The effects of mercury shock on the performance and microorganisms in biological wastewater treatment process

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Abstract. In this study, the effects of Hg²⁺ shock on the removal efficiencies for organic matters and nutrient, enzyme activities, and microbial community structures in lab-scale sequencing batch reactor (SBR) were investigated. The results showed that the short-term exposure (6 h) to Hg²⁺ at 0.05 mM significantly reduced the removal efficiencies of dissolved organic carbon (DOC), ammonium-nitrogen (NH₄⁺-N) and orthophosphate (PO₄³⁻-P). At lower concentration (0.01 mM) and prolonged (30 days) exposure condition, NH₄⁺-N removal was severely inhibited, and it was recovered after 15 days of restoration. Higher levels of reactive oxygen species (ROS), catalase (CAT) and superoxide dismutase (SOD) activities were detected in the Hg²⁺ shocked reactor and eventually restored to the control level after 60 days of restoration. No increase in the release of lactate dehydrogenase (LDH) was observed under both short-term and long-term shock conditions, indicating no irreversible damage to the cell membrane. The relative abundance of genus Zoogloea and Paracoccus were decreased after Hg²⁺ shock, which implies these microorganisms may be sensitive to heavy metal exposure.

1. Introduction

In the full-scale biological wastewater treatment processes, the activated sludge system may suffer from unregulated discharge of heavy metals [1,2]. Heavy metals, such as Cu²⁺, Pb²⁺, Cd²⁺, Hg²⁺, etc., enter wastewater from a variety of sources including chemical, metallurgical, electronic, pharmaceutical industries and domestic waste inputs [3]. High levels of heavy metals would inhibit the activities of the functionally relevant organisms, change the microbial community structures, and eventually affect the performance and stability for pollutant removal [4]. Many researches have shown that different heavy metals at different exposure levels could have various effects on the biological treatment systems. For example, Wang et al [5] found that short exposure time (24 h) or low dosage (5 mg/L) of Mn²⁺ could promote nitrification, while prolonged reaction time (48 h) or raised dosage (40 mg/L) reversed the effect to inhibition. Ong et al [6] noted that the removal of dissolved organic carbon (DOC) decreased with the increasing Ni²⁺ concentration in wastewater. Wu et al [7] studied the effects of nano zerovalent iron (nZVI) particles on the biological nutrient removal (BNR) process, and observed the increase of reactive oxygen species (ROS), release of lactate dehydrogenase (LDH) and...
decrease of ATP contents in the reactor, indicating the adversely effects of nZVI on microorganisms. Mowat [8] found that the microbial toxicities of heavy metals at 20 mg/L for 5 d were in the following order: Hg>Ag>Cr³⁺>Fe³⁺>Cu>Ni>Cd>Co>Cr⁶⁺>Sn>Zn. Bing [9] reported that Cu²⁺ and Hg²⁺ shocks had noticeable impacts on microbial community structures in biological systems and decreased the microbial diversities.

However, previous studies have primarily focused on the short-term impacts of heavy metals on effluent quality parameters, microbial enzyme activities, and toxicity indicators in activated sludge systems [10-13], the number of studies that investigated the impacts in a long-term perspective considering shock, adaption and restoration phases are very scarce.

In this study, the short-term and long-term operation of lab-scale sequential batch reactor (SBR) were performed with the goals to evaluate the impact of Hg²⁺ shock on contaminant removal performance (including DOC, ammonium-nitrogen (NH₄⁺-N) and orthophosphate (PO₄³⁻-P)), microbial activity, and community structure, as well as its resilience. The cytotoxicity of Hg²⁺ on activated sludge was studied by measuring the intracellular ROS production and the activities of antioxidant enzymes such as catalase (CAT) and superoxide dismutase (SOD). The effects of Hg²⁺ on microbial activity were evaluated by using soil dehydrogenase activity (sDHA), ATP content and LDH release as indicators. High-throughput 16S rRNA gene amplicon sequencing was used to investigate the changes in microbial community structure during the shock, adaptation and restoration phases.

2. Methods and material

2.1. Setup and operation of SBR reactor

Two self-built SBR systems with 4 L of working volume were used in this study (one as the control/blank reactor, the other as the Hg²⁺ shocked reactor). Activated sludge collected from NO.3 Municipal Wastewater Treatment Plant of Xi’an was used as the seeding sludge and mixed with the synthetic influent wastewater at a ratio of 1:1. The reactors were operated in a 6-hour cycle, including filling/anaerobic (50 min), aeration (240 min), settling (50 min), withdrawal (10 min) and idle (10 min) phases. The sludge retention time (SRT) was kept at ~10 d in each reactor. The concentration of mixed liquor suspended solids (MLSS) was maintained at ~4000 mg/L. The airflow rate was controlled to maintain the dissolved oxygen (DO) level in SBRs above 2.0 mg/L during aeration cycle. During the whole test, the pH and temperature in SBRs were constantly operated at ~7.0 and ~25°C. Glucose, ammonium chloride and potassium dihydrogen phosphate were used as carbon, nitrogen and phosphorus sources in the synthetic influent wastewater (table 1).

| Compounds | Concentration (mg/L) | Compounds | Concentration (mg/L) |
|-----------|----------------------|-----------|----------------------|
| C₆H₁₂O₆  | 813 (COD 650)       | MgSO₄     | 25                   |
| NH₄Cl     | 230 (NH₄⁺-N 30)     | FeSO₄     | 5                    |
| KH₂PO₄    | 50 (PO₄³⁻-P 5)      | CaCl₂     | 140                  |
| NaHCO₃    | 700                  | Trace element solution | 1 mL |

2.2. Analysis methods

Water quality parameters such as DOC, NH₄⁺-N, PO₄³⁻-P, MLSS and MLVSS were measured in accordance with Standard Methods. The intracellular ROS production, ATP content, LDH release and activities of sDHA, CAT and SOD in the Hg²⁺ shocked reactor were measured by using specific commercial kits, and represented as percentage of that in the blank reactor. All the analyses were run in at least duplicate.

2.3. DNA extraction and high-throughput sequencing

The DNA extraction method in the sludge sample was extracted using the PowerSoil® DNA
Extraction Kit. The extracted DNA was assayed for concentration using a Qubit dsDNA BR assay kit and a Qubit 2.0 fluorometer (Invitrogen, Life Technologies, Grand Island, NY, USA) and integrity was detected by 1% agarose gel electrophoresis. The DNA fragment for quality control analysis was subjected to 2×250 bp double-end sequencing using an Illumina MiSeq high-throughput analyzer (BGI Tech Solutions Co., Ltd., Shenzhen, China). Bioinformatics processing and statistical analysis of sequencing data were mainly implemented using Mothur (v.1.31.2) and QIIME (v1.8.0) software.

3. Results and discussion

3.1. Effect of short-term Hg$^{2+}$ shock

The short-term shock tests were conducted with activated sludge from the SBR reactor when performance is stable. The stock solution of Hg$^{2+}$ was added to the beaker to ensure a final Hg$^{2+}$ concentration of 0, 0.02, 0.05, 0.08, 0.11, 0.16 and 0.39 mM, respectively. The activated sludge experienced similar 6-hour cycle procedures as in the reactor. As shown in figure 1(a), all the removal efficiencies, including DOC, NH$_4^+$-N and PO$_4^{3-}$-P, drastically decreased when Hg$^{2+}$ concentration was 0.05 mM. Both DOC and PO$_4^{3-}$-P removal were completely inhibited when Hg$^{2+}$ concentration reached to 0.08 mM. The NH$_4^+$-N removal efficiency reduced to 14% when Hg$^{2+}$ concentration increased to 0.39 mM. It is indicated that, under the short-term shock with elevated Hg$^{2+}$ concentration, the activities of ordinary heterotrophic organisms (OHOs) and polyphosphate-accumulating organisms (PAOs) might be more easily inhibited compared to the activities of ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB).

![Figure 1](image_url)  
**Figure 1.** Effects of short-term Hg$^{2+}$ shock on (a) removal efficiencies of DOC, NH$_4^+$-N and PO$_4^{3-}$-P, (b) sDHA activity, ATP content, (c) Relative ROS production, and (d) LDH release.

The sDHA activity can reflect the degradation capacity of active microorganisms on organic matters in the system [14]. As shown in figure 1(b), the relative sDHA activity reduced to 70% at Hg$^{2+}$ shock concentration of 0.08 mM, and was undetectable at 0.11 mM and 0.39 mM, which is consistent
with the trend of DOC removal. As an important molecule that provides energy to living cells, ATP can be used as an indicator of biomass and microbial activity. Figure 1(b) shows that the ATP content significantly reduced to 18% at Hg²⁺ shock concentration of 0.05 mM, and was undetectable when shock concentration above 0.16 mM. Similar results were also reported by Wu et al [7] when studying the effects of nZVI particles on activated sludge and microorganisms. As shown in figure 1(c), the intracellular ROS production gradually increased with the increase of Hg²⁺ concentration. When Hg²⁺ concentration reached to 0.08 mM, the ROS production rapidly increased to 10 times that of the control. And it increased 38 times when Hg²⁺ concentration increased to 0.39 mM, which will cause oxidative damage to the cells. However, the LDH test (figure 1(d)) showed there is no detectable damage to microorganisms’ cell integrity by Hg²⁺.

3.2. Effect of long-term Hg²⁺ shock on performance

Based on the results of the short-term shock test, the Hg²⁺ concentration in the long-term shock test was set at 0.01 mM. As shown in table 2, after 30 days of continuous exposure of Hg²⁺ with low concentration, it had an obvious inhibition effect on NH₄⁺-N removal, while relatively less influence on the removal of other parameters (DOC and PO₄³⁻-P). The different observations in pollutant removal performance between short-term and long-term shock tests seem to indicate that, the Hg²⁺ accumulation in reactor have more severe inhibitory effect on the nitrification by autotrophic AOBs and NOBs, while Hg²⁺ concentration in influent have more impacts on the activities of heterotrophic OHOs and PAOs. Similarly, Li et al [15] found low concentrations of Hg²⁺ exposure could cause decreases in the nitrification rate. After 30 days of restoration, however, the NH₄⁺-N removal efficiency was restored to the initial level, which indicates the inhibitory effect is reversible.

| Parameter       | Before | Shock-adaptation | Restoration |
|-----------------|--------|------------------|-------------|
|                 |        | 0-15d            | 16-30d      | 0-15d       | 16-60d       |
| DOC Influent (mg/L) | 160.0  |                  |             |             |             |
| Effluent (mg/L)     |        | 7.8±1.5          | 6.7±0.7     | 6.1±1.5     | 7.7±1.4     | 11.8±2.2    |
| Removal efficiency (%) | 95.2±0.9 | 95.8±0.4         | 96.2±0.9    | 95.2±0.9    | 92.6±1.3    |
| NH₄⁺-N Influent (mg/L) | 30.0  |                  |             |             |             |
| Effluent (mg/L)     |        | 1.4±0.6          | 21.4±1.4    | 23.2±1.5    | 8.4±7.3     | 3.9±1.2     |
| Removal efficiency (%) | 95.3±1.9 | 28.7±4.8         | 22.7±4.9    | 72.1±24.3   | 86.9±3.8    |
| PO₄³⁻-P Influent (mg/L) | 5.00  |                  |             |             |             |
| Effluent (mg/L)     |        | 0.13±0.08        | 0.16±0.18   | 0.02±0.04   | 0.86±0.59   | 0.85±0.69   |
| Removal efficiency (%) | 97.5±1.5 | 96.8±3.7         | 99.6±0.9    | 82.9±11.9   | 83.0±13.7   |

3.3. Effect of long-term Hg²⁺ shock on microbial enzyme activities

As shown in figure 2(a), the relative sDHA activity in the Hg²⁺ shocked reactor decreased to 15% after 15 days of shock, gradually increased after 30 days of adaptation, and then almost recovered after 60 days of restoration, which agrees well with the changes in DOC removal efficiency, indicating the acclimatization of the heterotrophic microorganisms to low level of Hg²⁺ exposure. The intracellular ROS production was also measured during the shock-adaptation and restoration phases to examine the oxidative stress in the presence and absence of Hg²⁺. As shown in figure 2(b), although it is relatively lower than that in the Cu²⁺ shocked reactor [16], the level of ROS gradually increased compared to that in the blank reactor. In response to the elevated oxidative stress, the levels of SOD and CAT, which are two key antioxidant enzymes scavenging ROS for the protection of cellular homeostasis, increased during the shock-adaptation phase, and eventually restored to the control level after 60 days of restoration. Some studies reported various pollutants can retard the antioxidant responses, and therefore disturb the microbial metabolism. While in our case, it seems to indicate a high resistance
and fast recovery in the SBR system under the long-term Hg$^{2+}$ shock with low concentration. Han et al [17] reported the presence of extracellular polymeric substances (EPS) in activated sludge alleviates the negative impacts of NAOCl shock on CAT and SOD activities, and in turn reduced the intracellular ROS production, and maintained the ATP content and sDHA activity, which is similar with our enzyme activity results. Further studies are warranted to better understand the protective roles of EPS and the underlying mechanisms in resisting the environmental stress. Moreover, no increase in LDH release was observed under the long-term Hg$^{2+}$ shock conditions (data not shown), indicating there is no irreversible cell membrane damage or lysis.

![Figure 2](image_url)

**Figure 2.** Effects of long-term Hg$^{2+}$ shock on (a) sDHA activity, (b) intracellular ROS production, and CAT and SOD activities in different phases (S: shock-adaptation, R: restoration).

### 3.4. Effect of long-term Hg$^{2+}$ shock on microbial community structure

The changes in microbial community structure in the reactor during shock-adaptation and restoration phases are shown in figure 3. At the genus level, 49 genera were identified, 10 of which were dominant, including Zoogloea, Paracoccus, and Hydrogenophaga. During the 30 days of shock-adaptation phase, the relative abundance of Zoogloea, one important denitrifier implicated in floc formation and EPS production, decreased at the beginning and gradually recovered. The relative abundance of Paracoccus also decreased, while the relative abundance of Hydrogenophaga increased.
obviously, implying a higher resistance to the Hg\(^{2+}\) shock. The results of Alpha diversity (table 3), in terms of observed OTU numbers, Chao1, ACE, Shannon, and Simpson indices, revealed a decreased richness and evenness in the Hg\(^{2+}\) shocked reactor. Such less diverse microbial community was also observed after 60 days of the restoration, which might require a longer culturing time for recovery. Even though, the overall performance and enzyme activities in the reactor is comparable with that before the shock, implying that the microbial community shows resilience to the long-term Hg\(^{2+}\) shock due to its functional redundancy and complementation.

| Parameter | Before | Shock-adaptation | Restoration |
|-----------|--------|------------------|-------------|
| OTUs      | 563    | 610              | 497         |
| Chao      | 646    | 702              | 587         |
| ACE       | 636    | 687              | 597         |
| Shannon   | 4.16   | 4.34             | 4.03        |
| Simpson   | 0.041  | 0.033            | 0.037       |

4. Conclusions
This study investigated the changes in pollutant removal performance, microbial enzyme activities and community structure in the SBR during the Hg\(^{2+}\) shock-adaptation and restoration phases. Short-term exposure test showed that the removal efficiencies of DOC, NH\(_{4}\)\(^+\)-N and PO\(_4\)\(^{3-}\)-P significantly decreased when Hg\(^{2+}\) concentration was 0.05 mM. Long-term exposure tests indicated that continuous addition of 0.01 mM Hg\(^{2+}\) might have less influence on the activities of heterotrophic OHOs and PAOs, but an obvious negative impact on autotrophic AOBs and NOBs. In response to the Hg\(^{2+}\) shock, the activities of antioxidant enzymes elevated and eventually reduced the intracellular ROS production and maintained the ATP content and sDHA activity. According to the results of high-throughput 16S rRNA sequencing, the microbial community showed resilience to the long-term Hg\(^{2+}\) shock with low concentration. Among all dominant bacterial genera, Zoogloea and Paracoccus are sensitive to Hg\(^{2+}\) exposure, while Hydrogenophagae is more tolerant.

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References
[1] Battistoni P, Fava G and Ruello M L 1993 Heavy metal shock load in activated sludge uptake and toxic effects Water Res. 27 821-7
[2] Şengör S S, Gikas P, Moberly J G et al 2012 Comparison of single and joint effects of Zn and Cu in continuous flow and batch reactors J. Chem. Technol. Biotechnol. 87 374-80
[3] Cheng S 2003 Heavy metal pollution in China: Origin, pattern and control Environ Sci Pollut R. 10 192-8
[4] Madoni P, Davoli D, Gorbi G et al 1996 Toxic effect of heavy metals on the activated sludge protozoan community Water Res. 30 135-41
[5] Wang X H, Ren N Q, Wang A J et al Effect of ferrous and manganese ion on nitrification J. Harbin Institute of Technology 35 122-5 (in Chinese)
[6] Ong S A, Toorisaka E, Hirata M et al 2004 Effects of nickel (II) addition on the activity of activated sludge microorganisms and activated sludge process J. Hazard Mater. 113 111-21
[7] Wu D, Shen Y, Ding A et al 2013 Effects of nanoscale zero-valent iron particles on biological
nitrogen and phosphorus removal and microorganisms in activated sludge J. Hazard Mater. 262 649-55
[8] Mowat A 1976 Measurement of metal toxicity by biochemical oxygen demand J Water Pollut Control Fed. 48 853-66
[9] Xie B 2004 Effects of heavy metals on activated sludge microorganisms Shanghai Chem. Ind. 29 13-6 (in Chinese)
[10] Yin J, Xu H, Shen D et al 2015 Effect of Cu (II) shock loads on shortcut biological nitrogen removal in a hybrid biofilm nitrogen removal reactor Biodegradation 26 1-12
[11] Wang Y, Qin J, Zhou S et al 2015 Identification of the function of extracellular polymeric substances (EPS) in denitrifying phosphorus removal sludge in the presence of copper ion Water Res. 73 252-64
[12] Henriques I, Kelly R T, Dauphinais J L et al 2007 Activated sludge inhibition by chemical stressors-a comprehensive study Water Environ. Res. 79 940-51
[13] You S J, Tsai Y P and Huang R Y 2009 Effect of heavy metals on nitrification performance in different activated sludge processes J. Hazard Mater. 165 987-94
[14] Margesin R, Walder G, Schinner F 2000 The impact of hydrocarbon remediation (diesel oil and polycyclic aromatic hydrocarbons) on enzyme activities and microbial properties of soil Acta Biotechnol. 20 313-33
[15] Li J Y, Zhao Q X, Wang J et al 2009 Comparative study on microbial toxicity of heavy metals to activated sludge J. Environmental Pollution Control 31 17-20
[16] Wang D Q, Yang Z J, Liu T et al 2018 Short-term and long-term effects of copper on the performance and microorganisms in sequencing batch reactor IOP Conference Series: Earth and Environmental Science 191 012108
[17] Han X, Wang Z, Chen M et al 2017 Acute responses of microorganisms from membrane bioreactors in the presence of NaOCl: protective mechanisms of extracellular polymeric substances Environ. Sci. Technol. 51 3233-41