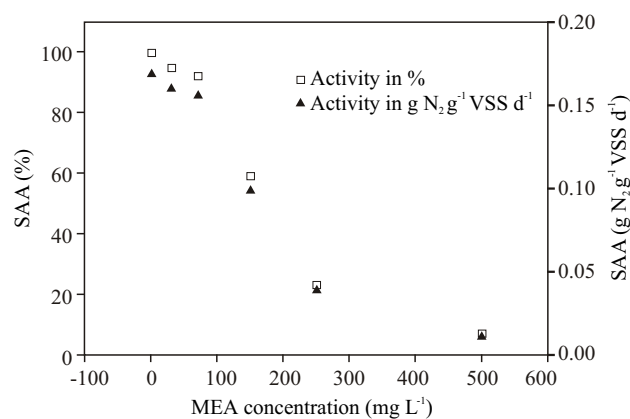


Fig. 1. Inhibition of Anammox activity caused by different concentrations of MEA.

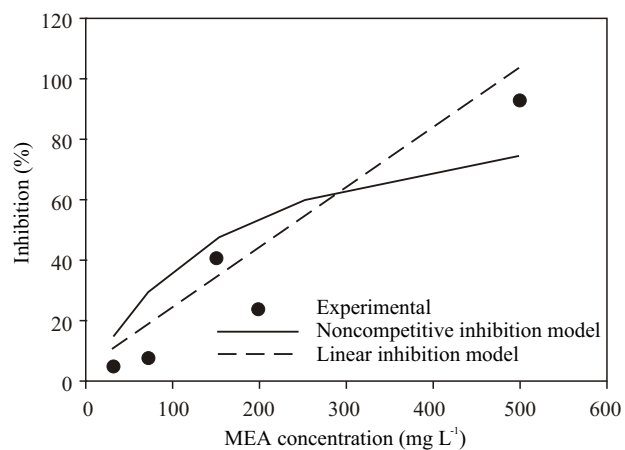


Fig. 2. Inhibition of Anammox activity as a function of MEA concentration: experimental data and best fit with noncompetitive inhibition and linear inhibition model.

Table 1. Experimental design to study the short-term effects of MEA on Anammox activity

Exp. set	Temperature ($^{\circ}\text{C}$)	MEA concentration (mg L^{-1})	$\text{NH}_4^+\text{-N}$ concentration (mg L^{-1})	$\text{NO}_2^-\text{-N}$ concentration (mg L^{-1})	MLVSS concentration (mg L^{-1})
A	25	0, 30, 70, 150, 250, 500	70	70	4000-5500

where inhibition is in %, $[I]$ is inhibitor (MEA) concentration in mg L^{-1} , K_i is half-inhibition coefficient [18].

MEA has both a primary alcohol and a primary amine group, which is slowly biodegraded to ammonia and acetaldehyde [9]. Further, degradation of acetaldehyde to acetate and ethanol has also been reported [10]. Dapena-Mora et al. [16] studied the short-term effects of acetate at different concentrations on Anammox activity. They observed 70% inhibition at 50 mM of acetate in batch tests [16]. Since, ammonia is one of the substrates of Anammox bacteria, the Anammox inhibition would be due to acetaldehyde or in other terms due to primary alcohol of the MEA.

The present study gives a basic idea about how Anammox reactor would behave if exposed to the MEA. A very high exposure of Anammox reactor by MEA would completely inhibit the Anammox bacteria, which should be avoided. Lin et al. [2] proposed a two-stage bioprocess (sequential hydrolysis reactor and Anammox reactor) to treat an organic nitrogen solvent. Similarly MEA should be treated in a separate reactor to avoid the inhibition of Anammox reactor. Moreover, the optimum conditions for Anammox activities should be maintained when treating wastewater containing MEA. However, further research is needed to evaluate the long-term effects of MEA on Anammox process.

2. Short-term Effect of Copper on the Activities of Anammox Bacteria

Copper is the most common heavy metal present in the nitrogen rich wastewater streams including leachate and swine wastewater. Therefore, before treating such wastewaters by Anammox process, it is important to study the effect of copper on Anammox activity. Copper is an important component of nitrite reductase enzyme in the Anammox bacteria and therefore, it is generally supplemented in the culture medium at trace levels to enhance the activities of Anammox process [14]. However, a higher concentration of copper can inhibit the Anammox activity by deactivating the nitrite reductase enzyme. As the inhibition effects of heavy metals depend on various

parameters, inhibitions at different concentrations of copper, initial biomass, and substrates were evaluated in this study. The inhibitions at different incubation temperatures and exposure time were also evaluated.

Figure 3 shows the effect of different copper concentrations on Anammox activity at various exposure times (Table 2, Experimental set I). The incubation temperature and biomass concentrations were $35\text{ }^\circ\text{C}$ and $> 2500\text{ mg VSS L}^{-1}$. It is quite evident from Fig. 3 that copper had stimulatory effects on Anammox activities at low concentrations ($< 5\text{ mg L}^{-1}$) and all exposure times (3-24 h). The SAAs were increased to 137 and 136% compared to negative control at copper concentrations of 1 and 2 mg L^{-1} , respectively after 24 h of exposure.

However, decreased in Anammox activities were observed at higher copper concentrations ($= 5\text{ mg L}^{-1}$) after 3 h of exposure. The SAA decreased to 57% at copper concentration of 10 mg L^{-1} after 3 h of exposure. The Anammox activities were recovered after longer exposure time (Fig. 3). The SAA in the presence of 10 mg L^{-1} of copper was recovered and reached to 105% after 24 h of exposure.

To see the influence of initial biomass concentration on Anammox activities under copper stress, experiments were repeated at MLVSS concentration of $< 2000\text{ mg L}^{-1}$ (Table 2, Experimental set II). Figure

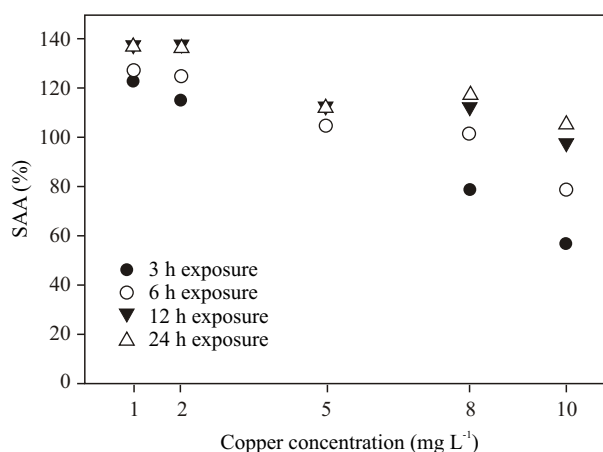


Fig. 3. Effect of different copper concentrations on Anammox activity at various exposure times.

Table 2. Experimental design to study the short-term effects of copper on Anammox activity

Exp. Set	Temperature ($^\circ\text{C}$)	Copper concentration (mg L^{-1})	$\text{NH}_4^+\text{-N}$ concentration (mg L^{-1})	$\text{NO}_2^-\text{-N}$ concentration (mg L^{-1})	MLVSS concentration (mg L^{-1})
I	35	0, 1, 2, 5, 8, 10	70	70	2700-3300
II	35	0, 1, 2, 5, 8, 10	70	70	1300-1900
III	25	0, 1, 2, 5, 8, 10	70	70	3500-4800
IV	15	0, 1, 2, 5, 8, 10	70	70	3000-3600
V	35	0, 5	0, 5, 10, 20, 30, 40, 50, 70, 90	0, 5, 10, 20, 30, 40, 50, 70, 90	3700-4400

4 data show that biomass concentration had significant impact on Anammox activity under copper stress condition. The Anammox activities significantly decreased to 53 and 9% in the presence of 2 and 8 mg L⁻¹ of copper, respectively after 24 h of exposure time (Fig. 4a). While at the same conditions (exposure time, 24 h; temperature, 35 °C; copper concentration, 2 and 8 mg L⁻¹), copper had stimulatory effect on Anammox activity (Fig. 4a). Figure 4b shows that the inhibition of Anammox activity caused by different concentrations of copper increased as exposure time increased at low biomass concentration (< 2000 mg L⁻¹). On the other hand, at higher biomass concentration (> 2500 mg L⁻¹), exposure time had positive effect on the inhibition of Anammox activity caused by different concentrations of copper (Fig. 3b).

The IC₅₀ value of copper was calculated to be 4.2 mg L⁻¹ using the results obtained from the experimental set II mentioned in Table 2. Lotti et al. [19] also studied the short-term effect of copper on Anammox activity and the IC₅₀ value of copper in their experiments was only 1.9 mg L⁻¹ [19], which is much lower compared to the results obtained in our study. In a recent study, the IC₅₀ value of copper for Anammox

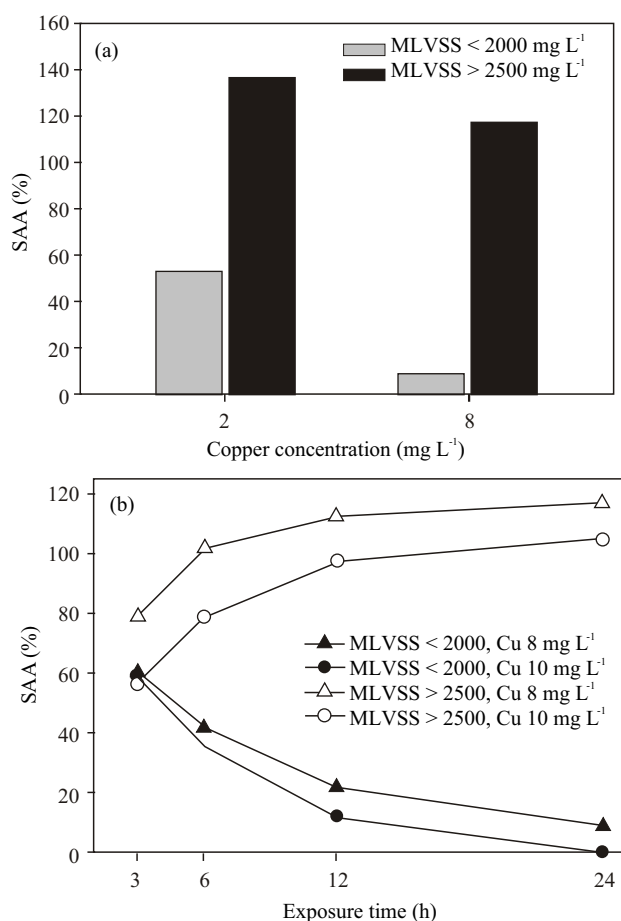


Fig. 4. Anammox activities under copper stress at different biomass concentrations (a) comparison of SAA after 24 h of incubation; (b) comparison of SAA at different exposure time.

mixed culture in a batch test was 12.9 mg L⁻¹ [14], which is much higher, compared to the results obtained by Lotti et al. [19] and our study. Use of different Anammox species, source of seed sludge, biomass concentration, copper exposure time, temperature, pH could be the most plausible reasons for these different levels of copper inhibition on Anammox activity. In case of experimental set III mentioned in Table 2, the IC₅₀ value of copper was calculated to be 9.0 mg L⁻¹. This difference between IC₅₀ values in case of experimental set II and III were due to the different conditions (temperature and biomass concentration). The IC₅₀ values for experimental sets I and IV were not calculated in the present study as inhibition was not observed in case of experimental set I, while complete inhibition was observed in case of experimental set IV.

Since temperature is one of the most important parameters to affect any biochemical process, its effect on Anammox activity under copper stress condition was evaluated at 35, 25 and 15 °C (Table 2, Experimental sets I, III and IV, respectively). At 15 °C, no Anammox activities were observed in control and tests (under copper stress) in batch tests. Figure 5 compares the effect of different copper concentrations on Anammox activity at 25 and 35 °C after 12 h of exposure time. The Anammox activities decreased as copper concentration increased in both temperatures. However, the effects of copper at tested concentrations (1-10 mg L⁻¹) were remained positive at 35 °C and negative at 25 °C compared to control. This could be explained as the optimum temperature range for the growth of Anammox bacteria has been reported to be between 30 and 40 °C [4,6]. At lower temperatures (below the optimum temperature range), the activities of metabolic enzymes of Anammox bacteria are reduce [20], therefore, lower Anammox activities are observed at 25 than 35 °C. This suggests that temperature would play an important role in copper containing nitrogen rich wastewater treatment by Anammox process.

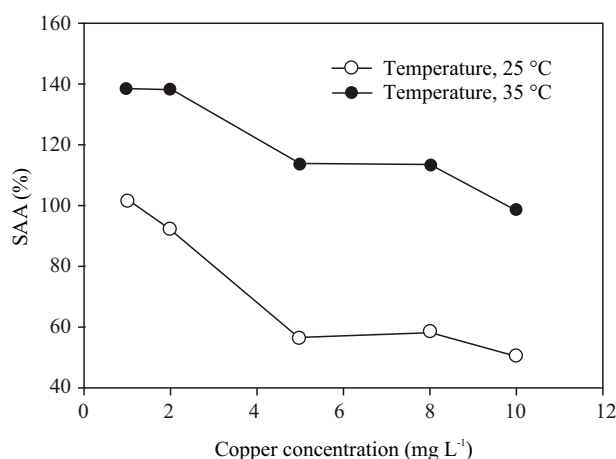


Fig. 5. Effect of different copper concentrations on Anammox activity at 25 and 35 °C.

The effects of initial substrate concentrations on Anammox activity under fixed copper concentration (5 mg L^{-1}) were also studied by varying the substrate (NH_4^+ and NO_2^-) concentrations between 0 to 90 mg N L^{-1} (Table 2, Experimental set V). The temperature was fixed at $35 \text{ }^\circ\text{C}$ and the headspace pressure was measured after 24 h of incubation. It is clear from Fig. 6 that gas pressure increased as initial substrate concentration increased in the serum bottle. At substrate (NH_4^+ or NO_2^-) concentration above 70 mg-N L^{-1} , the headspace pressure slightly decreased suggesting the substrate inhibition above this value.

The IC_{50} value of ammonium in batch tests reported by Dapena-Mora et al. is very high (770 mg L^{-1}) [16]. On the other hand, Strous et al. observed complete inhibition of Anammox activity at nitrite concentration of 100 mg-N L^{-1} [4]. Therefore, it could be concluded that the inhibition of headspace pressure was due to the nitrite concentration rather than ammonium in batch tests. However, compared to the control, the presence of copper (5 mg L^{-1}) significantly decreased the gas pressure in the serum bottles at all substrate concentrations tested in this study. The maximum headspace pressure was observed at 50 mg-N L^{-1} of nitrite and ammonium concentration, respectively. Overall, this study suggests that by maintaining the optimum conditions, treatment of copper containing nitrogen rich wastewater could be possible by Anammox process.

CONCLUSIONS

The effects of MEA and copper on Anammox activities were studied in short-term batch tests. The MEA, in all tested concentrations, inhibited the Anammox activity with IC_{50} concentration of 175 mg L^{-1} . The noncompetitive inhibition and linear inhibition models were fitted to the experimental data of

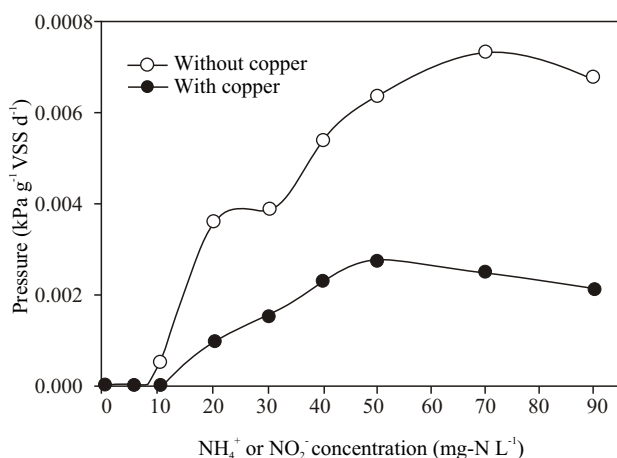


Fig. 6. Changes in gas pressure in the serum bottles at different substrate (NH_4^+ -N and NO_2^- -N) concentrations with or without copper inhibition.

Anammox inhibition caused by different MEA concentrations. In case of copper, its effect on Anammox activity depends on various parameters such as concentrations of copper, biomass and substrates, exposure time and temperature. At low metal concentrations and high biomass concentration ($> 2500 \text{ mg-VSS L}^{-1}$), copper had stimulatory effect on Anammox activity, while it had inhibitory effect at low biomass concentration ($< 2000 \text{ mg VSS L}^{-1}$). At lower temperature, copper had inhibitory effect on Anammox activity even at higher biomass concentration. Therefore, high biomass concentration and higher temperature (about $35 \text{ }^\circ\text{C}$) should be maintained in the reactor to treat copper containing nitrogen rich wastewater by Anammox process. However, further research is needed to study the long-term effects of MEA and copper on Anammox process.

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