Desalination and Water Treatment
www.deswater.com
doi: 10.1080/19443994.2015.1087339

57 (2016) 17836–17843
August

Taylor & Francis
Taylor & Francis Group

Using the dehydrogenase activity for alert of activated sludge system under different copper concentrations

Qian Feng[a,b,*], Yubing Xiao[a,b], Xiaochen Li[c], Zhaoxia Xue[a,b], Fang Fang[a,b], Jiasun Cao[a,b], James Oloche Oleyiblo[a,b], Zhirong Hu[d]

[a]*Key Laboratory for Integrated Regulation and Resources Exploitation on Shallow Lakes of Ministry of Education, Hohai University, Nanjing, Jiangsu 210098, P.R. China, Tel. +86 25 83787935; Fax: +86 25 83786701; emails: xiaofq@hhu.edu.cn (Q. Feng), xiaoyubing18 @163.com (Y. Xiao), xzhx223@163.com (Z. Xue), fangf65@hhu.edu.cn (F. Fang), Tel./Fax: +86 2583786701; email: caojiashun@163.com (J. Cao), Tel. +86 13852291540; Fax: +86 25 83786701; email: evangjamesa1@gmail.com (J.O. Oleyiblo)*
[b]*College of Environment, Hohai University, Nanjing, Jiangsu 210098, P.R. China*
[c]*Water Conservancy and Civil Engineering College, Shandong Agriculture University, Tai'an, Shandong 271018, P.R. China, Tel. +86 15866021886; Fax: +86 538 8241676; email: lixiaochen02@163.com*
[d]*EnviroSim Associates Ltd, McMaster Innovation Park, 175 Longwood Road South, Suite 114A Hamilton, Ontario L8P 0A1, Canada, Tel. +1 9054812607; Fax: +1 905 481 2610; email: zhironghu@yahoo.com*

Received 30 December 2014; Accepted 23 August 2015

ABSTRACT

The aim of this study is to evaluate the effectiveness of dehydrogenase activities as an early warning indicator in wastewater treatment process under the impact of Cu(II). The substrate removals in sequencing batch reactors (SBR) on different Cu(II) concentrations (0, 5, 10, and 20 mg L^{-1}) were investigated during long-term operation. Meanwhile, the dehydrogenase activities of activated sludge taken from SBR were observed in short-term bioassays. The results indicate that the inhibitory rates of Cu(II) on substrate removal efficiencies increased along with an increment of Cu(II) concentration. The dehydrogenase activities demonstrated similar trend. Logarithmic curves were used to describe the inhibitory effect of Cu(II) on dehydrogenase activities. Positive correlations were then obtained between the substrate removal efficiencies during long-term operation and dehydrogenase activities in short-term bioassays. Thus, 2-(*p*-iod-phenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT)-dehydrogenase activity test can be applied as an effective method to alert the activated sludge system performance under the impact of heavy metal. In addition, median inhibitory concentration (IC50) by INT-dehydrogenase test after 1-h and 24-h Cu(II) exposure (5.93 mg L^{-1} for 1 h exposure and 5.45 mg L^{-1} for 24 h exposure) were obtained. These results showed that IC50 after 24 h of Cu(II) exposure appeared more sensitive to copper than that after 1 h. Therefore, INT-dehydrogenase test after 24 h Cu(II) exposure could be better fit for the early warning of wastewater treatment performance.

Keywords: Activated sludge; Substrate removal; Copper; Dehydrogenase activities; Inhibition; Alert

*Corresponding author.

1. Introduction

Copper is a kind of common heavy metal in sewage, which is mainly contained in effluent of electroplating, printed circuit boards manufacturing, paints production, and plastic and alloy manufacturing [1,2]. As a key constituent of respiratory enzyme, different concentrations of copper have significant impacts on the biological systems. On one hand, microbial metabolism may be enhanced by low concentrations of copper. Carotenogenesis of *Blakeslea trispora* [3] and carotenoid yield of *Rhodotorula* strain [4] were improved by adding trace amounts of Cu(II). 1–2 μmol amount of copper greatly stimulated exopolyphosphatase activity in cell extracts from *Acidithiobacillus ferrooxidans* [5]. In activated sludge systems, copper (0.5 and 1 mg L^{-1}) increased the maximum specific growth rates [6] and biomass yields [7]. On the other hand, high concentrations of copper can cause the inhibition of microbial activity and growth. Wu and Rodgers [8] demonstrated that copper inhibition occurred at 0.07 mg L^{-1} in enhanced biological phosphorous removal system. The results of Zhou et al. [9] indicated that copper (1.0 mg L^{-1}) was toxic to the micro-organism in the biofilm after 24 h of exposure. In Anammox bioreactor, the half maximal inhibitory concentration of Cu(II) (1.9 mg L^{-1}) was calculated by an exponential inhibition model [10]. Higher inhibitory concentrations of copper were reported in fermentative methane production of anaerobic reactors [11,12], Anammox mixed culture [13], fixed microbial film leachate treatment systems [14], and activated sludge processes [15–18]. Owing to the important role of copper in microbial metabolism, the impacts of copper on biological systems have attracted many researchers' attentions.

It is vital to evaluate copper toxicity in wastewater treatment process. A large number of methods have been proposed for the assessment of copper toxicity in microbial systems. Respiratory rate is one of the most common indicators for toxicity assessment in aerobic micro-organism. Madoni et al. [19] used the response of specific oxygen uptake rate for studying the inhibitory effects of copper on activated sludge micro-organisms. Similar methods for copper toxicity assessment were utilized by other researchers [20–22], and especially oxygen concentration profile obtained by microelectrodes proved to be a reliable indicator in inhibition analysis [9]. Besides respiratory rate, microbial growth parameters [15,19,23] and substrate degradation kinetics [2,8,24,25] are also extensively used in biological wastewater treatment processes. The processes included the organic carbon removal, ammonium oxidization, nitrification, denitrification [2,14,18], phosphorous uptake [8], methane production [12], and Anammox [10]. Moreover, dehydrogenase activity test presents as an effective toxicological method [26]. Generally, three kinds of tetrazolium salts, 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), 2,3,5-triphenyltetrazolium chloride (TTC), and 2-(*p*-iod-phenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT) are commonly used in dehydrogenase activity test. Das et al. [27] applied CTC test to evaluate the effect of silver nanoparticles on metabolic activity of natural aquatic bacterial communities. CTC activity of bacteria in wastewater treatment plant [28], rivers [28], and seas [29] was also studied in detail previously. For TTC test, Wang et al. [30] observed the TTC activity of aerobic granules with a supplement of 5 mg L^{-1} Cu(II) for 2 months. During the period, a significant reduction of TTC activity was found after 2 d. Moreover, the relationship between Cu(II) concentrations and TTC activity inhibition was given by Bestawy et al. [31].

Although the effects of copper on micro-organisms in biological wastewater treatment process were studied, limited results demonstrated the relationship between Cu(II) concentration and dehydrogenase activity clearly. Not so much attention has been paid closely to the validity of dehydrogenase activity acting as an early warning indicator. Comparing these three methods for dehydrogenase activity test, CTC test turns out to be hard to perform at most wastewater treatment plant in China, since some of the essential equipment is too expensive such as fluorescence microscope and flow cytometry. By contrast, the equipment for TTC- and INT-dehydrogenase test is much cheaper and user-friendly. For the TTC test, the oxidation reduction potential (ORP) is up to +460 mV, which means TTC has low electron affinity, resulting in a weaker capability to obtain electrons than oxygen. Therefore, the excess oxygen needs to be removed during TTC-dehydrogenase test. Additionally, TTC-dehydrogenase test is not suitable for anaerobic and denitrifying sludge. In contrast, INT has lower ORP (+90 mV), which makes INT-dehydrogenase test free from the faults of TTC-dehydrogenase test. Hence, microbial activity in aerobic, anaerobic, and denitrifying systems can be measured by INT-dehydrogenase test [32]. Above all, INT-dehydrogenase activity is selected to reflect microbial activity in our study due to its simple procedures and wide application properties. Both the performance of sequencing batch reactors (SBRs) during long-term operation and the INT-dehydrogenase activities of activated sludge in short-term bioassays were investigated under different Cu(II) concentrations. Based on the analysis, the

relationships between the SBRs performances and INT-dehydrogenase activities of activated sludge were established. Besides, median inhibitory concentrations (IC50) determined by INT-dehydrogenase tests were further discussed under various Cu(II) exposure times. The results would be useful to gain an effective alerting method for the activated sludge system under impact of copper.

2. Materials and methods

2.1. Reactor setup

Four identical SBRs were used to evaluate long-term inhibitory effects of Cu(II) on the substrate removal performance in activated sludge processes. Each reactor with a working volume of 4 L was made of clear acrylic plastic. SBRs were all operated in cycles of 6 h each, consisting of five stages: FILL (30 min), REACT (240 min with aeration), SETTLE (60 min), DRAW (20 min), and IDLE (10 min). The volumetric exchange rate of the four reactors was uniform by 50%.

2.2. Wastewater and sludge

The seed sludge was taken from an aeration tank of Jiangning Development Zone Wastewater Treatment Plant located in Nanjing. Synthetic wastewater was used to simulate the composition of real domestic wastewater. The chemical composition of the influent was as follows: sucrose 647.1 mg L^{-1}, NH_4HCO_3 263.4 mg L^{-1}, K_2HPO_4 20.4 mg L^{-1}, KH_2PO_4 4.93 mg L^{-1}, $MgCl_2{\cdot}6H_2O$ 3.7 mg L^{-1}, $FeCl_2{\cdot}2H_2O$ 3.7 mg L^{-1}, $CaCl_2{\cdot}2H_2O$ 3.7 mg L^{-1}, $MnSO_4$ 0.057 mg L^{-1}, H_2MoO_4 0.031 mg L^{-1}, $ZnSO_4$ 0.046 mg L^{-1}, $CoSO_4$ 0.049 mg L^{-1}. During the experiment, the influent COD, NH_4^+-N, PO_4^{3-}, and pH were 586 ± 69 mg L^{-1}, 65.8 ± 7.8 mg L^{-1}, 14.8 ± 1.1 mg L^{-1}, and 6.7 ± 1.3, respectively.

2.3. Experimental procedures

Activated sludge was cultivated in the four reactors (SBR 1, SBR 2, SBR 3, and SBR 4). The SBRs were fed with synthetic wastewater. Cu(II) was added in the form of $CuSO_4{\cdot}5H_2O$ to provide a concentration of 0, 5, 10, and 20 mg L^{-1} in different reactors. The mixed liquor suspended solid (MLSS) in each SBR was around 5 g L^{-1}. Each reactor was operated at solids retention time of 6 d by discharging excess sludge. The temperature of the reactor was kept at 20 ± 1°C by a thermostatic bath. An air pump and a pipe joined to a porous stone in the bottom of the reactor were used to provide the dissolved oxygen (DO) at the level above 5.0 mg L^{-1}. The pH of SBRs was maintained at 7.0 ± 0.5 by adding a phosphate buffer. During the experimental period, COD and NH_4^+-N concentrations of influent and effluent were measured everyday. Activated sludge taken from the control system (SBR 1) was poured into the batch reactors for short-term batch assays where the dehydrogenase activities were analyzed under different Cu(II) concentrations.

2.4. Dehydrogenase activities assays

Batch assays were conducted in 10 identical cylindrical containers. Each container with a liquid volume of 1 L was kept in thermostatic water batch. The chemical composition of influent, temperature, DO, and volumetric exchange rate of containers were all the same as those in SBRs experiment. The initial concentrations of Cu(II) in the containers were 0, 1, 2, 4, 5, 8, 10, 16, 20, and 32 mg L^{-1}, respectively. In the experiment, sludge was regularly sampled for dehydrogenase activity test. Dehydrogenase activity (DHA), measured by 2-(*p*-iod-phenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT), was determined by Martinez [33] in the experiment. Total dehydrogenase activity (total DHA), which is related to the substrate removal efficiency, is the product of INT-dehydrogenase activity and MLSS in the container. Each test was replicated for three times.

2.5. Analytical methods

Measurements of COD, NH_4^+-N and MLSS were determined in accordance with the standard methods [34]. DO values were measured daily with an Orion 830A (Thermo Electron Company, USA).

The inhibitory rates of Cu(II) on the system performance and the inhibitory rates of Cu(II) on dehydrogenase activity were calculated by the following equation [35]:

$$IR = ((R_0 - R)/R_0) \times 100\% \quad (1)$$

where IR is the inhibitory rate, %; R_0 is the substrate removal efficiency or DHA of activated sludge in control reactor without Cu(II) feeding; and R is the corresponding value in the other reactors with Cu(II) feeding.

Linear and nonlinear regressions were applied in statistical analysis. The profile of COD or NH_4^+-N removal inhibitory rate with copper dosage was determined by nonlinear logistic regression model. The regression equations were also employed to estimate

median inhibitory concentration (IC50). In addition, we used linear correlation analysis to build relationships between the dehydrogenase activities and substrate removal efficiencies. All statistical analyses were processed in Excel 2007 and Origin 8.5.

3. Results and discussion

3.1. Inhibitory effects of Cu(II) on the performance of SBR

Fig. 1 shows the removal efficiencies of COD and ammonia nitrogen (NH_4^+-N) in SBRs under different Cu(II) concentrations. The control system without Cu (II) addition exhibits high removal efficiency (above 90%) of COD and NH_4^+-N during the whole experimental period. When the Cu(II) dosage was 5 mg L^{-1}, no remarkable variations were observed in the NH_4^+-N removal efficiencies, while approximately about 15% drops of COD removal efficiencies were found. Both COD and NH_4^+-N removal efficiencies significantly decreased with the Cu(II) addition high up to concentrations of 10 and 20 mg L^{-1}.

The average substrate removal efficiencies and the inhibitory rates of substrate removal efficiency were calculated for further analysis (Fig. 2). In Fig. 2, increases in Cu(II) concentration had a negative effect on substrate removal and resulted in the growth of the inhibitory rates on substrate removal efficiency. Without feeding Cu(II), the average removal efficiencies of COD and NH_4^+-N were 93.16 and 99.89%, respectively. When Cu(II) concentration increased to 5 mg L^{-1}, the average removal efficiencies of COD and NH_4^+-N dropped to 77.92 and 98.06%, respectively. Meanwhile, the inhibitory rates of COD and NH_4^+-N

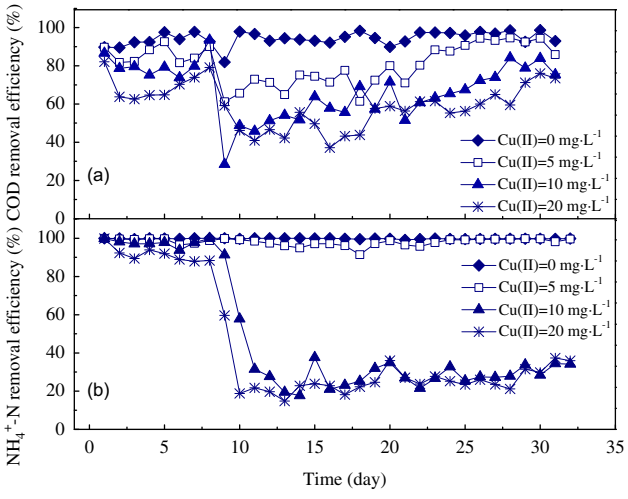

Fig. 1. The performance of SBRs under different Cu(II) concentrations: (a) COD removal efficiency (%); (b) NH_4^+-N removal efficiency (%).

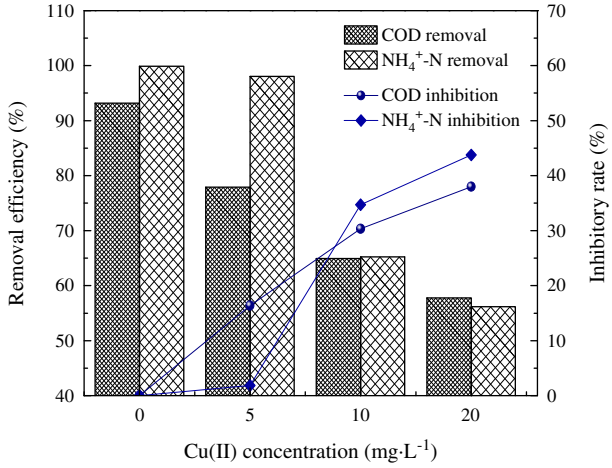

Fig. 2. The average substrate removal efficiency and inhibitory rate in SBRs under different Cu(II) concentrations.

removal efficiency were 16.36 and 1.83%. Low average substrate removal efficiencies (64.91% for COD and 65.20% for NH_4^+-N) were showed due to further addition of Cu(II) to 10 mg L^{-1}. The minimal substrate removal efficiencies (57.76% for COD and 56.18% for NH_4^+-N) were observed when Cu(II) concentration reached 20 mg L^{-1}. Correspondingly, the maximal inhibitory rates of substrate removal efficiency were obtained (38.0% for COD removal and 43.75% for NH_4^+-N removal).

The inhibitions of Cu(II) on substrate removal were determined by the concentration of Cu(II). Since copper is a basic component in enzymes related to nitrification, such as the primary enzyme, ammonia monooxygenase [36,37], the nitrification process does not deteriorate at a low concentration of Cu(II) [2,18]. However, significant inhibition of Cu(II) on nitrification was observed at a high concentration of Cu(II). These can be attributed to the inhibition of Cu(II) on the growth and activity of nitrifying bacteria [2,31]. As for the degradation of organics, the inhibition started at relatively low concentrations of Cu(II). When the concentration of Cu(II) reached relatively high level, serious instability would be observed in the biochemical system [15]. Our results compared well with some previous researchers [18,28,38]. The inhibition of Cu (II) on organic removal is due to cell damage and respiration inhibition of aerobic heterotrophic microorganism [39,40]. It is commonly accepted that all aerobic cells generate free radicals constantly in biological processes [41]. Excessive concentration of Cu (II) can simulate the production of free radicals mostly as reactive oxygen species, which will damage proteins, lipids and DNA of cells [42,43].

It was also found in Fig. 2 that the inhibitory rates of Cu(II) on the COD removal were lower than that on the NH_4^+-N removal at high concentrations of Cu (II) (10 and 20 mg L^{-1}). These results indicated that high concentrations of Cu(II) led to smaller inhibitory effects on the organic substrate removal process than the effects on the nitrification process. It was widely accepted that biological inhibition of nitrogen oxidation was more pronounced than that for organic carbon [2,14]. Our findings support this view at high concentrations of Cu(II), while opposite results were observed at low concentrations of Cu(II) (5 mg L^{-1}). The phenomena may be likely ascribed to the role of copper in microbial growth and metabolism. In this case, it can be concluded that inhibition of Cu(II) on aerobic heterotrophs is greater than that on nitrifier under low concentrations of Cu(II).

3.2. Inhibitory effects of Cu(II) on the dehydrogenase activity

Fig. 3 demonstrates the inhibitory effect of Cu(II) on the INT-dehydrogenase activity and inhibitory rate after 1 and 24 h of Cu(II) exposure. It can be clearly observed that the INT-dehydrogenase activity significantly dropped with the increasing of Cu(II) concentrations. In the batch reactors, the lowest INT-dehydrogenase activity (11.48 and 19 μg mg^{-1} h^{-1}) were observed on Cu(II) concentration of 32 mg L^{-1} after 1 and 24 h of Cu(II) exposure, respectively. It exhibited a dramatic growth of inhibitory rates with the increasing Cu(II) concentrations. When the Cu(II) concentration became 1 mg L^{-1}, the lowest inhibitory rates (1.10% for 1 h of Cu(II) exposures and 19.85% for 24 h) were obtained. The largest inhibitory rates were obtained with the highest level of Cu(II) concentration (32 mg L^{-1}). These findings were in agreement with those reported by Bestawy et al. [31] in activated sludge processes and Wang et al. [30] in aerobic granules reactor.

Logarithmic curves illustrated using following equations preferably describe the mode of Cu(II) inhibitory impact on dehydrogenase activities after 1 and 24 h of Cu(II) exposure, which was similar to the results obtained by Yin et al. [32]:

$$IR_{1\,h} = 0.2623\ \ln^{[Cu(II)]} + 0.03326 \quad R^2 = 0.937 \tag{2}$$

$$IR_{24\,h} = 0.1428\ \ln^{[Cu(II)]} + 0.2576 \quad R^2 = 0.949 \tag{3}$$

where $IR_{1\,h}$ and $IR_{24\,h}$ are the inhibitory rate of Cu(II) on the INT-dehydrogenase activity after 1 and 24 h of Cu(II) exposure respectively, and [Cu(II)] represents the Cu(II) concentration in the influent.

Dehydrogenase enzyme is a kind of important intracellular enzyme for microbial metabolism. The INT-dehydrogenase activity deserves much attention when studying cellar respiratory processes in aerobic and anaerobic reactions [44]. Rising of Cu(II) concentration negatively impacts the cellar respiration of microbe [45]. Due to the inhibition of microbial metabolism, the INT-dehydrogenase activity jumped down. The differences in INT-dehydrogenase activity between 1 and 24 h of Cu(II) exposures could be explained as the ability of microbial adaptation [18].

3.3. Relationships between biological activities and the performance in SBR

The relationships between the INT-dehydrogenase activities and substrate removal efficiencies are also

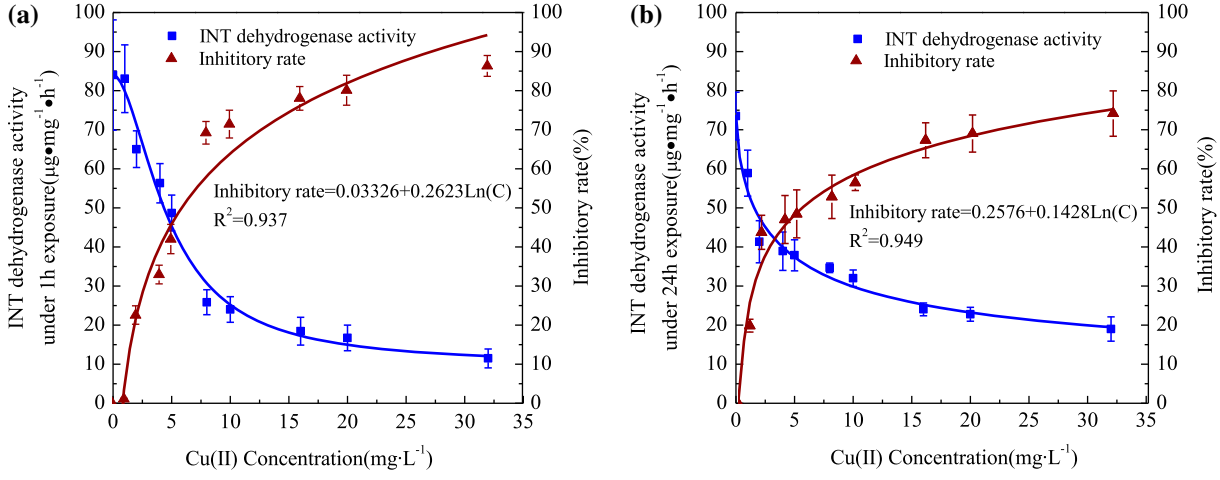

Fig. 3. Inhibitory effects and inhibitory rates of Cu(II) on the INT-dehydrogenase activity: (a) under 1 h exposure; (b) under 24 h exposure.

Table 1
Correlations between the substrate removal efficiencies and dehydrogenase activities

	INT-dehydrogenase activity (1 h)[b]	Total INT-dehydrogenase activity (1 h)[c]	INT-dehydrogenase activity (24 h)[b]	Total INT-dehydrogenase activity (24 h)[c]
NH_4^+-N[a]	$Y = 121.08x - 53.316$ $R^2 = 0.7997$	$Y = (1.0696x - 0.4766) \times 10^6$ $R^2 = 0.8029$	$Y = 76.762x - 19.751$ $R^2 = 0.6013$	$Y = (0.6856x - 0.1866) \times 10^6$ $R^2 = 0.6109$
COD[a]	$Y = 193.54x - 98.786$ $R^2 = 0.9859$	$Y = (1.7074x - 0.8765) \times 10^6$ $R^2 = 0.9870$	$Y = 135.98x - 58.331$ $R^2 = 0.9104$	$Y = (1.2087x - 0.5269) \times 10^6$ $R^2 = 0.9160$

[a]The removal efficiency of NH_4^+-N and COD, percent.
[b]The INT-dehydrogenase activity, μg mg^{-1} h^{-1}.
[c]The total INT-dehydrogenase activity, μg L^{-1} h^{-1}.

shown in Table 1. The dehydrogenase activity illustrates the microbial oxygen uptake in aerobic metabolism [46]. Therefore, positive correlation is identified between the substrate removal efficiency and dehydrogenase activity.

Total INT-dehydrogenase activity was correlated with substrate removal efficiency too. Because of the effects of microbial concentration, total INT-dehydrogenase activity demonstrated better correlations with substrate removal efficiency than INT-dehydrogenase activity. Moreover, the correlation coefficient of INT-dehydrogenase activity and COD removal efficiency was higher than that of INT-dehydrogenase activity and NH_4^+-N removal efficiency. These differences might result from different adaptability of autotroph and heterotroph under various concentrations of Cu(II).

The inhibitory concentrations (IC_{20}, IC_{50}, and IC_{80}) obtained from INT-dehydrogenase test after 1 and 24 h of Cu(II) exposure are shown in Table 2. The median inhibitory concentrations (IC_{50}) of Cu(II) via INT-dehydrogenase test after 1 and 24 h of exposure were 5.93 and 5.45 mg L^{-1}, respectively. The value range was consistent with previous researches. Anderson et al. [47] showed that IC_{50} of Cu(II) for INT-dehydrogenase activities was 2.5–5.1 mg L^{-1}. Yin et al. [32] indicated that IC_{50} of Cu(II) was 5.52 mg L^{-1} for INT-dehydrogenase activities and 1.52 mg L^{-1} for TTC-dehydrogenase activities. Additionally, IC50 via INT-dehydrogenase test after 1 h of exposure was higher than that after 24 h of exposure. This result showed that IC50 after 24 h of exposure was more sensitive to copper than it after 1 h of exposure. Based on the comparison, INT-dehydrogenase test after 24 h of Cu exposure could be better fit for the early warning of wastewater treatment performance.

Table 2
The inhibitory concentrations of Cu(II) for dehydrogenase activities (mg L^{-1})

INT-dehydrogenase activity	IC_{20}	IC_{50}	IC_{80}
1 h of exposure	1.89	5.93	18.6
24 h of exposure	0.67	5.45	44.7

4. Conclusion

When the Cu(II) concentration increased from 0 to 20 mg L^{-1}, the inhibitory effects of copper on substrate removal efficiency and dehydrogenase activity increased. Meanwhile, the relationships between the concentration of Cu(II) and dehydrogenase activity of activated sludge can be described well as logarithmic curves model. Hence, meaningful findings demonstrate positive correlations between the dehydrogenase activity and the substrate removal efficiency. These kinds of correlations prove that INT-dehydrogenase activity analyzing in short-term bioassays can be applied as an effective method to alert the activated sludge system performance under loading of heavy metal. In addition, comparison of IC_{50} presents that INT-dehydrogenase test after 24 h of Cu exposure can be better fit for the early alert of wastewater treatment performance. However, different correlations and formulas of dehydrogenase activity and substrate removal may be obtained in activated sludge system treating practical wastewater, due to the variation of some parameters such as organic toxicant, pH, and temperature. Further works are needed in activated sludge system treating practical wastewater.

Acknowledgements

This work was supported by the Natural Science Foundation of China (51208173), Major Science and Technology Program for Water Pollution Control and Treatment (2012ZX07101-003). A Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions.

References

[1] S.N. Sin, H. Chua, W. Lo, P.H. Yu, Effects of trace levels of copper, chromium, and zinc ions on the performance of activated sludge, Appl. Biochem. Biotechnol. 84–86 (2000) 487–500.

[2] R.X. Jiang, S.J. Sun, K. Wang, Z.M. Hou, X.C. Li, Impacts of Cu(II) on the kinetics of nitrogen removal during the wastewater treatment process, Ecotoxicol. Environ. Saf. 98 (2013) 54–58.

[3] N.S. Govind, A.R. Amin, V.V. Modi, Stimulation of carotenogenesis in *Blakeslea trispora* by cupric ions, Phytochem. Photobiol. 21 (1982) 1043–1044.

[4] K. Mahattanatavee, S. Kulprecha, Production of β-carotene by *Rhodotorula* sp. Y1621, Microbial. Utiliz. Renew. Resour. 7 (1991) 295–300.

[5] S.L. Alvarez, C.A. Jerez, Copper ions stimulate polyphosphate degradation and phosphate efflux in *Acidithiobacillus ferrooxidans*, Appl. Environ. Microbiol. 70 (2004) 5177–5182.

[6] F. Dilek, Ü. Yetis, Effects of heavy metals on activated sludge process, Water Sci. Technol. 26 (1992) 801–813.

[7] N. Beyenal, T. Özbelge, H. Özbelge, Combined effects of Cu^{2+} and Zn^{2+} on activated sludge process, Water Res. 31 (1997) 699–704.

[8] G.X. Wu, M. Rodgers, Inhibitory effect of copper on enhanced biological phosphorus removal, Water Sci. Technol. 62 (2010) 1464–1470.

[9] X.H. Zhou, T. Yu, H.C. Shi, H.M. Shi, Temporal and spatial inhibitory effects of zinc and copper on wastewater biofilm from oxygen concentration profiles determined by microelectrodes, Water Res. 45 (2011) 953–959.

[10] T. Lotti, M. Cordola, R. Kleerebezem, S. Caffaz, C. Lubello, M.C. van Loosdrecht, Inhibition effect of swine wastewater heavy metals and antibiotics on Anammox activity, Water Sci. Technol. 66 (2012) 1519–1526.

[11] K. Mori, M. Hatsu, R. Kimura, K. Takamizawa, Effect of heavy metals on the growth of a methanogen in pure culture and coculture with a sulfate-reducing bacterium, J. Biosci. Bioeng. 90 (2000) 260–265.

[12] D.L. Wu, Z.Z. Yang, G.M. Tian, Inhibitory effects of Cu(II) on fermentative methane production using bamboo wastewater as substrate, J. Hazard. Mater. 195 (2011) 170–174.

[13] G.F. Yang, R.C. Jin, The joint inhibitory effects of phenol, copper(II), oxytetracycline (OTC) and sulfide on Anammox activity, Bioresour. Technol. 126 (2012) 187–192.

[14] J. Scullion, M. Winson, R. Matthews, Inhibition and recovery in a fixed microbial film leachate treatment system subject to shock loading of copper and zinc, Water Res. 41 (2007) 4129–4138.

[15] A. Cabrero, S. Fernandez, F. Mirada, J. Garcia, Effects of copper and zinc on the activated sludge bacteria growth kinetics, Water Res. 32 (1998) 1355–1362.

[16] T.Y. Pai, S.C. Wang, H.M. Lo, C.F. Chiang, M.H. Liu, R.J. Chiou, W.Y. Chen, P.S. Hung, W.C. Liao, H.G. Leu, Novel modeling concept for evaluating the effects of cadmium and copper on heterotrophic growth and lysis rates in activated sludge process, J. Hazard. Mater. 166 (2009) 200–206.

[17] J. Li, X. Liu, Y. Liu, J. Ramsay, C. Yao, R. Dai, The effect of continuous exposure of copper on the properties and extracellular polymeric substances (EPS) of bulking activated sludge, Environ. Sci. Pollut. Res. Int. 18 (2011) 1567–1573.

[18] V. Ochoa-Herrera, G. Le ón, Q. Banihani, J.A. Field, R. Sierra-Alvarez, Toxicity of copper(II) ions to microorganisms in biological wastewater treatment systems, Sci. Total Environ. 412–413 (2011) 380–385.

[19] P. Madoni, D. Davoli, L. Guglielmi, Response of sOUR and AUR to heavy metal contamination in activated sludge, Water Res. 33 (1999) 2459–2464.

[20] M.D.C. Oviedo, D.S. Márquez, J.M.Q. Alonso, Toxic effects of metals on microbial activity in the activated sludge process, Chem. Biochem. Eng. Q 16 (2002) 139–144.

[21] S.A. Ong, P.E. Lim, C.E. Seng, Effects of adsorbents and copper(II) on activated sludge microorganisms and sequencing batch reactor treatment process, J. Hazard. Mater. 103 (2003) 263–277.

[22] E.U. Cokgör, S. Özdemir, Ö.K. Özgün, G. Insel, D. Orhon, Critical appraisal of respirometric methods for metal inhibition on activated sludge, J. Hazard. Mater. 139 (2007) 332–339.

[23] Y. Liu, Effect of chemical uncoupler on the observed growth yield in batch culture of activated sludge, Water Res. 34 (2000) 2025–2030.

[24] M.Y. Pamukoglu, F. Kargi, Mathematical modeling of copper(II) ion inhibition on COD removal in an activated sludge unit, J. Hazard. Mater. 146 (2007) 372–377.

[25] T.A. Özbelge, H.O. Özbelge, P. Altınten, Effect of acclimatization of microorganisms to heavy metals on the performance of activated sludge process, J. Hazard. Mater. 142 (2007) 332–339.

[26] R. Barrena, F. Vazquez, A. Sanchez, Dehydrogenase activity as a method for monitoring the composting process, Bioresour. Technol. 99 (2008) 905–908.

[27] P. Das, C.J. Williams, R.R. Fulthorpe, M.E. Hoque, C.D. Metcalfe, M.A. Xenopoulos, Changes in bacterial community structure after exposure to silver nanoparticles in natural waters, Environ. Sci. Technol. 46 (2012) 9120–9128.

[28] K. Sawaya, N. Kaneko, K. Fukushi, J. Yaguchi, Behaviors of physiologically active bacteria in water environment and chlorine disinfection, Water Sci Technol. 58 (2008) 1343–1348.

[29] L. Alonso-Saez, J.M. Gasol, T. Lefort, J. Hofer, R. Sommaruga, Effect of natural sunlight on bacterial activity and differential sensitivity of natural bacterioplankton groups in northwestern Mediterranean coastal waters, Appl. Environ. Microbiol. 72 (2006) 5806–5813.

[30] X.H. Wang, L.H. Gai, X.F. Sun, H.J. Xie, M.M. Gao, S.G. Wang, Effects of long-term addition of Cu(II) and Ni(II) on the biochemical properties of aerobic granules in sequencing batch reactors, Appl. Microbiol. Biotechnol. 86 (2010) 1967–1975.

[31] E. El Bestawy, S. Helmy, H. Hussein, M. Fahmy, Optimization and/or acclimatization of activated sludge process under heavy metals stress, World J. Microbiol. Biotechnol. 29 (2013) 693–705.

[32] J. Yin, X.J. Tan, N.Q. Ren, Y.B. Cui, L. Tang, Evaluation of heavy metal inhibition of activated sludge by

TTC and INT-electron transport system activity tests, Water Sci. Technol. 152 (2005) 231–239.
[33] R. Martínez, Psychrophilic and psychrotrophic respiratory metabolism in antarctic microplankton, Polar Biol. 16 (1996) 483–489.
[34] APHA, Standard Methods for the Examination of Water and Wastewater, twentieth ed., American Public Health Association, Washington, DC, 1998, pp. 2-55–2-61.
[35] Z. Kong, P.A. Vanrolleghem, W. Verstraete, An activated sludge-based biosensor for rapid IC50 estimation and on-line toxicity monitoring, Biosens. Bioelectron. 8 (1993) 49–58.
[36] H.A. Painter, A review of literature on inorganic nitrogen metabolism in microorganisms, Water Res. 4 (1970) 393–450.
[37] S.V. Antonyuk, R.W. Strange, G. Sawers, et al., Atomic resolution structures of resting-state, substrate- and product-complexed Cu-nitrite reductase provide insight into catalytic mechanism, Proc. Natl. Acad. Sci. USA 102 (2005) 12041–12046.
[38] Y.P. Ting, H. Imai, S. Kinoshita, Effect of shock-loading of heavy metals on total organic carbon and phosphate removal in an anaerobic–aerobic activated sludge process, World J. Microbiol. Biotechnol. 10 (1994) 308–312.
[39] Y. Kurmaç, The impact of toxicity of metals on the activity of ureolytic mixed culture during the precipitation of calcium, J. Hazard. Mater. 163 (2009) 1063–1067.
[40] R.A. Neumegen, A.R. Fernandez-Alba, Y. Chisti, Toxicities of triclosan, phenol and copper sulfate in activated sludge, Environ. Toxicol. 20 (2005) 160–164.
[41] V.B. Djordjević, Free radicals in cell biology, Int. Rev. Cytol. 237 (2004) 57–89.
[42] G.J. Brewer, Copper toxicity in the general population, Clin. Neurophysiol. 121 (2010) 459–460.
[43] J.J. Harrison, H. Ceri, R.J. Turner, Multimetal resistance and tolerance in microbial biofilms, Nat. Rev. Microbiol. 5 (2007) 928–938.
[44] J.P. Obbard, Measurement of dehydrogenase activity using 2-p-iodophenyl-3-p-nitrophenyl-5-phenyltetrazolium chloride (INT) in the presence of copper, Biol. Fert. Soils 33 (2001) 328–330.
[45] E. Frangipani, V.I. Slaveykova, C. Reimmann, D. Haas, Adaptation of aerobically growing *Pseudomonas aeruginosa* to copper starvation, J. Bacteriol. 190 (2008) 6706–6717.
[46] C. Aragón, M.D. Coello, J.M. Quiroga, Effect of manganese(II) on the respiratory activity of biological sludge from wastewater treatment plant, Chem. Eng. Res. Des. 88 (2010) 641–646.
[47] K. Anderson, B. Koopman, G. Bitton, Evaluation of int-dehydrogenase assay for heavy metal inhibition of activated sludge, Water Res. 22 (1988) 349–353.