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RESEARCH PAPER

The Role of Extracellular Polymeric Substances in Reducing Copper Inhibition to Nitrification in Activated Sludge

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Abstract This study presents the role of extracellular polymeric substances (EPS) in reducing the inhibitory effects of copper towards nitrifying activated sludge. EPS could ameliorate copper toxicity in activated sludge by binding metal ions. A series of copper inhibition experiments were conducted with activated sludge taken from laboratory-scale sequencing batch reactor (SBR) systems operated under different feast-famine cycles, which provided the different conditions to the EPS-producing heterotrophic populations. The toxicity of copper to both nitrifiers and heterotrophs decreased with increasing the feast-famine period. Average EPS contents were substantially higher in SBR sludge with longer feast-famine periods indicating that the decrease in the toxicity of copper at a longer feast-famine period was attributed to the presence of higher amounts of EPS. Comparison of the responses of nitrifiers and heterotrophs to free copper suggests that nitrifiers were no more sensitive to copper than heterotrophs.

Keywords: copper toxicity, nitrification, extracellular polymeric substances

1. Introduction

Autotrophic microbial oxidation of ammonia to nitrate is the key process in the removal of ammonia from wastewater. Because of the low growth rate of nitrifying bacteria and their sensitivity toward a wide variety of inorganic and organic compounds, nitrification process is often considered to be more susceptible to toxic compounds in wastewater. Nitrifying bacteria are sensitive to a wide variety of inorganic and organic chemicals [1-3]. Copper is one of the heavy metals that are known to inhibit the activity of nitrifiers [4,5]. However, copper is also known to be an essential micronutrient to all organisms as an important cofactor required for enzyme activity [6]. The toxicological response of nitrifying populations to copper has not been well understood and the information in the literature is contradictory. The reported concentrations of copper for the inhibition of nitrification in activated sludge varied from 1 to 40 mg/L, and the inhibition concentration of copper to nitrification was dependent on bioreactor type as shown in Table 1 [7-10]. Of nitrifiers, ammonia oxidizing bacteria (AOB) is known to be more susceptible to copper inhibition than nitrite oxidizing bacteria (NOB) [11].

One of the possible reasons for this large variability is that the concentration of the copper ions in bulk may be lowered by physical and chemical reactions, such as adsorption or complexation, in activated sludge [12,13]. Another reason may be due in part to the difference in biological conditions and community structures found in biological treatment process [14]. Nitrifying activated sludge established on domestic wastewaters consists of a complex mixture of microbial species of bacteria, yeast, fungi, protozoa and other higher trophic organisms. This complex nature of activated sludge can explain the differences between the degrees of inhibition with copper in pure culture and in sludge, and sometimes between

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Table 1. Inhibition effect of copper on nitrification in various bioreactor types

Bioreactor type	Concentration of copper (mg/L)	% inhibition	Reference
A_2O	20 ~ 40	40 ~ 97	[7]
CSTR	5 ~ 30	26 ~ 48	[8]
Biofilm	1	37	[9]
batch	16 ~ 34	20 ~ 80	[10]

sludges from different sources. However, most of the reported studies on the effect of copper on nitrification were based on results from either pure cultures or short-term batch mixed culture studies while the impact of heterotrophic communities present in mixed culture was rarely considered.

A large body of environmental literature demonstrates that substantial quantities of soluble metals can be adsorbed to extracellular polymeric substances (EPS) present in activated sludge processes [15-19]. Various types of bacteria including denitrifiers, polyphosphate-accumulating bacteria, and filamentous bacteria are known to produce EPS in wastewater treatment plant [20], and these EPSs have been shown to adsorb a variety of metal ions [16,21]. The EPS has a relatively high adsorption affinity towards Cu despite a slightly lower than Zn and Fe [22,23]. Friedman and Dugan [24] reported that *Zoogloea ramigera* 115, which produce an extensive extracellular zoogloeal matrix, can adsorb metals two times more than *Z. ramigera* I-16-M, which produces no zoogloeal matrix. Su *et al.* [17] showed that the biomass harvested from an activated sludge process with aerobic selector had significantly higher sorption capacity than did the biomass from a conventional system. A distinctive feature of the activated sludge morphology in aerobic selector systems was the presence of large amorphous zoogloeal colonies [25], which indicated that aerobic selector provided a favourable condition to the EPS-producing bacteria, *Z. ramigera* [26]. Ozdemir *et al.* [27] isolated EPS-producing bacterium, *Ochrobactrum anthropi* from activated sludge for biosorption tests. Dead cells of *O. anthropi* exhibited high adsorption capacity for chromium, cadmium, and copper. Also, it was reported that the EPSs produced from anaerobic sludge under sulfate-reducing conditions readily removed cadmium from solution through complexation [28].

The goal of this study was to investigate the role of EPS-producing heterotrophic populations on the inhibitory effects of copper toward nitrification. Based on the previous studies mentioned above, it was hypothesized that the presence of large quantities of EPS, which can bind soluble metals, will play a significant role in ameliorating copper toxicity in activated sludge. Specific objectives are (1) to investigate the toxic effects of copper on the performance of nitrifiers and heterotrophs grown in sequencing batch reactor (SBR) with different feast-famine cycles, (2) to quantify the abundance of EPS produced from the SBR depending on the feast-famine period, and (3) to estimate the inhibitory effects of free copper unbound to EPS on nitrifiers and heterotrophs.

Table 2. Medium composition of synthetic wastewater

Component	Concentration (mg/L)
Peptone	160
Meat extract	110
Urea	30
Potassium phosphate, dibasic (K_2HPO_4)	28
Sodium chloride (NaCl)	7
Calcium chloride ($CaCl_2 \cdot 2H_2O$)	4
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	2

2. Materials and Methods

2.1. Chemicals

All chemicals used in media preparation were reagent grade or better (Fisher Scientific, Pittsburgh, PA, USA and Sigma-Aldrich, St. Louis, MO, USA). Commercial measurement kits were used for ammonia and nitrate analysis (Hach Company, Loveland, CO, USA). All glassware, polyethylene and polypropylene plastic labwares were soaked in 10% HNO_3 (v/v) for at least 48 h before the use.

2.2. Bench-scale sequencing batch reactors (SBRs)

The activated sludge used in this study was obtained from a local domestic wastewater treatment plant in Choongnam province. The activated sludge was acclimated in bench-scale sequencing batch reactors (SBRs) by feeding synthetic wastewater of which composition is shown in Table 2. The bench-scale SBRs were made up of transparent acrylic material. The cylindrical SBRs had working volume of 4 L, and were stirred by impeller mixers. The dissolved oxygen (DO) concentration in each SBR was maintained above 5.0 mg/L by supplying air through an aquarium air pump and porous stone diffuser. Temperature in each reactor was controlled at 25°C with Visi-therm heaters (Mentor, OH, USA) in the reactor. The pH of the reactor was maintained in the range of 7.3 ~ 7.5 using a pH recorder/controller (Fisher Scientific, Pittsburgh, PA, USA) and base (0.2 N NaOH) addition. A mean cell residence time (MCRT) was maintained at 10 days in each reactor by daily wasting of activated sludge during aeration period of the SBR.

Three different feast-famine cycles (6, 12, and 24 h) were given to each SBR to find out the favourable conditions for EPS-producing heterotrophs by comparing the amount of EPS produced. This will give information about the role of

EPS on ameliorating copper inhibition towards nitrifying populations. Each SBR operation cycle consisted of fill, react, settle, and decant phases and the equivalent hydraulic retention time (HRT) in all SBRs was 24 h.

2.3. Copper inhibition test

Inhibition of copper to nitrifiers and heterotrophs was assessed in terms of changes in nitrification and TOC removal rates of the harvested activated sludge samples. Activated sludge samples were harvested from each reactor under steady state condition and transferred to a batch inhibition test vessel (250 mL Erlenmeyer flask). A steady state condition was defined when daily MCRT, aeration basin TSS, effluent nitrate and TOC concentrations, and nitrification rate did not vary more than 10% after elapsing three feast-famine cycles (6, 12, and 24 h). Activated sludge samples from the each SBR were washed and resuspended in the batch test vessels containing predetermined concentrations of copper. After exposing the activated sludge samples to copper for 1 h, 50 mL of either ammonium medium or organic medium was added to the test vessels to determine nitrification and TOC removal rates, respectively. The TSS concentrations in the test vessels were 1,000 ± 200 mg/L. Ammonium medium solution was prepared by dissolving 2.65 g $(NH_4)_2SO_4$ and 5.04 g $NaHCO_3$ in 1 L of deionized water. This medium was diluted 10 times in the vessels to give the final ammonia concentration of 56 mg N/L. Organic medium solution with TOC concentration at 140 mg/L had the same composition as the synthetic wastewater used for the SBR operation only excluding ammonium chloride. The test vessels were placed on a platform shaker (New Brunswick Scientific, Edison, NJ, USA) and continuously stirred at 125 rpm. One mL of sample was taken every hour, and the residual ammonium and TOC were measured after filtration using GF/C filter. The rates of ammonia oxidation (mg NH_4^+-N/h/g MLSS) and TOC removal (mg TOC/L/h/g MLSS) were determined by calculating the declining slopes of ammonia and TOC concentrations, which were used as indicators for nitrifying and heterotrophic activities, respectively. In nitrification inhibition test, the degree of inhibition was calculated as

$$\frac{R_0-R_t}{R_0}\times 100 = \%\ \text{Inhibition}$$

where R_0 is the nitrification rate of the control and R_t is the nitrification rate of reactors receiving copper. The same equation was used to measure heterotrophs inhibition rate.

2.4. Analytical methods

EPS was extracted from the activated sludge samples taken from each SBR reactor and was quantified by the alkaline extraction and ethanol precipitation method [29]. Fifty mL of samples were collected from each reactor and centrifuged at 3,500 rpm for 20 min. The supernatant was discarded and 20 mL of NaOH was added to the pellet. The samples were then agitated at 125 rpm for 5 h under room temperature condition and then were again centrifuged to recover the supernatant. Ethanol was added to a pink-colored and highly viscous extracts to give a final concentration of 60% (v/v) and the mixture was stored overnight in a refrigerator. A white cotton-like precipitate was then resuspended with small amount of water and then filtered through a GF/C filter. After drying the filter, the mass of remaining EPS extract was measured by weight difference to determine the EPS content in sludge (mg EPS/g MLSS).

TOC was measured with a DC-190 TOC analyzer (Tekmar-Dohrmann, Cincinnati, OH, USA). Mixed liquor suspended solids (MLSS) and Mixed liquor volatile suspended solids (MLVSS) were determined according to Standard Methods [30]. Nitrate and ammonia were measured by pre-set cadmium reduction method and Nessler method a Hach DR2010 Spectrometer (Hach Company, Loveland, CO, USA), respectively.

Concentrations of free copper ions were measured at the end of each batch inhibition test (feast-famine cycles of 6, 12, and 24 h) by Spectro Flame-EOP ICP (Spectro Analytical Instruments, Kleve, Germany). Equal aliquot of samples were collected from each reactor and filtered through pre-acid washed 0.45 μm filter and acidified for ICP measurement. The free copper ion activity was determined by an Orion model 94-29 cupric ion selective electrode (Cu-ISE, USA) and a model 90-02 Ag/AgCl double junction reference electrode (Orion, MA, USA). The solution pH was determined by an Orion pH meter/electrode (model 9105, MA, USA).

3. Results and Discussion

3.1. Copper inhibition in SBR depending on feast-famine cycle time

Once the operation of the SBR reactors reached a steady state, three SBRs with different feast-famine cycles were exposed to various concentrations of copper. The activated sludge harvested in each SBR was taken and the inhibition of copper to nitrifiers and heterotrophs were assessed by the inhibition test as mentioned above. Fig. 1 shows the short-term effects of copper on the activities of nitrifiers in SBR depending on the feast-famine cycle time. As the feast-famine cycle time increased from 6 to 24 h, toxicity curves shifted down implying that the tolerance of nitrifiers to copper increased. Fig. 2 shows the short-term effects of copper on the activities of heterotrophic populations. Similarly, the inhibitory effect of copper to heterotrophs

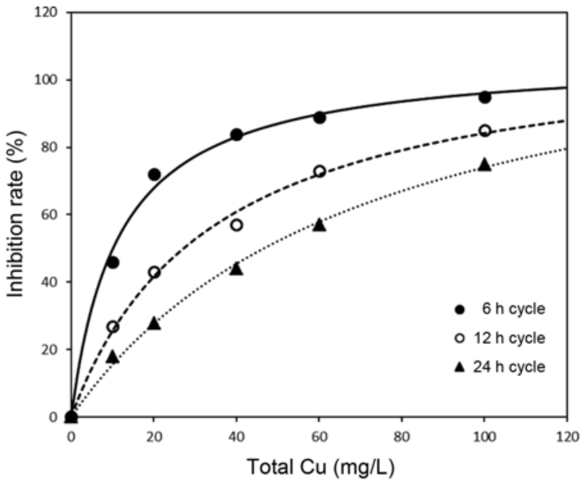

Fig. 1. Short-term effect of copper on the activity of nitrifiers in SBR.

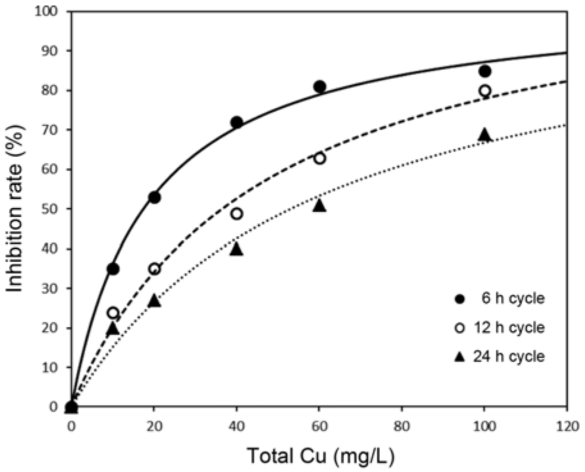

Fig. 2. Short-term effect of copper on the activity of heterotrophs in SBR.

Table 3. $IC_{50}{}^a$ values of copper for nitrifiers and heterotrophs

Activated sludge populations	Fill-and-draw cycle (h)		
	6	12	24
Nitrifiers	10.1	27.2	46.6
Heterotrophs	17.4	36.5	53.0

[a] The concentration of copper where the removal rates of ammonia or TOC were reduced by half, expressed as mg/L.

decreased with increasing the feast-famine period. Table 3 summarized the IC_{50} values of copper for nitrifiers and heterotrophs as a function of feast-famine cycles of SBR. IC_{50} values for nitrifiers ranged from 10.1 to 46.6 mg/L of total copper while those values for heterotrophs were between 17.4 and 53.0 mg/L. The values of IC_{50} were determined from the curves of observed percentage inhibition rate and its corresponding copper concentration, which were fitted to the nonlinear concentration-effect model using

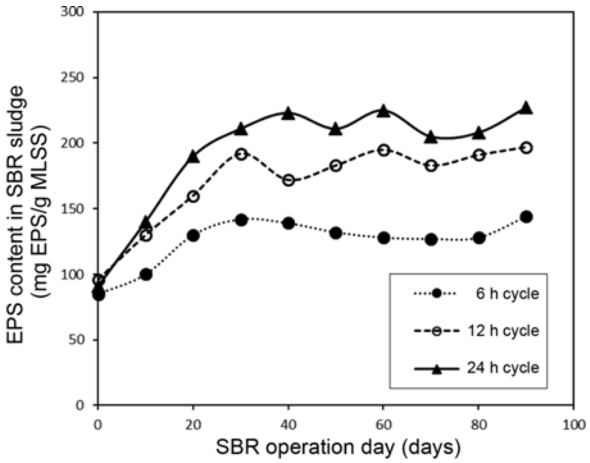

Fig. 3. EPS content in activated sludge of SBR systems at different feast-famine cycles.

Sigmaplot software v 10.0 (Systat software Inc., CA, USA). The results demonstrated that the toxicity of copper to both nitrifiers and heterotrophs decreased with increasing the feast-famine period. The IC_{50} values for heterotrophs were slightly higher than those for nitrifiers indicating that nitrifiers might appear to be a little more susceptible to copper than heterotrophs under short-term exposure conditions.

3.2. EPS content in the SBR depending on feast-famine cycle

In order to investigate the role of EPS for ameliorating copper toxicity in activated sludge, the EPS contents in the activated sludge samples taken from the SBRs were quantified by the alkaline extraction method. Fig. 3 compares the contents of EPSs in activated sludge of SBR operated with different feast-famine cycles. The EPS contents in SBR sludge gradually increased and levelled off after about 30 d of operation regardless of feast-famine conditions. Average EPS content in the SBR after 30 days of operation increased as the cycle period increased: 134.3, 187.6, and 215.7 mg EPS/g MLSS for the SBR with 6, 12, and 24 h cycles, respectively.

Many researchers found that the EPS-producing bacteria were enriched in activated sludge by employing a feast-famine condition [25,26,31,32]. It could be surmised that a longer feast-famine period provided a favourable condition to the EPS-producers in activated sludge leading to more production of EPSs, which in turn reduced the toxicity of copper to both nitrifiers and heterotrophs (Table 3). Various type of di- and tri-valent heavy metals including copper could be largely removed by adsorption to EPS so that toxicity of metals on microorganisms could be reduced [33]. According to Sheng *et al.* [13], protein and humic substances in EPS had thermodynamically favourable characteristics to bind copper ion.

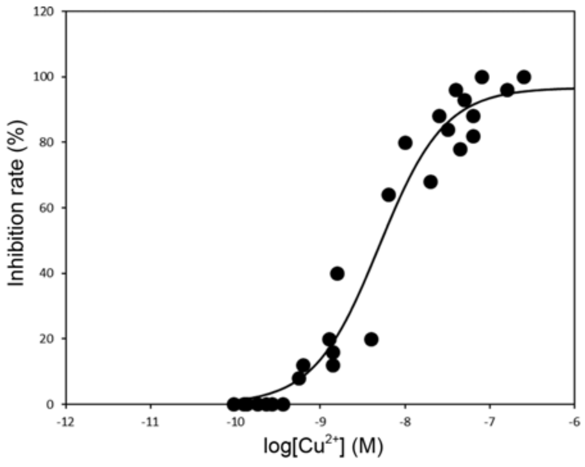

Fig. 4. Normalization of copper toxicity to nitrifiers as a function of free copper concentrations.

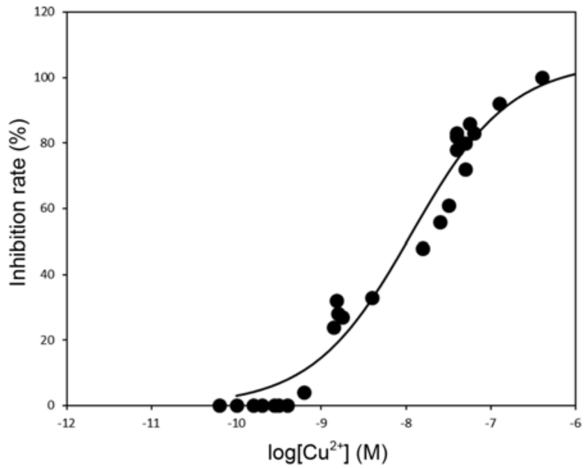

Fig. 5. Normalization of copper toxicity to heterotrophs as a function of free copper concentrations.

Relative to a conventional activated sludge process, biosolids produced from a system with feast-famine regimes (*e.g.*, aerobic selector, SBR, *etc.*) had a greater adsorption capacity for metals [3,17]. You *et al.* [3] demonstrated that SBR sludge had the higher adsorption capacity of heavy metals than A_2O (anaerobic-anoxic-oxic) process sludge. Su *et al.* [17] described that the EPS-producers were found more in activated sludge process with aerobic selector than conventional process and sludge in the former showed a better adsorption capacity of metals.

3.3. Effects of free copper on nitrifiers and heterotrophs

Since the toxicity of copper is directly correlated to the concentration of free copper rather than the concentration of total copper [34,35], free copper ion concentrations were measured after the toxicity test in each batch test set. Free copper ion represents the copper ion out of the EPS binding region. Free copper ion concentration measured by a selective ion electrode varied in the range between $10^{-10.5}$ and $10^{-6.5}$ M. Figs. 4 and 5 show the normalization of all copper toxicity data for nitrifiers and heterotrophs as a function of free copper ion concentrations, respectively. Inhibition of free copper on both populations exhibited a 'S-curve' behaviour, which clearly described severe inhibition of nitrifiers and heterotrophs took place at higher free copper ion concentration. It was interestingly found that the activities of free copper ions were dependent on the SBR operating conditions, *i.e.* feast-famine cycles, which also governed the content of EPS in activated sludge as discussed above.

Overall results demonstrated that the concentration of free copper and the percentage inhibition of both nitrifying and heterotrophic communities decreased with the increase in the feast-famine period. The IC_{50} values for free copper ions were determined at 5.3×10^{-9} and 9.9×10^{-9} M for nitrifiers and heterotrophs, respectively. Toxic responses of nitrifiers and heterotrophs to free copper concentration were statistically analyzed by F-tests with 0.05 confidence interval using GraphPad Prism v 4.0. The entire inhibition curves for nitrifiers and heterotrophs shown in Figs. 4 and 5 were analyzed. The responses of these two populations were not significantly different, which indicates that degree of inhibition by copper to nitrifiers was not significantly higher than heterotrophs. In general, nitrifiers are known to be more susceptible to copper than heterotrophs, however it might be attributed to lower population and less diversity of nitrifiers than heterotrophs in activated sludge. Furthermore, the inhibition of copper to both microbial groups could be reduced under a particular condition like more presence of EPS which plays a role of binding and inactivating copper ion in activated sludge.

4. Conclusion

A series of copper inhibition tests demonstrated that the inhibition of copper to nitrifiers was significantly dependent on the feast-famine cycle time in the SBR as well as copper concentration. As the feast-famine cycle increased, the contents of EPS in activated sludge increased, which in turn reduced copper toxicity to either nitrifiers or heterotrophs by lowering the activity of free copper ion concentration. The responses of nitrifiers and heterotrophs to copper ion were similar each other suggesting that nitrifiers were no more sensitive to copper than aerobic heterotrophs. The results of this study will enhance the level of understanding of the inhibitory effects of copper on nitrifying activated sludge and also provide meaningful engineering information such as operational strategy for the SBR to respond the inhibition of copper for both nitrification and organic removal by manipulating the feast-famine time.

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