Short-Term and Long-Term Inhibitory Effects of Copper on Anammox Process †

Cigdem Kalkan Aktan †*, Ayse Ekin Uzunhasanoglu †, Kozet Yapsakli † and Bulent Mertoglu 2

1 Department of Environmental Engineering, Faculty of Engineering, Marmara University, Goztepe, Istanbul 34722, Turkey; ekin.uzunhasanoglu@gmail.com (A.E.U.); kyapsakli@marmara.edu.tr (K.Y.)
2 Department of Bioengineering, Marmara University, Goztepe, Istanbul 34722, Turkey; bulent.mertoglu@marmara.edu.tr
* Correspondence: cigdemkalkan@marmara.edu.tr; Tel.: +90-533-724-06-10
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Abstract: The main goal of this study is to evaluate the short-term and long-term inhibitory effects of Cu (II) on Anammox process. To investigate the short-term inhibition level, four different concentrations (1, 2.5, 5 and 10 mg L−1 as Cu2+) were tested in batch reactors. IC50 levels for short-term exposure deduced as 4.57mg L−1 (R2: 0.97) from the modified non-competitive inhibition model. Lab-scale continuous flow up-flow fixed bed reactor with Kaldness biofilm carriers was operated 240 days with gradually increased Cu concentrations (from 0.2 to 8 mg L−1). To identify the IC50 levels in case of prolonged exposure of Cu(II), experimental data were fitted with a modified non-competitive inhibition model, and calculated as 6.77 mg L−1 (R2: 0.95). The results show that the IC50 level for copper in long-term exposure was higher than in short-term exposure and the possible reason for that is the self-adaptation of Anammox bacteria.

Keywords: anammox; copper; inhibition; nitrogen removal; heavy metal

1. Introduction

Nitrogen is the most common and important problem in industrial and domestic wastewater streams [1]. Therefore, there is an emerging concern to develop new technologies to get rid of nitrogen compounds [2]. For the past twenty-five years, there has been a rapid rise in the field of research about Anaerobic Ammonium Oxidation (Anammox) process which has a crucial potential to treat high nitrogen loaded wastewater streams. In the anammox process ammonium is oxidized to dinitrogen gas in anoxic conditions with hydrazine as an intermediate [3]. The Anammox process is found as promising alternative and cost-effective because of low aeration requirements and less sludge production comparing to conventional nitrogen removal process [4,5]. Due to the low growth rate with a doubling time 11 days (at pH 8) [6] of anammox bacteria efficient biomass retention is needed for the cultivation and enrichment period [7]. In order to provide high biomass concentration in the reactor systems, some solid support materials like non-woven biomass carriers [8,9], Kaldnes rings [10,11] or glass beads [12] were used in the literature.

Anammox process is applicable for especially ammonium-rich wastewaters such as landfill leachates [13,14], anaerobic digester rejects water [15,16], wastewater from semiconductor factories [17] and sludge liquors [18]. However, this process has been restricted by environmental and operational conditions due to vulnerable structure and slow growth rate of Anammox microorganisms [4,19]. Consequently, many studies have been focused on a great variety of inhibitory factors over the Anammox process in wastewater streams including organic matter [20], substrates [11,21,22], salts [23] etc. So far, however, little attention has been paid to inhibitory effects
of heavy metals [24–28] and little is known about the short and long-term effect of copper on Anammox activity.

Ammonium-rich wastewater streams such as landfill leachates may contain heavy metals at high concentration like; cadmium (Cd²⁺), chromium (Cr³⁺), copper (Cu²⁺), lead (Pb²⁺), nickel (Ni²⁺), and zinc (Zn²⁺) [29]. Although some of the heavy metals are fundamental for microbial cell production, they are also common inhibitors for biological processes in high concentrations. Although some specific heavy metals have been studied Cd(II), Cu(II), Ni(II), Zn(II), Hg, Pb, Ag on Anammox activity [24–28,30–34], there has been little agreement on inhibition doses of these heavy metals. However, the experimental data are rather controversial, and there is no general agreement about inhibitory concentrations. A number of studies have examined the short-term inhibition level of Cu(II) on Anammox activity. Even though there were some studies published about the short-term effects of Cu(II), little is known about the long-term response of Cu(II) on the Anammox process [26,30]. Consequently, it is necessary to evaluate the inhibition level of Cu on the Anammox process for both short and long-term exposure, and further studies should be carried out.

Therefore, the present paper aims to analyze the short and long-term effects of Cu on the Anammox system. In order to determine the short-term effect of Cu batch tests were conducted for 24 h. Lab-scale continuous flow up-flow fixed bed reactor was operated to investigate the effect of prolonged exposure of Cu on the Anammox process.

2. Material and Methods

2.1. Anammox Seed Sludge and Synthetic Wastewater

The seed sludge was taken from ongoing lab-scale up-flow column reactor that enriched over six years. Reactors inoculated with Anammox culture containing approximately 1000 mg L⁻¹ MLVSS concentration.

Synthetic wastewater was prepared to feed the reactors which composed macro and micronutrients that was described at Egli et al. [14] and contains 1:1.1 ratio ammonium to nitrite, as 100 mg L⁻¹ NH₄⁻N and 110 mg L⁻¹ NO₂⁻N for batch studies and for continuous reactor 300 mg L⁻¹ NH₄⁻N and 330 mg L⁻¹ NO₂⁻N. Besides, nitrate was added as a concentration of 50 mg L⁻¹ NO₃⁻N in order to ensure anoxic condition and to avoid decaying of Anammox biomass.

2.2. Experimental Setup Long-Term Experiment

For long-term experiments, a plexiglass continuous flow up-flow reactor was designed which has an active volume of 2.31 L with diameter 7 cm and height of 60 cm. The average flowrate of the system was 1800 mL d⁻¹ and hydraulic retention time was 1.28 d. The reactor was half full filled up with Type K1 Kaldnes rings packing material to provide abundant biomass retention. The carrier materials were provided by Anox Kaldnes Company and made of polyethylene (PEHD) material with nominal length and diameter 7.2 mm and 9.1 mm, respectively. The reactor was operated under 35 ± 1 °C to provide constant temperature water bath was used around the reactor. To enable the anoxic conditions inside the reactor 90% N₂ + 10% CO₂ gas combination was supplied from the bottom of the column. By this way, required inorganic carbon source was supplied by CO₂ that is found in the gas mixture.

2.3. Batch Studies

In order to determine the effect of short-term exposure of Cu(II) batch tests were carried out in side armed glass reactors with 100 mL total volume and 50 mL liquid phase volume. 100 mg/L NH₄⁻N and 110 mg/L NO₂⁻N were added to the mineral medium. To provide seed sludge for batch experiments, Anammox biomass was enriched in a sequencing batch reactor (SBR) operated at a sludge retention time of 50 days. After steady-state conditions and 90%, nitrogen removal efficiencies were achieved in the SBR, mixed liquor was taken from the reactor for batch inhibition studies. The reactors were inoculated with Anammox biomass approximately as 800 ± 100 mg L⁻¹ MLVSS concentration. The anoxic condition was provided by purging nitrogen gas inside the
reactors. The reactors were incubated at 35 ± 1 °C and 150 rpm. Batch system configuration is shown in Figure 1. Samples were obtained every 3 h and analyzed for ammonium and nitrite nitrogen during the 24-h period.

![Graph](image1.png)

**Figure 1.** (a) The effect of Cu on the removal efficiency of ammonium-nitrogen. (b) The effect of Cu on the removal efficiency of nitrite-nitrogen

2.4. Analytical Methods

High-Pressure Liquid Chromatography (HPLC) was used to determine nitrite and nitrate concentrations. The ammonium concentrations were measured by using Nesslerization method. Volatile suspended solids (VSS) and suspended solids (SS) measurements were done by using gravimetric methods according to standard methods [35].

Cu concentrations were measured using Perkin Elmer Analyst 400 Flame Atomic Absorption Spectrometry (AAS). In batch tests, copper concentrations were measured after 24 h exposure time.

2.5. Evaluation of IC50 Values

In order to calculate median inhibition concentration (IC50), both linear and nonlinear regression models were applied using GraphPad Prism 7 software. IC50 values were calculated based on total nitrogen (ammonium nitrogen + nitrite nitrogen) oxidation rate and modified non-competitive inhibition model was chosen.

- Modified non-competitive inhibition model
\[
I\% = 100 \times \left(1 - \frac{1}{1 + \left(\frac{HM}{a}\right)^b}\right)
\]

where \(I\%\) is the inhibition response, \(a\) is the concentration causing 50% inhibition in nitrogen removal rate, \(b\) is a fitting parameter.

NRR was calculated based on the fast initial rate and was calculated by using Equation (2).

\[
NRR \left(\frac{mgN}{mgVSS \; h}\right) = \frac{(TN_{(inf)} - TN_{(eff)})}{t \times VSS}
\]

where \(TN_{(inf)}\) and \(TN_{(eff)}\) were initial and final total nitrogen concentrations. Percent inhibition values were calculated according to Equation (3).

\[
\% \; inhibition = 100 - \left(\frac{NRR_{w/1HM} \times 100}{NRR_{blank}}\right)
\]

3. Results and Discussion

3.1. Short-Term Effect of Cu on Anammox

In order to evaluate the IC50 levels and nitrogen removal rates (NRR) for short-term responses, batch tests were performed by using increasing level of heavy metal concentrations. To monitor the ammonia nitrogen and nitrite nitrogen removal rates samples were taken every three hours and analyzed during the 24-h time period. Four different Cu metal concentration (1 ppm, 2.5 ppm, 5 ppm and 10 ppm) were tested to determine the level of inhibition and the effect of metal concentration on ammonia and nitrite nitrogen removal were shown in Figure 1.

To analyze inhibition level of heavy metal on Anammox bacteria various inhibition models were studied as non-competitive inhibition model, modified non-competitive inhibition model, linear model and 4-point logistic method (Figure 2). The consistency of the inhibition model to the experimental data was fitted with minimum squared errors method and it was found that modified non-competitive is the most representative model. Also, some researchers used the modified non-competitive model to express the heavy metal inhibition on the biochemical process [27,34,36,37]. Therefore, IC50 values for short-term inhibition was determined by modified non-competitive inhibition model.

IC50 levels for short-term exposure calculated as 4.57 mg L\(^{-1}\) (R\(^2\): 0.97 according to 95% confidence interval) from the modified non-competitive inhibition model based on total applied Cu concentration. The removal rate was calculated as 37 mg TN/mgVSS/h for the control samples, however, NRR was reduced to 11 mg Nmg\(^{-1}\) VSSh\(^{-1}\) for the 10 ppm Cu added samples. Figure 3 illustrates the decrease on the NRR with increasing Cu concentrations.

Pertinent literature concerning the inhibitory effect of Cu reveals the toxic effect of Cu on Anammox process. Lotti et al. [33] reported the heavy metal concentration which causes the 50% decrease in Anammox activity(IC50) as 1.9 mg L\(^{-1}\), on the other hand Zhang et al. reported that the short-term inhibition IC50 level as 32.5 mg L\(^{-1}\) and Van del Rio et al. [38] found for the same level of inhibition concentration as 19.3 mg L\(^{-1}\). The variability of the results may be explained by the different Anammox species that take role in the conversions. Therefore, Anammox species that exposed the heavy metals should be also determined for further analysis.
3.2. Long-Term Effect of Cu on Anammox

Prior the heavy metal exposure, continuous flow system was operated approximately one year and enrichment of the reactor was achieved with 94% nitrogen removal efficiency. Afterward, in order to test the long-term inhibitory effect of Cu on the reactor performance, the system operated for 240 days under gradually increased Cu loadings (Figure 4). Heavy metal concentration was increased stepwise from 0.2 mg L\(^{-1}\) to 8 mg L\(^{-1}\) and the average removal rate of nitrogen was found at 97% to 6 ppm Cu addition. The enhancement effect of Cu addition at low concentrations has been reported by many researchers [27,39]. In literature, the presence of some heavy metals at low concentrations play a crucial role to stimulate metabolism of microorganisms. Cu is one of the essential micronutrients for many enzymes of microorganisms. Chen et al. [39] reported that Cu concentration should be kept below 4 mg L\(^{-1}\) to provide good reactor capacity and the optimum dosage to improve the specific anammox activity indicated as 1.18 mg L\(^{-1}\). In our study, we observed the enhancement of the nitrogen removal rate until Cu concentration reached up to 6 ppm. As it was shown in Figure 5 the removal efficiency started to decrease after 180 days at 6 ppm Cu addition.

The nitrogen removal rate between 0.2 mg L\(^{-1}\) to 6 mg L\(^{-1}\) was calculated as 19.10 ± 0.60 mg NL\(^{-1}\) h\(^{-1}\) (according to 95% confidence interval) and after that point removal rates started to decrease. Figure 5b shows the nitrogen removal rates under stepwise increase of Cu concentrations. The nitrogen removal rates decreased to 2.2 mg NL\(^{-1}\) h\(^{-1}\) when Cu concentration reached up to 8 ppm.

To identify the IC50 levels for the long-term exposure of Cu the experimental data were fitted with the modified non-competitive inhibition model and calculated as 6.77 mg L\(^{-1}\) (R\(^{2}\): 0.95 according to 95% confidence interval) based on removal efficiency of nitrogen. Figure 5a shows the applied Cu
concentration versus the percent inhibition values according to modified non-competitive inhibition model.

![Figure 4. Influent and effluent ammonia and nitrite nitrogen profiles during Cu exposure.](image)

![Figure 5. (a) Relative Percent inhibition vs. Cu concentration (b) Nitrogen removal rates during long-term exposure.](image)

Yang et al. [26] reported that the IC50 value as 12.9 mg L\(^{-1}\) for the short-term inhibition, while they investigated the long-term inhibition in upflow anaerobic sludge blanket reactor and found 94% activity loss when 5 mg L\(^{-1}\) Cu added to the system. Zhang et al. [40] also report 65% reduction in NRR when Cu concentration was increased 5 to 8 mg L\(^{-1}\). For both of the studies, they observed higher tolerance to Cu in short-term exposures rather than long-term. In contrast to earlier findings, we have obtained higher median inhibition levels in long-term exposure. The possible reason for that starting from very low concentrations of Cu in our study may have provided suitable conditions for adaptation of the microorganisms.

4. Conclusions

Results have shown that the IC50 level on Anammox of short-term Cu effect was 4.57 mg L\(^{-1}\) while in long-term exposure the value increased to 6.77 mg L\(^{-1}\). This difference can be attributed to the self-adaptation of Anammox bacteria in the continuous flow reactor in a time period of 240 days.

**Author Contributions:** C.K.A. is the corresponding author of the paper. She is responsible from the experimental setup, AAS measurements, and evaluation of the IC50 values by using GraphPad Prism program. A.E.U. is a master student she was responsible from some analytical measurements like ammonium, volatile suspended solid, suspended solids and HPLC measurements. K.Y. is the advisor of this study and B.M. is the co-advisor. They are responsible from the examination and interpretation of the results.

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