Short-term and long-term effects of copper on the performance and microorganisms in sequencing batch reactor

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Abstract. The present work investigated the short-term and long-term impacts of copper ion (Cu²⁺) on the variation of microbial community structures and pollutant (carbon, nitrogen, and phosphorus) removal performance in sequencing batch reactor (SBR). The results showed that the short-term exposure (within 1 cycle) of 7 mg/L or higher concentration of Cu²⁺ decreased the removal efficiencies of dissolved organic carbon (DOC) and orthophosphate (PO₄³⁻-P), as well as the ATP content and dehydrogenase activity. In the long-term exposure test (30 days), the addition of Cu²⁺ at 3 mg/L significantly compromised the removal performance of DOC, ammonia-nitrogen (NH₄⁺-N) and PO₄³⁻-P. Higher production of intracellular reactive oxygen species (ROS) was observed along with increasing activities of catalase (CAT) and superoxide dismutase (SOD). The increase of lactate dehydrogenase (LDH) release was detected at the end of the experiment, indicating the occurrence of membrane damage and cell leakage. The microbial diversity and community structure changed with time, which would also be another important factor that affects the pollutant removal performance in SBR.

1. Introduction
The occurrence of shock load (i.e., heavy metals, salinity, hazardous materials) and abnormal operational events (i.e., loss of aeration, feed starvation, washout of biomass) in biological wastewater treatment systems would detrimentally affect its performance and stability for pollutant removal and thus pose potential threats to the water quality of receiving waters [1]. There have been a number of studies previously reporting the impact of heavy metals on the activated sludge systems [2-4], which mainly focused on the short-term effects on the water quality parameters, extracellular polymeric substances (EPS), microbial enzyme activities and toxicological indicators [5,6]. Previous research has shown that different heavy metals at different concentrations could have varied impact on the biological sewage treatment systems. Chen et al found that when Cu²⁺ concentration was higher than 40 mg/L, the removal rate of chemical oxygen demand (COD) in the sewage treatment system was lowered to <10%, indicating the compromised system performance [7]. Rong et al studied the tolerance of biological nitrogen removal system to heavy metals [8]. When Cu²⁺ concentration was above 0.5 mg/L, the removal of NH₄⁺-N was significantly affected and the nitrification process is inhibited. It was reported by Zheng et al that the short-term exposure of ZnO nanoparticles (NPs)
could lead to an increase of generation of reactive oxygen species (ROS) in the activated sludge [9]. Hou et al. found that the short-term exposure at 50 mg/L CeO$_2$ NPs could affect the ROS production and lactate dehydrogenase (LDH) release in sequencing batch biofilm reactors (SBBR) [10]. Madoni et al. found that the influence of heavy metals on microbial communities was Cd>Cu>Pb>Zn>Cr [11]. However, little is known on the mechanism of effects of heavy metals on inhibiting microorganisms and their close relationship with wastewater treatment efficiency. Meanwhile, few studies evaluated the difference between the short-term and long-term effects of heavy metals on the activated sludge systems, especially in terms of the variation of microbial community structures.

The aim of this study was to investigate the changes of pollutant removal performance, microbial activity and community structure in sequencing batch reactor (SBR) after short-term and long-term exposure of copper, as well as its recovery ability. The cytotoxicity of Cu$^{2+}$ to activated sludge was characterized based on the generation of ROS and the activities of antioxidant enzyme activities (AEA) including catalase (CAT) and superoxide dismutase (SOD). The ATP content and release of LDH were used as indicators to evaluate the effects of Cu$^{2+}$ on microbial activity. High-throughput 16s rRNA gene amplicon sequencing was used to evaluate the effect of Cu$^{2+}$ on microbial community structure.

2. Methods and material

2.1. Setup and operation of SBRs

Activate sludge collected from a local municipal wastewater treatment plant (WWTP) in Xi’an City (Shaanxi province, China) was used as the seeding sludge for the SBRs. The reactors were operated in 6-hour cycle, including filling, aeration, settling, withdrawal and idle for 50, 240, 50, 10 and 10 min, respectively. The airflow rate was controlled to maintain the dissolved oxygen (DO) level in SBRs above 2.0 mg/L during aeration cycle. The sludge retention time (SRT) of the sludge was kept at 10 d. During the entire test, the reactors were operated at 25°C constantly. The synthetic wastewater was composted of glucose, NH$_4$Cl, KH$_2$PO$_4$ and trace element, with the average COD, NH$_4$$^+$-N and PO$_4^{3-}$-P of 650, 30 and 11 mg/L, respectively. Then, wastewater containing 0 (control), 1, 3, 5, 7, 10, and 25 mg/L Cu$^{2+}$ were prepared by adding the certain Cu$^{2+}$ stock solution into the synthetic wastewater. In the long-term exposure test, Cu$^{2+}$ was continuously added to the system for 30 days as the shock phase, and then the addition of Cu$^{2+}$ was stopped and run for additional 60 days as the recovery phase.

2.2. Analysis methods

Water samples were collected and analyzed for dissolved organic carbon (DOC), NH$_4$$^+$-N, NO$_2$$^-$-N, NO$_3$$^-$$-N$, PO$_4^{3-}$-P, MLSS and MLVSS at the end of each cycle using standard methods. The intracellular ROS production was measured according to Wu et al [12] and the same analysis kits (Beyotime Institute of Biotecnology, China) were used. LDH activity and ATP content was determined by using LDH cytotoxicity detection kit and ATP assay kit (Beyotime Institute of Biotecnology, China), respectively. The activities of CAT and SOD were measured by CAT and SOD assay kits provided by Jiancheng Bioengineering Co. Ltd. (Nanjing, China). The protein contents were examined by the BCA protein assay kit (Sangon Biotech, China).

2.3. DNA extraction and high-throughput sequencing

Total DNA of the sludge samples were extracted using an UltraClean Microbial DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) according to the manufacturer’s protocol. The extracted DNA was qualified on 1.0% agarose gel and quantified on a Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA) using a Qubit dsDNA BR Assay Kit. Then, paired-end sequencing (2×250 bp) was conducted on the Illumina MiSeq platform by BGI Tech Solutions Co., Ltd. (Shenzhen, China). Processing and analysis of the sequencing data was performed using Mothur (v.1.31.2) and QIIME pipeline (v1.8.0). All raw reads were preprocessed to eliminate the adapter pollution and low quality reads. Clean reads were then merged and clustered to operational taxonomic units (OTU) at 97%
sequence similarity.

3. Results and discussion

3.1. Effect of short-term shock of Cu\(^{2+}\) on performance and microorganisms

Short-term shock loading tests were conducted in beakers using matured sludge from the SBRs after their performance was stabilized. After taken from the reactors during the idle period into the beakers, the sludge were washed using tap water and then synthetic wastewater for three times. Afterwards, the sludge experienced similar cycle procedures as in the SBRs with a test time of 6 h. The Cu\(^{2+}\) stock solution was added into the beakers, ensuring the final concentrations of Cu\(^{2+}\) to 0, 1, 3, 5, 7, 10, and 25 mg/L, respectively. Figure 1(a) shows that when the Cu\(^{2+}\) shock concentration was less than 7 mg/L, the concentration of DOC, NH\(_4^+\)-N and PO\(_4^{3-}\)-P in the effluent of the SBR system were relatively low, and the removal efficiencies were not affected substantially. However, when the Cu\(^{2+}\) shock concentration was above 10 mg/L, the effluent PO\(_4^{3-}\)-P concentration increased by 30%. It indicated that when the SBR system was exposed to short-term shock of Cu\(^{2+}\) at 10 mg/L or higher, the activities of the polyphosphate accumulating organisms (PAOs) was most likely to be suppressed, thereby reducing the ability of the system to remove PO\(_4^{3-}\)-P. Similar results were also reported by Wang et al. [13] that the abundance of PAOs decreased significantly under the influence of Cu\(^{2+}\). Upon exposure to 25 mg/L Cu\(^{2+}\) for 6 hours, the effluent DOC concentration increased to 20 mg/L and the removal efficiency reduced by 18%. However, there is no significant change observed in NH\(_4^+\)-N removal efficiency (95%) with the increased dose of Cu\(^{2+}\).

![Figure 1](image_url)

**Figure 1.** Effects of Cu\(^{2+}\) on the variations of DOC, NH\(_4^+\)-N, PO\(_4^{3-}\)-P (a), ROS production and ATP content (b).

When microorganisms experienced certain environmental stress, the rate of ROS generation will increase, which will thereby induce oxidative stress in the cells, and lead to cytotoxicity and damage of membranes [14]. To eliminate the excess ROS, antioxidant enzymes (i.e., SOD and CAT) would be produced as the self-defense mechanism of the cell. ATP, as the most important energy currency molecule of the cells, plays a critical role in various physiological processes. ATP content can be therefore used as an indicator of viable biomass and the microbial activity [15]. As shown in figure 1(b), similar to the trends of water quality parameters, there was no significant change in intracellular ROS level when the Cu\(^{2+}\) concentration was less than 7 mg/L. When the Cu\(^{2+}\) concentration increased to 25 mg/L, the ROS level increased to about 10 times than that in control group. At the same time, the ATP content gradually decreased with the increasing Cu\(^{2+}\) level. At 10 mg/L of Cu, the ATP content decreased to 40% of the control group. The results showed that the short-term shock of Cu\(^{2+}\) above certain concentration induced oxidative stress to microorganism, causing the increase of ROS production and the decrease of ATP content. The similar trend was observed previously on the effect
of nanoscale zero-valent iron particles (NZVI) on biological nitrogen and phosphorus removal and microorganisms [12] that the increasing NZVI concentration resulted in the increasing ROS production but decreasing ATP. Generation of ROS thereby induces oxidative stress in the cells, which could damage cell membranes, leading to cellular injury and reduced metabolic activity [16]. Consequently, less ATP will be needed to maintain the cell activity, which in turn, could lead to the compromised pollutant removal performance.

3.2. Effect of long-term shock of Cu\(^{2+}\) on performance
Based on the results of the short-term exposure test, the concentration of Cu\(^{2+}\) for the long-term exposure test was set to 3 mg/L. As shown in table 1, the continuous dosing with low concentrations of Cu\(^{2+}\) inhibited the ability of microorganisms to degrade organic matter. On the 15th day of shock phase, the effluent DOC concentration increased and the removal efficiency decreased by 7%. The effluent NH\(_4^+\)-N concentration significantly increased, and the removal efficiency decreased to 24% on the 30th day of shock phase. This indicates that the long-term exposure of Cu\(^{2+}\) strongly inhibited the activity of ammonia oxidizing bacteria (AOB), resulted in the accumulation of NH\(_4^+\)-N in the system. Meanwhile, the PO\(_4^{3-}\)-P removal efficiency was significantly inhibited on the 15th day of shock phase. The data on the 60th day of recovery phase showed that the system removal capacity for DOC, NH\(_4^+\)-N and PO\(_4^{3-}\)-P were gradually recovered. However, the activity of nitrite oxidizing bacteria (NOB) was not fully recovered, resulting in the accumulation of nitrite in effluent (data not shown).

| Table 1. Pollutant removal performance in different phases (unit: mg/L). |
|----------------------|----------------------|----------------------|----------------------|
| Phase               | Initial              | Shock               | Recovery             |
|                     |                      | 15\(^{th}\) | 30\(^{th}\) | 60\(^{th}\) |
| DOC                 |                      |                      |                      |
| Influent            | 160.00               | 7.34                | 18.34                | 15.67                | 13.63                |
| Effluent            | 18.34                | 15.67               | 13.63                |
| Removal efficiency (%) | 95.41              | 88.54               | 90.21               | 91.48               |
| NH\(_4^+\)-N        |                      |                      |                      |
| Influent            | 30.00                | 1.34                | 19.58                | 22.75                | 3.64                |
| Effluent            | 19.58                | 22.75               | 3.64                |
| Removal efficiency (%) | 95.53              | 88.54               | 90.21               | 91.48               |
| PO\(_4^{3-}\)-P     |                      |                      |                      |
| Influent            | 5.00                 | 0.18                | 4.02                | 2.20                | 0.93                |
| Effluent            | 4.02                 | 2.20                | 0.93                |
| Removal efficiency (%) | 96.40              | 19.60               | 56.00               | 81.40               |

3.3. Effect of long-term shock of Cu\(^{2+}\) on microbial enzymes
As shown in figure 2(a), the intracellular ROS level gradually increased during the shock phase. On the 15th day of shock phase, ROS level increased to about 3 times higher than that of control group. The corresponding SOD and CAT activities were increased by 231% and 131%, respectively. There is no inhibition of antioxidant enzyme activities observed in this study, which may be related to the continuous but low dose of Cu\(^{2+}\) that alleviating its negative impacts on the antioxidant enzymes [17]. The release of LDH was used as an important indicator of cell membrane integrity. As shown in figure 2(b), LDH release reached to 286% of the control group after 30 days of Cu shock, indicating that the long-term exposure of Cu\(^{2+}\) in the system not only inhibited the growth and metabolic activities of microbial cells, but also resulted in damage to cell integrity. Combined with short-term shock results, it can be concluded that the cytotoxicity of heavy metal to microorganisms in the activated sludge was highly time-dependent. The inhibitory effect on the system's functional enzymes is recoverable.
3.4. Effect of long-term shock of Cu$^{2+}$ on community structure

Alpha diversity analysis based on 16S rRNA gene sequencing showed that the number of OTUs was significantly reduced from 565 to 265 on 30th day of shock phase (table 2). Chao1, ACE, Shannon and Simpson indices displayed a drastically lower richness and evenness in the shock phase compared to the control. The results of community structure analysis showed that the distribution of microbial communities at the genus level had a significant change after Cu$^{2+}$ shock. As showed in figure 3, a total of 49 genera were identified in the sludge samples, 12 of which were dominant. As the most dominant genus, the relative abundance of Zoogloea decreased significantly on the 15th day of shock phase. Zoogloea has been considered as the typical activated sludge bacterium with nitrite reduction capability and responsible for the formation of activated sludge flocs. In contrast, genus of Ca. Xiphinematobacter and Sphingobium increased significantly on the 15th day after shock. Dechloromonas also significantly increased on the 30th day of shock phase. Similar trend was also previously reported [18] that the multi-walled carbon nanotubes (MWCNTs) reduced the abundance of Bacteroidetes and Nitrosomonas in the studied activated sludge system.

### Table 2. Summary of alpha diversity analysis.

| Samples               | OTUs | Chao   | ACE    | Shannon | Simpson |
|-----------------------|------|--------|--------|---------|---------|
| Before the shock      | 565  | 676.44 | 669.35 | 3.95    | 0.047   |
| 6h after shock        | 626  | 709.90 | 702.06 | 4.46    | 0.025   |
| 15th day of shock     | 226  | 317.44 | 419.09 | 2.16    | 0.228   |
| 30th day of shock     | 265  | 304.20 | 304.87 | 2.70    | 0.160   |

![Figure 3. Taxonomic classification of the 16S rRNA gene sequencing data at genus level.](image)
4. Conclusions
This study investigated the effects of short-term and long-term exposure of Cu\(^{2+}\) on the pollutant removal performance, microbial enzyme activities, and the microbial community structures in sequencing batch reactor (SBR). Short-term exposure test showed that after a high dose of Cu\(^{2+}\) (>7 mg/L), the removal efficiencies of DOC and PO\(_4^{3-}\)-P decreased, along with the increase of ROS production and decrease of the ATP content. Long-term exposure test showed that the continuous addition of Cu\(^{2+}\) at 3 mg/L also reduced the removal efficiencies of DOC, NH\(_4^+\)-N and PO\(_4^{3-}\)-P. According to the ROS, CAT, SOD and LDH results, the exposure of Cu\(^{2+}\) not only inhibited the microbial activities but also caused the damage to cell integrity. According to the high throughput 16S rDNA sequencing results, the richness and diversity of microbial communities were significantly reduced after long-term exposure to Cu\(^{2+}\). Among all dominant bacterial genera, Zoogloea and Flavobacterium were most sensitive to Cu\(^{2+}\) exposure. The main cause for these phenomena may be related to the toxicity of heavy metals that inhibiting different types of microbial enzymes, resulting in the changes of microbial species, functional genes and community structure in the system.

Acknowledgments
The project was supported by National Natural Science Foundation of China (No. 51409209 and 31600421), Natural Science Basic Research Plan in Shaanxi Province of China (No. 2017JM5070), China Postdoctoral Science Foundation (No. 2014M562439), Shaanxi Postdoctoral Science Foundation, Scientific Research Program Funded by Shaanxi Provincial Education Department (No. 17JS097) and The Open Project of State Key Laboratory of Eco-hydraulics in Northwest Arid Region of China (Xi’an University of Technology) (No. 2016KFKT-2).

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